Assessment of Microbiological Safety of Some Traditionally Processed Meat and Fish Products of Sikkim

A Thesis Submitted

To

Sikkim University

In Partial Fulfilment of the Requirement for the

Degree of Doctor of Philosophy

By

Meera Ongmu Bhutia

Supervisor: Professor Dr. Jyoti Prakash Tamang

Co-supervisor: Dr. Namrata Thapa

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

JUNE 2020

DECLARATION

I declare that the present Ph.D thesis entitled **"Assessment of Microbiological Safety of Some Traditionally Processed Meat and Fish Products of Sikkim"** submitted by me for the award of the degree of **Doctor of Philosophy in Microbiology** of Sikkim University under the supervisions of **Professor Dr. Jyoti Prakash Tamang,** Professor, Department of Microbiology, School of Life Sciences, Tadong, Sikkim University and **Dr. Namrata Thapa,** Head and Associate Professor, Department of Zoology, NBBD College (Sikkim University), Tadong, Sikkim, 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.

Meera

.....

Meera Ongmu Bhutia
Registration No: 14/PhD/MCB/05
Research Scholar
Department of Microbiology
School of Life Sciences
Sikkim University (www.cus.ac.in)
Gangtok

Date: 15th June 2020
Place: Tadong, Gangtok

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

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

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

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

CERTIFICATE

This is to certify that the PhD thesis entitled **"Assessment of Microbiological Safety of Some Traditionally Processed Meat and Fish Products of Sikkim"** submitted to **SIKKIM UNIVERSITY** in partial fulfilment for the requirement of the Doctor of Philosophy in Microbiology, embodies the work carried out by **Miss Meera Ongmu Bhutia** 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
Prof. Dr. Jyoti Prakash Tamang
ICIMOD Mountain Chair
PROFESSOR
CENTRE Laboratory, Department of Microbiology
SIKKIM UNIVERSITY (central university)
Tadong 737102, Gangtok, Sikkim, INDIA

CO- SUPERVISOR

Dr. Namrata Thapa
Associate Professor and Head
Department of Zoology
NBBD College
(Sikkim University)
Tadong 737102, Sikkim, INDIA

Dr.NAMRATA T. TAMANG
H.O.D.
Department of Zoology
N.B.B.D. College, Tadong

Head of the Department
Dr. Hare Krishna Tiwari
Associate Professor
Department of Microbiology, School of Life Sciences
Sikkim University

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

Place: Tadong, Gangtok
Date: 15th 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: 12th 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 **0% tolerance rate**, within the permissible limit (below 10% tolerance rate) as per the norm of Sikkim University.

"Assessment of Microbiological Safety of Some Traditionally Processed Meat and Fish Products of Sikkim"

Submitted by **Miss Meera Ongmu Bhutia** under the supervisions of **Professor Dr. Jyoti Prakash Tamang,** Professor, Department of Microbiology, School of Life Sciences, Sikkim University, Tadong 737102, Sikkim, India and **Dr. Namrata Thapa,** Head and Associate Professor, Department of Zoology, NBBD College (Sikkim University), Tadong 737102, Sikkim, India.

Meera

.....

Meera Ongmu Bhutia

Signature of the candidate

.....

Professor Dr. Jyoti Prakash Tamang

Signature of Supervisor

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

.....

Dr. Namrata Thapa

Signature of Co-Supervisor

Dr.NAMRATA T. TAMANG
H.O.D.
Department of Zoology
N.B.B.D. College, Tadong

.....

Professor A.S. Chandel

Librarian, Sikkim University

Librarian
Teesta-Indus Library
SIKKIM UNIVERSITY
6th Mile, Samdur, PO: Tadong
Gangtok-737102, Sikkim India

URKUND

Document Information

Analyzed document	Meera Bhutia-PhD Thesis-Plagariasm (20).docx (D74166354)
Submitted	6/5/2020 5:08:00 PM
Submitted by	Prof A S Chandel
Submitter email	librarian@cus.ac.in
Similarity	0%
Analysis address	librarian.sikkim@analysis.urkund.com

Sources included in the report

SA	URL: Rekha R.docx Fetched: 3/20/2018 6:55:00 AM	1
SA	URL: Yati_Vaidya_Bioscience_Thesis.pdf Fetched: 5/4/2017 7:42:00 AM	1
W	URL: https://cyberleninka.org/article/n/531610 Fetched: 6/5/2020 5:19:00 PM	3
W	URL: https://www.frontiersin.org/articles/492547 Fetched: 5/13/2020 7:27:18 AM	1

A Chandel

Librarian
Teesta-Indus Library
SIKKIM UNIVERSITY
6th Mile, Samdur, PO: Tadong
Gangtok-737102, Sikkim India

ACKNOWLEDGEMENTS

Having invested a wholesome of years in this project, I have in fact lived a lifetime in it. Doctorate is something beyond acquiring theoretical or practical knowledge but how we leverage and contribute to human welfare and Mother Nature. This project wasn't an exception. The journey of my doctoral research would not have been possible without the guidance and encouragement of many special people and their contributions will always be cherished. Hence, I would like to take this opportunity to express my indebtedness to those who have assisted me in a myriad of ways.

First, I would like to express my sincere gratitude to my mentor and supervisor Professor Dr. Jyoti Prakash Tamang, Department of Microbiology, Sikkim University. He has always been a torch-bearer, supportive and considerate advisor I could have never asked for. There were several moments where I had reached the 'crossroads' but each time he was there to steer me towards the right path and made me realize that my work mattered. Prof. Tamang's willingness and commitment to offer his valuable time and intellect has been a key driver, this thesis was completed. Also, I am grateful to my co-supervisor Dr. Namrata Thapa, Head and Associate Professor, Department of Zoology, NBBD College, Tadong for supporting me in all the ways in carrying out my preliminary work at research laboratory of NBBD College and offering me several opportunities to participate in various workshops under Biotech-hub, Department of Science and Technology. She has in fact been a great emotional support either, guiding me throughout personally and professionally.

My sincere thanks to all the faculty members; Dr. Hare Krishna Tiwari, Head and Associate Professor, Dr. Buddhiman Tamang, Dr. Nagendra Thakur, Dr. Bimala Singh and Dr. Anil Kumar Verma, Assistant Professors of Department of Microbiology, Sikkim University for their kind suggestions and support. Additionally, special thanks to the laboratory staff members for their support.

I also take this opportunity to acknowledge Govt. of India for providing Maulana Azad National Fellowship and DAICENTER (DBT-AIST International Center for Transnational & Environmental Research) for funding the research work.

I thank my DAICENTER team members (Dr. Shankar Prasad Sha, Ranjita Rai, Pooja Pradhan, Anu Anupma, H. Nakibapher Jones Shangpliang, Pynhunlang Kharnaior, Priyambada Pariyar, Souvik Das and Tanupriya Kumari Sha) for the stimulating discussions, for the sleepless nights we were working together before deadlines and for all inevitable memories we have had in the last couple of years. I also acknowledge the help and support of my other PhD scholar friends in Department of Microbiology.

The journey of life would not be possible without the support from my family. I wish to dedicate this work to my dear father Late Shri Tenzing Bhutia, Indian Army whose blessing has inspired me for life and will always do. My deepest gratitude to dear mother for all the sacrifices she has made to create the person I am today and also to my siblings for supporting me in every possible way.

Finally, I thank almighty for letting me through all the difficulties and giving me strength, knowledge, ability and opportunity to undertake this research study and to persevere and complete it satisfactorily. Without his blessings, this achievement would not have been possible.

…….

Date: 15/06/2020 **Meera Ongmu Bhutia**

CONTENTS

INTRODUCTION

Dietary Culture

Every nation in this world has unique ethnic foods and beverages with distinct dietary cultures and habits based on agro-climatic conditions and accessibility to edible raw resources of plant/animal origins (Tamang et al. 2020). Fermented foods are produced from raw or boiled materials or substrates of plant or animal sources mostly by natural or spontaneous fermentation and also few products by black-slopping method or additional of traditionally prepared starter culture(s) containing functional microorganisms which convert the biochemical structures of raw or boiled materials into edible products with acceptable sensory properties (Tamang 2010a; Tamang et al. 2016a). Microbiota convert the bio-chemical components present in substrates of raw/boiled plant or animal sources during fermentation resulting into tasty, palatable, eatable product as fermented food and beverages with nutritional value, and health-benefits to consumers including vitamins and minerals, antioxidant and probiotic functions (Tamang et al. 2016b). Religion has a strong influence on food habits such as taboos and other restrictions which are imposed on a wide range of foods and beverages including consumption of animal flesh and fish and alcoholic drink (Tamang and Samuel 2010). Consumption of domesticated animal flesh and river/sea-fish is a part of dietary culture of many people in the world, except in India and some Asian countries, where a majority of Hindu and Buddhists exclude meat and fish from their diet due to religion taboos (Tamang et al. 2016a).

Traditional Meat Products

Perishable flesh of domesticated animals is traditionally processed into several meat products by fermentation (Tamang et al. 2016a), smoking (Plavsic et al. 2015), sun-drying (Aksoy et al. 2019) and salting (Uğuz et al. 2011) to increase the shelf-life and

also to enhance the taste and texture as cultural foods. Traditionally processed meat and fish products are important food ecosystems where microorganisms dominate the ecological niche (Tamang et al. 2020). Preparation and eating of various types of traditional meat products is food culture of many ethnic communities in the world such *jerky* of America (Nummer et al. 2004), *pastrima* and *sucuk* of Turkey (Kaban 2013), *botillo* of Spain (Fontán et al. 2007), *alheira* of Portugal (Ferreira et al. 2006), *nham* of Thailand (Santiyanont 2019), and *biltong* of South Africa (Petit et al. 2014). During 1500 BCE, the Babylonians used to prepare sausages by stuffing minced animal flesh in intestines (Adams 2010; Franciosa et al. 2018), later making of sausage-like products spread during the Roman era throughout southern Europe and other areas near the Mediterranean Sea (Pederson 1979; Hutkins 2019). Dominant bacteria present in meat fermentations are some lactic acid bacteria including strains of *Lactobacillus sakei, Lb. plantarum, Lb. curvatus, Enterococcus faecium, Pediococcus pentosaceus, Leuconostoc carnosum*, *Leuc. gelidum, Leuc. pseudomesenteroides, Weissella* spp. etc. (Nguyen et al. 2013; Dias et al. 2015; Laranjo et al. 2017; Bartkiene et al. 2019). The other main group of bacteria are *Kocuria*, micrococci, and coagulase-negative staphylococci (Marty et al. 2011; Sánchez et al. 2017), which reduce nitrate to nitrite and also contribute flavor in fermented sausages (Lücke 2015; Sánchez et al. 2017). Enterobacteriaceae family, which has a largest numbers of Gram-negative pathogenic bacteria such as *Klebsiella, Enterobacter, Citrobacter, Salmonella, Escherichia coli, Shigella, Proteus, Serratia* and other species (Morales-López et al. 2019) are present in meat products (Karoki et al. 2018; Gelbíc˘ová et al. 2019).

Traditional Fish Products

Perishable fish caught from sea, lakes, rivers and streams are mostly preserved by fermentation (Zang et al. 2019), sun drying (Thapa 2016a), smoking (Olaleye and Abegunde 2015), and salting (Tamang et al. 2016a) for consumption as seasoning, condiments, curry and side dish. Traditionally processed fish products are country-specific with various vernacular names for products such as *jeot kal* in Korea, *shottsuru* and *shiokara* in Japan, *yucha* and *suanyu* in China, *patis* in Philippines, *plaa-som, nam pla* and *pla ra* in Thailand (Wu et al. 2000; Saithong et al. 2010; Lee et al. 2014; Zhang et al. 2016; Koo et al. 2016; Tamang et al. 2016a; Wang et al. 2017; Song et al. 2018; Zang et al. 2019) are commonly prepared as sauce in South Asia except in India, Bangladesh, Nepal and Bhutan (Tamang 2020). Sea as well as river fishes are also traditionally fermented into various fish products in Africa (Anihouvi et al. 2012) and in few European countries (Skåra et al. 2015). Several dominant bacterial genera have been reported in many fermented, dried and smoked fish products which include are *Enterococcus, Lactobacillus, Weissella, Pediococcus*, *Tetragenococcus*, *Bacillus, Micrococcus, Staphylococcus*, *Haloanaerobium, Halomonas, Salinivibrio* and *Salimicrobium* (Lee at el. 2014; Wang et al. 2017; Song et al. 2018).

Traditional Meat and Fish Products in India

In India, farming, pastoral, animal husbandry, fishery and agrarian system of traditional agriculture has been traced back to Indus Valley Civilization since 8000 years ago (Fuller 2015). Since then mixed-agricultures farming in all geo-morphological under different agro-climatic conditions in modern India is being practiced by farmers in the field of animal husbandry, forestry, fishery, etc. are to supplement daily food resources in local diets (Tamang 2020). Though the majority of Indian communities are

vegetarians, however, sizable numbers of Indian populaces is also non-vegetarians and consumes fresh meat and river/sea-fish as well as traditionally processed meat and fish products (Tamang et al. 2016c). The people of the Indian Himalayas and North East India prepare and consume varieties of traditionally processed meat products as side dish or curry in mail meal, some of them have been reported earlier such as *chartayshya, jamma* and *arjia* of Uttarakhand (Oki et al. 2011), *kargyong, satchu* and *suka ko masu* of Sikkim (Rai et al. 2009, 2010a,b), and *sa-um* of Mizoram (De Mandal et al. 2018). Similarly, traditionally processed sea-fish products are prepared and consumed in the coastal regions of India (Tamang 2020), and traditionally river/lake fish products are prepared and consumed in North East India (Thapa 2016b) such as fermented fish products - *ngari* (Thapa et al. 2004; Abdhul et al. 2014; Devi et al. 2015; Majumdar et al. 2009, 2015b; Singh et al. 2018) of Manipur, *hentak* of Manipur (Thapa et al. 2004; Aarti et al. 2016, 2017; Singh et al. 2018)*, tungtap* of Meghalaya (Thapa et al. 2004; Rapsang et al. 2011; Rapsang and Joshi 2012)*, sheedal* of Tripura (Majumdar et al. 2009, 2015b; Kakati and Goswami 2013a,b; Muzaddadi and Basu 2003, 2013; Muzaddadi 2015), and *lona ilish* of Tripura (Majumdar and Basu 2010; Das et al. 2020); sun-dried and smoked fish products- *namsing* of Assam (Chowdhury et al. 2019), *sukuti* of Sikkim and Darjeeling hills (Thapa et al. 2006; Thapa and Pal 2007)*, sidra* of Sikkim and Darjeeling hills (Thapa et al. 2006; Thapa and Pal 2007), *gnuchi* of Sikkim and Darjeeling hills (Thapa et al. 2006)*, bordia, karati* and *lashim* of Assam (Thapa et al. 2007; Thapa 2016a).

Dietary Culture in Sikkim

Sikkim is a tiny and hilly state of India with 7096 sq. km area and variable elevation ranging from 300 m to 8500 m located in the Eastern Himalayas (Figure. A). Sikkim

borders with China in the north and northeast, Bhutan in the east, Nepal in the west, and Gorkhaland Territorial Administration (GTA) in the state of West Bengal in the south.

Figure A. Map showing different parts of Sikkim along with neighbouring countries and state.

Administratively, Sikkim has four districts viz. East district, West district, South district and North district with a population of 610577 (www.census2011.co.in). About 80% ethnic community in Sikkim is Gorkha or Nepali followed by Bhutia and Lepcha (Tamang 2005). The Gorkha or Nepali community has several castes including Limboo, Tamang, Rai, Chettri, Sansyasi/Giri, Bahun, Magar, Pradhan/Newar, Gurung, Bhujel, Dewan, Sunwar, Khagatey, Sherpa, Kami, Damai, Sarki, Maji (Thapa and Tamang 2020). Livestock mostly plays a subsidiary role in the mixed farming system in Sikkim which includes poultry, cow, ox, pigs, goats, sheep, yaks, etc., mainly used for meat, milk and fermented dairy products. Unique high-mountain domesticated

animal called yak (*Bos grunniens*) is reared mostly on alpine and sub-alpine regions of Sikkim at more than 2100 m altitude for milk, milk products, wool, meat and meat products (Balaraman and Golay 1991). More than 44 species of fish have been reported from two major rivers of Sikkim viz. Teesta and Rangeet River and also their tributaries (Tamang 2002). The people living nearby rivers, streams, lakes and ponds catch the available local varieties of fishes for consumption, and some of them are traditionally preserved by smoking and sun-drying (Thapa 2016a).

Based on preference of diets, about 88.3% of people are non-vegetarians and 11.7% people are vegetarians in Sikkim (Tamang et al. 2007). Bhutia, Lepcha, Tibetan and Sherpa and non-Brahmin Gorkha/Nepali are non-vegetarians, eat chicken, mutton, buffalo, pork and beef, and also yak in North Sikkim. Beef is taboo to a majority of the Gorkha/Nepali except Tamang and Sherpa. Newar prefers buffalo meat. About 163.8 g of fermented foods and beverages are consumed daily in Sikkim representing 12.6 % of fermented foods in daily intake of meal (Tamang et al. 2007). Culinary and cuisine of different ethic people in Sikkim are unique and unparalleled since Sikkim is wedged in between Nepal and Bhutan with vast expanse of the Tibetan Plateau in north (Tamang 2005). Probably the food culture of these regions is a fusion of the Gorkha and Tibetan cuisines with modifications based on preference, acceptability, adaptability and other social ethos (Thapa and Tamang 2020). Modern methods of preservation of perishable meat and fish are still uncommon to majority of ethnic people of Sikkim; they prepare the traditional way of preservation of locally available meat and fish by fermentation, smoking, drying and salting. Dishes prepared from traditionally processed meat and fish products constitute a unique cuisine and represent the traditional culinary culture of Sikkim (Thapa and Tamang 2020).

Previous Reports on Traditional Meat and Fish Products from Sikkim

Traditional methods and their culinary of smoked and sun-dried meat products of Sikkim such as *shakampo, sukula, suka ko masu, satchu, kargyong, chilu* and *kheuri* have been documented (Rai et al. 2009; Tamang 2010b). Nutritional values of some of them have also been reported (Rai et al. 2010b). Bacterial species from beef *kargyong* and *satchu* prepared from beef meat were previously reported, based on phenotypic characteristics and biochemical tests, which included *Lactobacillus sake, Lb. sanfransisco, Lb. curvatus, Lb. divergens, Lb. carnis, Leuconostoc mesenterioides, Enterococcus faecium, Bacillus subtilis, B. thuringiensis, B. mycoides, Staphylococcus aureus* and *Micrococcus* spp. (Rai et al. 2010a). Traditional methods of preparation and their culinary of smoked and sun-dried fish products of Sikkim such as *sidra, sukuti, suka ko maacha,* and *gnuchi* have been documented (Thapa 2016a). Proximate composition of smoked and sun-dried fish products of Sikkim has also been analyzed (Thapa and Pal 2007). *Enterococcus durans*, *E. faecalis*, *E. faecium*, *E. hirae*, *Leuconostoc citreum*, *Leu. mesenteroides*, *Pediococcus pentosaceus* and *Weissella cibaria* were reported from some traditionally processed meat products such as *arjia, chartayshya, jamma* or *geema*/*juma* of Uttarakhand located in Western Indian Himalayas based on 16S rRNA and phenylalanyl-tRNA synthase (*pheS*) genes sequencing (Oki et al. 2011). Species of some lactic acid bacteria viz. *Lactococcus, Leuconostoc, Enterococcus, Pediococcus,* and *Weissella* were reported earlier from *suka ko maacha, sidra* and *sukuti* based on limited phenotypic and biochemical tests (Thapa et al. 2006).

Research Gap

Earlier reports on traditionally processed meat and fish products of Sikkim restricted only on lactic some species of lactic acid bacteria (LAB), and few non-LAB genera and species based on limited phenotypic and biochemical tests (Rai et al. 2010a; Thapa et al. 2006). However, the presence of pathogenic bacteria in these traditionally processed meat and fish products of Sikkim have not been assessed for safety measures which is the concern in food microbiology. The Rome Declaration on World Food Security of 1996 includes the right of everyone to have access to safe and nutritious food (World Food Summit 1996), and the World Summit on Food recognized the link between food safety and quality in meat products (Panea and Ripoli 2018) and in fish products (Teklemariam et al. 2015). Foodborne pathogens are causing a great number of diseases with significant effects on human health and economy (Bintsis 2017). Hence, the present Thesis emphasizes on assessment of microbiological safety of some traditionally processed meat and fish products of Sikkim.

Experimental Designs to study the Objectives

Various rapid detection methods of pathogenic bacteria in traditionally processed meats and fish using selective media are reported (Mandal et al. 2011; Priyanka et al. 2016). However, conventional method of identification of pathogenic and other spoilage bacteria are not reliable, hence application of 16S rRNA gene sequencing method using PCR products is more reliable (Clarridge III 2004; Srinivasan et al. 2015). Sanger Sequencing method or Chain-termination DNA (Sanger et al. 1977) is applied by the modified automation method to check the sequence (Heather and Chain 2016). An enzyme-linked immunosorbent assay (ELISA) has been developed for detection of various enterotoxins in foods such as staphylococcal enterotoxins (Giletto and Fyffe

1998; Bennett 2005), *Bacillus cereus* enterotoxins (Tallent et al. 2015) and *Salmonella* enterotoxins (Alahi and Mukhopadhyay 2017). Staphylococcal enterotoxins (SEs) cause food poisoning with emesis (Argudin et al. 2010). Various virulent genes of *Staphylococcus* such as SEA-SEE and SEG-SElY have been reported from different foods (Ono et al. 2015). Toxin producing *Bacillus cereus* causes two types of food poisoning: diarrhea and emesis (Ehling-Schulz et al. 2006). Non-haemolytic enterotoxin (*Nhe*) which is composed of *NheA, NheB* and *NheC* (Yu et al. 2020) is one of two three-component enterotoxins responsible for the diarrhoeal food-poisoning syndrome caused by *Bacillus cereus* (Lindbäck et al. 2004). Pathogenicity of *Salmonella* strains related to virulence genes such as as *invA* and *hilA,* which are present in the chromosomal *Salmonella* pathogenicity islands (SPIs) (Nayak et al. 2004), allow *Salmonella* to invade epithelial cells (Thung et al. 2018). Antibiotic susceptibility or sensitivity test specifies effective antibiotic dosage that will be most effective against the specific types of bacteria (Khan et al. 2019). Antibiotic resistance is defined as the genetic ability of microorganism to encode the resistance genes that inhibit the potential antibiotics for survival (Blair et al. 2015).

Application of sequence-based taxonomy or metataxonomic tool by high-throughput sequencing (Cox et al. 2017) together with updated bioinformatics tools, such as highly accurate pair-end read merger (PEAR) software (Zhang et al. 2014) and Quantitative Insights into Microbial Ecology (QIIME2) (Bolyen et al. 2019), has been increased for profiling bacterial communities in fermented foods with more accuracy (Ercolini 2013; Tamang et al. 2020). High-throughput sequencing method has been applied to reveal the colossal diversity of bacterial community mostly belonging to phylum Firmicutes in various meat products (Połka et al. 2015; Wang et al. 2018a,b), and also inferred functional roles of dominance bacteria using bioinformatics (Wilkinson et al. 2018).

Similarly, bacterial communities in some fermented and sun-dried fish products of different countries have been profiled by using high-throughput sequencing tools (Song et al. 2018; Du et al. 2019; Jiang et al. 2019). Application of PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software (Douglas et al. 2019) computational approach predicts gene functionality of cultured and uncultured bacterial communities in different ecosystems (Langille et al. 2013; De Filippis et al. 2017). Abundance of taxonomic groups in bacteria can now be easily assayed and visualized using Browser Utility for Relating micRobiome Information on Taxonomy and functiOn (BURRITO) software, an interactive multi-omics tool for visualizing taxa–function relationships in microbiome data (McNally et al. 2018).

In the present Thesis, we selected four different types of traditionally processed meat products of Sikkim viz. beef *kargyong* and pork *kargyong*, smoked beef/yak meat product *satchu,* and fermented yak meat product *khyopeh*; and three different traditionally processed fish products viz. *suka ko maacha, sidra* and *sukuti* of Sikkim. Based on above mentioned research gaps we designed the experiments and conducted our studies to fulfill the following objectives on:

- Isolation of potential bacterial pathogens.
- Their identity by culture-dependent method (phenotypic and Sanger sequencing of 16S rRNA gene sequence) and culture-independent technique (high-throughput sequence).
- Their roles in enterotoxin production, virulence gene dete axction in some genera, antibiotic susceptibility
- Their predictive gene functionality to detect metabolic pathways and probable disease profile in some traditional fermented meat and fish products of Sikkim.

OBJECTIVES OF THE THESIS:

- To determine and enumerate the microbial population present in samples.
- To isolate and identify the potential bacterial pathogens from traditionally processed meat and fish products of Sikkim by phenotypic and genotypic characterization.
- To determine the bacterial toxins by using ELISA technique.
- To determine the antibiotic susceptibility test.

REVIEW OF LITERATURE

Food products are highly susceptible to microbial contamination that may affect their quality attributes and reduce their nutritional value (Machado-Moreira et al. 2019). Bacteria, viruses, yeast and molds have the ability to grow and multiply in food which may cause food spoilage and foodborne diseases (Lorenzo et al. 2018). Among the four major groups, bacteria are considered the most important in food microbiology because of their ubiquitous presence and rapid growth rate (Ray and Bhunia 2007). Moreover, the possible presence of microbial toxins or pathogenic microorganisms such as *Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus, Campylobacter, Clostridium perfringens*, *and Aspergillus niger* may even endanger consumer safety and contribute to foodborne illness (Nummer et al. 2012). Meat and fish products are an excellent source of dietary proteins, vitamins and minerals; however, they are highly susceptible to microbial spoilage and can be an agent for transmission of array of infections and intoxications (Adam 2010; Mhango et al. 2010; Hathwar et al. 2012). Physical factors like temperature, pH, moisture and water activity (a_w) play important roles for the growth, multiplication and survival of microorganisms (Sautour et al. 2001). Moreover, the adoption of preservation techniques like low temperature, low pH and low weak acid preservative are leading towards the adaptation of foodborne microorganisms to more stressful environmental conditions thereby increasing the problems in the control of food spoilage and food poisoning (Beales 2004). To ensure the safety and palatability of meat and fish products, it is very important to understand the microbiological, technological and biochemical processes during fermentation (Ricke et al. 2007). Microbiota present in the food eco-systems convert the biochemical constituents of raw or boiled substrates during fermentation into acceptable food products with acceptable aroma, flavor, palatable texture, contributing nutritional value and health-promoting benefits (Tamang 2010b; Tamang 2020). In the Himalayan

regions of India, traditionally processed meat and fish products are preserved by drying, smoking, salting and fermentation which are common methods of food preservation (Rai et al. 2009; Thapa 2016a). There are several different types of meat and fish products in the world which are often consumed as a regular diet.

Fermented/Smoked/Dried Meat Products

Some common fermented/smoked/dried meat products of the world, raw materials used, region of origin and the culinary practices are described in Table A.

Table A. Some common fermented/smoked/dried meat products of the world

Product	Substrate/raw materials	Organoleptic characters and culinary	Country	References
Alheira	Pork/chicken/duck/veal meat, traditional heat bread, fats and spices	Dry/smoked sausage	Portugal	Ferreira et al. (2006)
Androlla	Pork meat, fats, salt and spices	Dry/smoked/ cured sausage	Spain	Fontann et al. (2007)
Arijia	Large intestine of chevon	Sausage, curry	India, Nepal	Oki et al. (2011)
Biltong	Beef meat muscles, vinegar, salt	Dry meat	South Africa	Petit et al. (2014)
Botillo	Pork meat, fats, salt and spices	Dry/smoked/ cured sausage	Spain	Cachaldora et al. (2013)
Chartayshya	Chevon	Dried/smoked meat, curry	India	Oki et al. (2011)

Charqui	Beef lean meat, salt	Dry meat	Brazil	Shimokomaki et al. (2016)
Chorizo	Pork meat, salt, additives and spices	Dry sausage	Spain	Casquete et al. (2012)
Jerky	Beef meat, brown sugar, salt	Dry meat	United States	Konieczny et al. (2007)
Jinhua ham	Whole pork leg, salt	Sun dried cured ham	China	He et al. (2008)
Kilishi	Beef meat, salt	Dry meat	Nigeria	Inusa and Said (2017)
Lap cheong	Ground pork and back fat	Semi dried	China	Wu et al. (2010a)
(Musom)	Pork meat, pork skin, rice, garlic and salt	Fermented sausage	Thailand	Santiyanont et al. (2019)
Nemchua	Lean pork, skin, garlic, cooked rice	Fermented sausage	Vietnam	Nguyen et al. (2013)
Nham	Pork meat, pork skin, rice, garlic and salt	Fermented sausage	Thailand	Santiyanont et al. (2019)
Pastirma	Meat muscles (beef/ lamb/buffalo/goat/camel) highly seasoned, not smoked.	Dry/cured meat	Turkey, Egypt, Russia	Abdallah et al. (2017); Gagaoua et al. (2018)
Soppressata	Pork meat and fat, potassium chloride and seasonings	Dry sausage	Italy	Di et al. (2016)
Salciccia	Chopped pork meat, spices and salt	Dry/semidry sausage	Italy	Vignolo et al. (2010)

Sucuk	Beef meat with fats, curing salts, nitrites and others spices	Dry sausage	Turkey	Soncu et al. (2018)
Salchichon	Pork meat, NaCl, additives and spices	Dry sausage	Spain	Casquete et al. (2012)
Skerpikjøt	Shank or legs of Sheep	Wind dried	Denmark	Sørensen et al. (2018)
Tocino, Tapa Longganisa	Pork, salt, sugar, potassium nitrate	Fermented cured pork	Philippines	Solidum et al. (2013)
Urutan	Ground pork and pork fat	Semi dried sour sausage	Indonesia	Antara et al. (2004)

Fermented meat products are produced either in the form of sausage (Adam 2010) or dried meat/ jerky (Ojha et al. 2017). In Europe, there is a greater diversity and consumption of fermented meat products like Italian *salami*, *chorizo*, *fuet* and *botillo* (Spain) and *alheira* (Portugal) (Collignan et al. 2001; Ferreira et al. 2006; Fontana et al. 2007; Quijada et al. 2018). Popular fermented meat products found in other parts of the world are *biltong* (South Africa), *kilishi* (Sahel), *charque* and *carne do sol* (Brazil), *pastrima* and *sucuk* (Turkey), Nham (Thailand) and *boucane* (Réunion) (Collignan et al. 2001; Kaban, 2013; Petit et al. 2014; Santiyanont, 2019). Mediterranean sausages like *chorizo*, *salshichon* and Italian *salami* are fermented at low temperature followed by ripening phase at longer period of time in the absence of smoking (Toldra 2004). On the other hand, Northern-European products like Hungarian and German-style salami are smoked and processed in a shorter period of time (Leistner 1992). These meat products generally consist of a mixture of minced meat and fat (pork/beef), salt with or without nitrite/nitrate, which is stuffed into a casing, fermented, dried or smoked and

consumed raw with no need for further smoking or cooking (Toldra 2004). *Skerpikjøt,* a wind-dried mutton is a delicacy of the Faroe Islands in Denmark which is prepared by hanging the shanks or legs of a sheep mixed with salt in a hjallur- a drying shed ventilated by the wind for 5-9 months, causing the meat to have different consistencies, distinct smell and taste (Sørensen et al. 2018).

Traditional fermented meat products have a long history in the regions of Asia, especially in China, South-East Asia and the Himalayan regions with typical characteristics of addition of salt/sugar as an ingredient which not only enhances the fermentation processes but also reduces water activity thereby extending the product shelf life (Chen et al. 2014). In China, the meat products are divided into two broad categories: Chinese fermented/dry-cured ham which involves green ham preparation, washing, salting, soaking, sun drying and ripening for 8-10 months (He et al. 2008) and Chinese-style fermented sausage (pork with 30:70 ratio of back fat to lean meat) which are semidry with added wine, sugar and sodium nitrite processed by sun drying for 10-14 days depending on the temperature and relative humidity (Wu et al. 2010). "Sour meat" is one of the traditional fermented foods produced in South East Asia and several species of lactic acid bacteria (LAB) contribute to fermentation imparting sour taste to meat (Chen et al. 2014). These uncooked meat products have different local names depending on the countries in which they are consumed. The Thai *nham* which is a traditionally fermented pork sausage is made up of minced lean raw pork, precooked pork skin cut into long thin straps and cooked rice, with other ingredients like salt, sugar, garlic, pepper and sodium nitrite and packed in a banana leaves for 3 days (Visessanguan et al. 2005). A similar meat product is *nem-chua* of Vietnam which consists of lean pork, shredded boiled pork rind, spices, sugar, salts and other additives, packed in banana leaves to provide anaerobic conditions and produce a special aroma

(Ho et al. 2009). The meat paste is formed into cubes upon which thin slices of chili and garlic are added for flavor and the fermentation lasts about 3-4 days at room temperature (Tran et al. 2011). *Urutan* is another fermented pork sausage of Indonesia which is made of lean pork, fats, spices, sugars and salt with or without nitrite that enhances the growth of LAB (Aryanta 2000). The mixture is stuffed into a natural casing followed by sun-dried and aging for few weeks at 10℃ to improve the flavor (Antarta et al. 2004). *Jamma* and *arijia* are traditional meat products of Uttarakhand in India prepared by mixing chopped chevon meat with pepper, salt, finger millet followed by boiling for 20 min and dried for 15-20 days (Rai et al. 2009).

The traditional fermentation of meat products depends on the native microflora whose growth is promoted by the environmental conditions of processing unit and contaminated raw materials during slaughtering (Talon et al. 2003). In a recent decade, global demand in meat products has increased drastically which is leading to target interest in traditional food for innovation (Salter 2017). Meat has a great importance in human nutrition due to high content of essential amino acids, fatty acids and vitamins as well as iron and zinc (Pereira and Vicente 2013). Fermented meats are also reported to cause a variety of pathologies associated with cardiovascular diseases, hypertensions, cancer and obesity due to high content of saturated fats and salt (Fernández-Ginés et al. 2005; Adam 2010). However, attempt is being made to develop healthier meat products with incorporation of probiotic strains to improve the quality and safety of the products (Bis-Souza et al. 2019).

Microbiology of Fermented Meat Products

Fermented foods are generally considered safe due to low water activity (aw) and pH which inhibits the growth of pathogenic bacteria that occurs during processing and

storage (Ferreira et al. 2006). However, there is a probability that some of the pathogenic organisms may be present in the final product by exhibiting an increased tolerance to heat and acidic environment during processing which is a greater challenge for several research groups (Beuchat et al. 2013). Several studies have been carried out on the microbial diversity of fermented foods focusing on the functionality of lactic acid bacteria as it contributes to the major microbiota at the end of the ripening stage of fermented food which has earned the 'generally regarded as safe' status (Talon et al. 2007; Mokoena et al. 2016). Several dominant bacteria reported from fermented meat are *Lb. sakei*, *Lb. curvatus* and *Lb. plantarum* in *androlla* of Spain (Fontán et al. 2007); *Lb. plantarum*, *Lb. brevis*, *Lb. curvatus*, *Lb. rhamnosus*, *Lb. paracasei*, *Enterococcus faecium*, *Leuc. Mesenteroides*, *Ped. pentosaceus*, *Ped. acidolactici*, *W.cibaria*, *W. viridescens* in *alheira* of Portugal (Albano et al. 2009); *Lb. platantarum*, *Lb. sakei*, *Pediococcus*, *Micrcoccus*, *S. xylosus*, *S. carnosus* in *pastirma* of Turkey (Aksu and Kaya 2005); *Lb. plantarum, Lb, sakei, Ped. pentosaceous* and *Ped. acidilactici* in *nham* of Thailand (Chokesajjawatee et al. 2009); *Lb. pentosus*, *Lb. plantarum*, *Lb. brevis*, *Lb. paracasei*, *Lb, fermentum*, *Lb acidipiscis*, *Lb. namurensis*, *Lc lactis, Leuc. citreum, Leuc. Fallax, Ped. acidilactici, Ped. stilessi, W. cibaria* and *W. paramesenteroides* in *nem-chua* of Vietnam (Nguyen et al. 2013); *Lb. sakei*, *Lb. plantarum*, *Lb. curvatus*, *Ped. pentosaceus, Leuc. carnosum* and *Leuc. gelidum* in *soppressata* of Italy (Parente et al. 2001). The microbial diversity of various meat products of India were found quite similar to the microbiota of other meat products around the world such as *Lb. sakei*, *Lb. divergens, Lb. carnis, Lb. curvatus, Leuc. mesenteroides, Enterococcus faecium, Micrococcoccus, Bacillus subtilis, Staphylococcus,* and yeasts *Debaromyces hansenii* and *Pichia anomala* in *kargyong* (Rai et al. 2010); *E. faecalis*, *E. faecium*, *E. hirae*, *Leuc. citreum*, *Leuc. mesenteroides*, *Ped. pentosaceus* and *W. cibaria* in *arijia* (Oki et al.

2011); *Lb. carnis*, *L. plantarum*, *B. subtilis*, *B. thuringiensis*, *Staphylococcus*, *Micrococcus, Debaromyces hansenii* and *P. burtonii* in *suka ko masu* (Rai et al. 2010). Application of sequence-based taxonomy or metataxonomic tool by high-throughput sequencing (Cox et al. 2017) together with updated bioinformatics tools, such as highly accurate pair-end read merger (PEAR) software (Zhang et al. 2013) and Quantitative Insights Into Microbial Ecology (QIIME2) (Bolyen et al. 2019), has been increased for profiling bacterial communities in fermented foods with more accuracy (Ercolini 2013; Tamang et al. 2020). High-throughput sequencing method has been applied to reveal the colossal diversity of bacterial community mostly belonging to phylum Firmicutes in various meat products (Połka et al. 2015; Wang et al. 2018a) and also inferred functional roles of dominance bacteria using bioinformatics (Wilkinson et al. 2018). Bacterial community structures in fermented sausages and meats of other countries have been extensively studied (Fontana et al. 2016; De Filippis et al. 2017; Ferrocino et al. 2018; Wang et al. 2018a,b; Cauchie et al. 2020; Mu et al. 2020; Tamang et al. 2020).

Fermented/Smoked/Dried Fish Products

Some common fermented/smoked/dried fish products of the world, raw materials used, region of origin and the culinary practices are described in Table B.

Table B. Some common fermented/smoked/dried fish products of the world

Product	Substrate/raw materials	Organoleptic characters and culinary	Country	Reference
Bakasang	Fish, shrimp	Paste, condiment	Indonesia	Huda (2012)
Belacan	Shrimp, salt	Paste, condiment	Malaysia	Huda (2012)
Bordia	Fish (*Gudushia chapra, Pseudeutropius atherinoides, Cirrhinus reba*), salt	Dried, salted, side dish	India	Thapa et al. (2007)
Budu	Marine fishes, salt, sugar	Muslim sauce, fish sauce	Thailand, Malaysia	Kanjan and Sakpetch (2020)
Burong Bangus	Milkfish, rice, salt, vinegar	Fermented milk fish, sauce	Phillipines	Arcales et al. (2018)
Burong isda	Fish, rice, salt	Fermented fish, sauce	Phillipines	Olympia et al (1995)
Gnuchi	Fish (*Schizothorax richardsonii*), salt, turmeric powder	Smoked and dried fish, eat as curry	India	Thapa (2016a)
Gulbi	*Pseudosciaena manchur- ica* and salt	Dried yellow corvina	Korea	Gwak and Eun (2010)
Hentak	Finger sized fish (*Esomus danricus*)	Fermented fish	India	Thapa et al. (2004)
Hout-Kasef	Fish, salt	Salt fermented fish	Saudi Arabia	Gassem (2019)

Ika-Shiokara	Squid, salt	Fermented squid	Japan	Kitamura et al. (2016)
Jeotkal	Fish	High-salt fermented, staple	Korea	Cho et al. (2002)
Karati	Fish (*Gudushia chapra, Pseudeutropius atherinoides, Cirrhinus reba*), salt	Dried, salted, side dish	India	Thapa et al. (2007)
Kusaya	Horse Mackeral, salt	Fermented dried fish	Japan	Kanno et al. (2012); Fujii et al. (2016)
Lanhouin	Fish (*Pseudotolithus* sp.), salt	Fermented cassava fish, condiment	Benin	Anihouvi et al. (2007)
Lashim	Fish (*Gudushia chapra, Pseudeutropius atherinoides, Cirrhinus reba*), salt	Dried, salted, side dish	India	Thapa et al. (2007)
Myulchijeot	Small sardine, salt	Fermented sardine	Korea	Kuda (2015)
Nam pla	*Solephorus* sp., *Ristelliger* sp. *Cirrhinus* sp., water, salt	Fish sauce	Thailand	Garnjanagoon chorn (2016)
Ngari	Fish (*Puntius sophore*), salt	Fermented fish	India	Thapa et al. (2004)
Ngan pyan yea	Fish, shrimp	Fermented fish sauce	Myanmar	Kobayashi et al. (2016)
Nuoc mam	Marine fish, salt	Fermented fish	Vietnam	Dang et al. (2017)

Pla-som (Pla-khao-sug)	Marine fish, salt, boiled rice, garlic	Fermented fish, condiment	Thailand	Sanpa et al. (2019)
Ræstur fiskur (Turrur)	Marine fish	Air dried fermented	Denmark	Sørensen et al. (2018)
Saba-narezushi,	Horse Mackerel, salt	Fermented dried fish	Japan	Kanno et al. (2012); Fujii et al. (2016)
Surströmming	Fish	Fermented herrings	Sweden	Nygaard (2019)
Tungtap	Fish, salt	Sun-dried	Meghalaya	Thapa et al. (2004)
Yegyo ngapi	Small/medium sized fish, salt	Dried fish	Myanmar	Kobayashi et al. (2016)

Fermented fish products are known for its medicinal value and rich in bio-nutrients, minerals and act as appetizer (Zang et al. 2019). Fermented fish products are generally categorized into two groups viz. natural fermentation and fermentation by using starter culture(s) (Devi et al. 2015). Spontaneous fermentation applies back-slopping method where a small quantity of previously performed successful fermentation was retained and added to the new batch resulting in the introduction of a substantial inoculum (Leroy and De Vuyst 2004). Fermentations using starter culture method applies the addition of functional cultures directly to the raw materials for the standard quality characteristics (Yongsawatdigul et al. 2007). In Asia, traditionally processed fish products are country-specific with various vernacular names for products such as *jeot kal* in Korea, *shottsuru* and *shiokara* in Japan, *yucha* in China, *patis* in Philippines, *nam pla* and *pla ra* in Thailand and are commonly prepared in the form of sauce in South

Asia except in India, Bangladesh, Nepal and Bhutan (Devi et al. 2015; Koo et al. 2016; Zang et al. 2019; Tamang et al. 2016; 2020). Fermentation of fish collected from sea as well as rivers are also commonly practiced in Africa (Anihouvi et al. 2012) but less common in European countries (Skåra 2015). Fish sauce called garum were ubiquitous condiments for the Romans, who had adopted them from the Greeks (Adams 1998). Norwegian *gravlaks*, or buried salmon, is a traditional, relatively mild tasting product; more heavily fermented products, *rakefisk* or *surfisk*, the most popular varieties of which are rakörret, fermented trout, in Norway and *surströmming*, made from herring in Sweden (Riddervold 1990; Kobayashi et al. 2000). Preparation of fish sauce takes many months of fermentation processes; hence the quality of raw materials is of great importance (Salampessy et al. 2010). There are different ways of production of fish and seafood sauces. *Bakasang* is prepared by using fish guts which are cut into smaller piece and mixed with salt in the ratio 3 parts and 5 parts, kept at nearby fire place for fermentation up to 3-6 weeks resulting into the formation of thick, salty, brown liquid with a characteristic aroma and flavor (Ijong and Ohta 1996). The Vietnamese fish sauce *nuoc-mam* is prepared by mixing fish with salt (3:1) in earthen jars and pressed by hand layer by layer, closely tight and buried in the ground for 6-18 months to allow for fermentation (Beddows 1985). The Thai fish sauce *nampla* is prepared by mixing the fish with salt and transferred into the fermentation tank like wooden barrels and earthen jars, placed in between two bamboo mats loaded with heavy weight to keep the fish in the brine produced from osmotic dehydration of fish which is fermented for up to 18 months (Wongkhalaung 2004). *Jeotkal*, a Korean fermented fish sauce are fermented at low temperatures (20ºC) for 2-3 months which is mainly used as an ingredient in *kimchi* preparation or as condiment (Lee 1993). The Japanese *narezushi* is prepared by mixing cooked rice, fish and salt which is used as condiment and if the

whole or partial part of fish is preserved with salt, it is called *ika shiokara* which is most fermented seafood in Japan (Fuji et al. 2016; Kitamora et al. 2016). Fish sauce production is an anaerobic process and high concentration of salt controls the pathogenic organism and favor the halotolerant and halophilic bacteria (Lopetcharat et al. 2001).

Indian fish products are slightly different from other parts of the world. The ethnic people of Bhutan, Nepal and North east India catch the available fish from rivers, streams and lakes which are either consumed fresh or traditionally preserved using indigenous knowledge of fermentation/drying/smoking without using any chemicals (Thapa 2016). Many villagers are economically dependent on these products by selling them in a market area which is consumed as a daily diet by the local people. The various types of fish products found in India are *karati, bordia* and *lashim* (sundried and salted), *shidal* of Tripura, *ngari* and *hentak* (fermented) of Manipur (Thapa 2016). Fermentation of fish is restricted to *ngari*, *hentak* in Manipur, *tungtap* in Meghalaya and *sidal* in Tripura whereas *karati, lashim* and *bordia* of Assam are either dried or smoked (Thapa et al. 2004, 2006). Environment is the main source of both beneficial and pathogenic microorganisms in traditional fish fermentation (Lee et al. 2014; Zhang et al. 2016), which metabolize the complex organic compounds into simpler molecules for the formation of distinctive flavour, texture, and taste in fermented/smoked/dried fish products (Wang et al. 2017; Bao et al. 2018).

Microbiology of Fermented Fish Products

Several species of bacteria and yeasts have been reported from fermented and traditionally preserved fish products of the world viz. *Haloanaerobium alcaliphilum in surströmming* of Sweden (Kobayashi et al. 2000); *Pseudomonas*, *Enterobacter*,

Moraxella, *Micrococcus*, *Streptococcus, Lactobacillus*, *Pseudomonas*, *Moraxella*, *Staphylococcus* and *Pediococcus* spp. in *bakasang* of Indonesia (Ijong and Ohta, 1996); *Corynebacterium kusaya, Spirillum* sp.*, C. bifermentans, Penicillium* sp. in *kusaya* of Japan (Satomi et al. 1997); Species of *Micrococcus, Pediococcus, Staphylococcus, Streptococcus*, *Sarcina, Bacillus, Lactobacillus, Corynebacterium, Pseudomonas* and some novel species such as *Lentibacillus salicamp, Lentibacillus juripiscarius, Bacillus piscicola, Halococcus thailandensis, Tetragenococcus halophilus and Tetragenococcus muriaticus* in *nam pla* of Thailand (Daroonpunt et al. 2016); *Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Strep. faecalis, Micrococcus* sp. in *burong bangus* of Philippines (Olympia et al. 1992); *Ped. cerevisiae, Staphylococcus* sp.*, Bacillus* sp. and *Micrococcus* sp. in *Myulchijeot* of Korea (Kuda 2015). Various dominant microorganism present in traditionally processed fish products of India are *Lb. plantarum, Lc. lactis, Leuc. Mesenteroides, Ent. faecium, Ent. faecalis, Ped. pentosaceus, Cand. chiropterorum, Cand. bombicola, Saccharomycopsis* sp. in *gnuchi* (Thapa et al. 2006); *Lb. plantarum, Lb. fructosus, Lb. coryniformis, Lb. amylophilus, Lc. lactis, Ent. faecium, B. subtilis, B. pumilus, Micrococcus* sp.*, Candida* sp.*, Saccharomycopsis* sp. in *hentak* (Thapa et al. 2004); *Lc. lactis, Leuc.mesenteroides, Lb. plantarum, B. subtilis, B. pumilus, Candida* sp. in *karati, bordia* and *lashim* (Thapa et al. 2007); *Lb. plantarum, Lb. amylophilus, Lb. pobuzihii*, *Lb. fructosus, Lb. coryniformis, Lc. lactis, Ent. faecium, B. subtilis, B. pumilus, Bindicu, Micrococcus* sp.*, Staphy. cohnii* subsp. *cohnii*, *Staphy. carnosus, Tetragenococcus halophilus* subsp. *flandriensis*, *Clostridium irregular, Azorhizobium caulinodans, Candida* sp. and *Saccharomycopsis* sp. in *ngari* (Thapa et al. 2004; Devi et al. 2015); *Lc. lactis* subsp. *cremoris, Lc. Plantarum, Lb. fructosus, Lb. amylophilus, Lb. corynifomis* subsp. *torquens, Lb. plantarum*, *Lb. puhozi, Ent. faecium, B. subtilis*, *B. pumilus*, *Micrococcus,*

yeasts-species of *Candida, Saccharomycopsis* in *tungtap* (Thapa et al. 2004; Rapsang et al. 2011); *Lc. lactis, Lb. plantarum, Leuc. mesenteroides, Ent. faecium, Ent. facalis, Ped. pentosaceus, W. confusa, Cand. chiropterorum, Cand. bombicola, Saccharomycopsis* sp. in *sidra*; *Lact. lactis, Lb. plantarum, Leuc. mesenteroides, Ent.faecium, Ent.faecalis, Ped.pentosaceus, Cand. chiropterorum, Cand. bombicola, Saccharomycopsis* sp. in *suka ko maacha* and *Lc. lactis, Lb. plantarum, Leuc. mesenteroides, Ent. faecium, Ent. faecalis, Ped. pentosaceus, Cand. chiropterorum, Cand. bombicola,* and *Saccharomycopsis* sp. in *sukuti* (Thapa et al. 2006). Several researchers have recently studied the microbial community structures in fermented fish products of many countries using high-throughput sequencing tools (Roh et al. 2010; Marui et al. 2015; Zhao et al. 2016; Song et al. 2018; Zang et al. 2018; Du et al. 2019; Jiang et al. 2019; Zhao and Eun 2020; Tamang et al. 2020).

Safety of Traditional Meat and Fish Products

Food borne pathogens are growing concern for human illness and death in developing countries (Fratamico et al. 2005; Alahi et al. 2017). The occurrence of pathogens in meat and fish products such as Shiga toxin–producing Enterohemorrhagic *Escherichia coli, Staphylococcus aureus, Salmonella, Campylobacter* spp., *Vibrio cholerae* and *Listeria monocytogenes* have been reported by many researchers worldwide (Busani et al. 2005; Bohaychuk et al. 2006). The demand for ensuring safe food supply is increasing. Food borne diseases due to microbial pathogens, biotoxins, and chemical contaminants represent serious threats in human's health with 1 in 10 people fall ill after eating contaminated food and 420000 die every year, resulting in the loss of 33 million healthy life years (DALYs) (Tirado et al. 2010; WHO 2020). Contaminated raw meat is one of the main sources of food-borne illnesses (Bhandare et al. 2007). The risk

of the transmission of zoonotic infections is also associated with contaminated meat (Podpecan et al. 2007). Another source of pathogens like *Vibrio, Salmonella, Shigella, Clostridium, Listeria* and Hepatitis A are from seafood (Feldhusen 2000). Fish and shellfish may also be a vehicle for pathogenic bacteria naturally occurring in aquatic environments, referred to as indigenous or derived from polluted waters and/or from post-capture contamination (Herrera et al. 2006). Outbreaks of foodborne disease are attributed to the emergence of new pathogens and reemergence of some old pathogens (Bhunia 2018). *Staphylococcus aureus* produces a wide range of staphylococcal enterotoxins (SEs) which are a major cause of food poisoning that typically occurs after ingestion of contaminated food particularly meat and fish products (Blaiotta et al. 2004; Salem et al. 2015). Among all the different types of enterotoxins, SEA is the most common cause of staphylococcal food poisoning around the world (Argudin et al. 2010). Although *S. aureus* can tolerate salt and nitrite, the risk of formation of enterotoxin is low at low pH (<5.3), low temperature (<25℃) and under anaerobic conditions (Lucke 1998). Traditional detection methods of virulence genes are simple but need bacteria enrichment and longer detection time (Baniardalan, et al. 2017). Recently, PCR techniques have been developed and applied to detect foodborne pathogens (Zhao et al. 2020). Enterotoxigenic *S. aureus* has been isolated from various meat products like dry fermented sausage of Italy (Quaglia 2019); *nham* of Thailand (Chokesajjawatee et al. 2009); *Chouriço* sausages of Portugal (Silva et al. 2018). Among the fish products, enterotoxigenic *S. aureus* was isolated from dried fish of Korea (Moon et al. 2017); in fish products of Iran (Arfatahery et al. 2016); *ngari* of India (Keisam et al. 2019). *B. cereus* causes self-limiting (24-48 h) food-poisoning syndrome viz. diarrheal type and emetic type but may also cause non-gastrointestinal disease like endocarditis and endophthalmitis (Logan and Rodrigez-Diaz 2006). The

accurate number of food poisonings caused by *B. cereus* in different countries is not known because it is not a reportable illness and is not always diagnosed (Kotiranta et al. 2000). *Salmonella enterica* is commonly acquired from contaminated food and is an important cause of illness worldwide (Hendriksen et al. 2011). *Vibrio parahaemolyticus* is a human pathogen that is widely distributed in the marine environments. This organism is frequently isolated from a various shellfish (Su and Liu 2007). *Clostridium perfringens* is another common cause of food borne illness because of its enterotoxin producing ability, short generation time, spore-forming ability, and resistance towards elevated temperature with meat and fish as common vehicle for outbreak (El-Shorbagy et al. 2012; Labbe and Juneja 2017). *Klebsiella pneumoniae* is a colonizer of livestock which causes extra-intestinal infections in humans and with the extensive use of antibiotics in food-animal production, antibiotic-resistant strains of *K. pneumonia* are becoming more prevalent among hospital as well as community acquired infections (Davis and Price 2016). Biogenic amines are commonly found in fermented foods as the suitable environmental conditions takes place during fermentations and favors the activity of microorganisms bearing decarboxylase enzymes resulting into accumulation of biogenic amines (Sarkadi 2017). The main factors that determines the decarboxylase enzyme activity are composition of raw meat, size of sausage, water content, additives used, fermentation time and storage conditions which strongly influences the microbial growth and interaction among microbial communities as well as acidification and proteolysis (Latorre-Moratalla et al. 2012). Histamine has been detected in high amount in fermented fish and tyramine has been commonly found in fermented meat sausage (De Mey et al. 2014; Ordóñez et al. 2016). High level of biogenic amines causes diseases with food poisoning symptoms such as stimulating the nerves and blood vessels in human's and also known as possible

precursor of carcinogenic nitrosamines (Biji et al. 2016). Introduction of competitive LAB starter strains in meat and fish products is important method to retard the formation of biogenic amines by amine-producing bacteria which leads to health-related benefits (Ammor and Mayo 2007). Many different methods to reduce biogenic amines content in fermented meat and fish are under way and one of the novel approaches is the addition of wine to fermented food which showed positive effects by reducing the level of biogenic amines (Coloretti et al. 2014).

Starter Cultures

Starters are often used in the fermented food to improve the quality, manufacturing and reproducibility in addition to the spontaneous ecosystem and selection of starter strains are based on different technological properties (Laranjo et al. 2017, 2019). This includes production of lactic acid or alcohol to carry out the main fermentation; galactosidases impacting sensorial properties in metabolic or enzymatic functionalities; production of antimicrobial compounds like bacteriocin, enterocin and degradation of anti-nutritive factors (Tamang et al. 2016b: Bintsis 2018; Waché et al. 2018). Lactic acid bacteria and *Micrococcus* or *Kocuria* belonging to the coagulase negative group are referred as technological microbiota as they are involved in the development of hygienic and sensory qualities of the final product (Talon et al 2004; Aquilanti et al. 2016; Rezac et al. 2018). The distinct color and flavor of fermented meat products are contributed by *Staphylococcus* and *Kocuria* which degrade free amino acids and inhibit the oxidation of unsaturated free fatty acids whereas lactic acid bacteria are mainly involved through their acidification corresponding to decreased pH (Talon and Leroy 2006; Fontana et al. 2016; Kumar et al. 2017). *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus lactis, Pediococcus pentosaceus*,

Lactobacillus casei, and *Pediococcus acidilactic* are the most common LAB strains in starter cultures which prevails in the sausage during fermentation and control the growth of undesired microbiota (Työppönen et al. 2003; Ammor and Mayo 2007; Pasini et al. 2018). Pediococci are less frequently isolated from European sausage but they are more commonly found in fermented sausage of United States and are used as a starter cultures to facilitate the acidification of meat batter (Kołożyn and Dolatowski 2009; Nie et al. 2014). A probiotic food has received market interest as health-promoting, functional foods and is introduced in to a large scale of food industries (De Vuyst et al. 2008; Zhao et al. 2019). Dry fermented meat products are generally not heated or mildly heated which is sufficient for the transfer of probiotic bacteria into human gastrointestinal tract (Kołożyn and Dolatowski 2012). Many researches have been conducted worldwide on the functional properties of lactic acid bacteria isolated from various traditional meat products. *Lb. plantarum, Lb. sakei* and *Lb. curvatus* isolated from Greek dry-fermented sausage was shown to enhance its hygienic quality by inhibiting *Listeria monocytogenes* and *Staphylococcus aureus* (Papamanoli et al. 2003; Comi et al. 2020). *Lb. acidophilus, Lb. paracasei* and *Bifidobacterium lactis* isolated from Hungarian salami showed antimicrobial activity against *E. coli* O111 and *Listeria monocytogenes* (Pidcock et al. 2002). *Lb. plantarum* and *Lb. pentosus* strains isolated from Scandinavian type fermented sausage were able to survive in an environment similar to human GIT, inhibited potential pathogenic bacteria in vitro and considered safe to be used with regard to their antibiotic resistance pattern (Klinberg et al. 2005). *Lb. fermentum, Lb. reuteri* and *Pediococcus acidilactici* strains isolated from Iberian dry fermented sausage showed promising probiotic meat starter culture suitable for its production. *P. pentosaceus* isolated from *nham* fermented sausage of Thailand exhibited anti-Listeria activity by producing bacteriocin and a useful functional starter

culture (Kingcha et al. 2012). It has been reported that meat and meat products are considered to cause a variety of pathologies due to higher content of saturated fatty acids, cholesterol and their association with cardiovascular disease, obesity and cancer (Boada et al. 2016). However, the trends towards functional food has led to the production of one or more functional ingredients in meat products like addition of vegetable/fish oils, fibers, vitamins, antioxidants, modification of fatty acids and cholesterol level, control of sodium chloride which is changing its image in these health conscious days (Fernández-Ginés et al. 2005).

LAB isolated from fermented fish has been reported to improve the organoleptic qualities, aroma and suppress the growth of spoilage microflora (Yin et al. 2002). Fermented fish produces many biologically active substances mainly derived from the extensive degradation of protein that have been linked with several beneficial biological properties (Je et al. 2005; Elegado et al. 2016;). *Lb. plantarum* isolated from *suan-yu*; *Lb. paracasei* isolated from *budu* and *Lac*. *lactis* isolated from *hukuti maas* have been found to produce bacteriocins with wide antimicrobial activity against many food-borne pathogens (Pringsulaka et al. 2012; Abbasiliasi et al. 2014; Kumar et al. 2017). The strains of *Virgibacillus* and coagulase negative *Staphylococcus* isolated from fish sauce has the ability to produce proteinases that increased the desirable volatile compounds (Yongsawatdigul et al. 2007). *Tetragenococcus halophilus* has an important role in improving the flavor characteristic of fish sauce as reported by Thongsanit et al. (2002). *Weissella* strains isolated from Thai fermented fish (*Plaa som*) were able to produce antibacterial compounds and folate which might be promoted for their functional properties (Deatraksa et al. 2018). *Leuconostoc mesenteroides* isolated from *saba-narezushi* was found to have antioxidant properties which promotes nitric oxide (NO) generation on murine macrophage, making it an ideal probiotic to improve

inflammatory bowel disease (Kuda et al. 2014). Recently, the concept of protective cultures for bio-preservation of perishable meat and fish products (Holzapfel et al. 1995; Benabbou et al. 2018; Said et al. 2019; Goel et al. 2020) and probiotics cultures for health benefits (Tamang 2015; Rezac et al. 2018) have been applied in the production of some fermented meat products and fish products (Laranjo et al. 2019; Aspri et al. 2020; Tamang et al. 2020).

MATERIALS AND METHODS

Media Used

1.	Petrifilm Aerobic Count Plates	(6400, 3M, USA)
2.	Baird Parker Agar Base	(M043, HiMedia, Mumbai)
3.	Nutrient Agar	(M012, HiMedia, Mumbai)
4.	Nutrient Broth	(M002, HiMedia, Mumbai)
5.	Plate Count Agar	(M091, HiMedia, Mumbai)
6.	Bacillus Cereus Agar	(M833, HiMedia, Mumbai)
7.	Xylose-Lysine Deoxycholate Agar	(M031, HiMedia, Mumbai)
8.	Eosin Methylene Blue Agar	(M317, HiMedia, Mumbai)
9.	Violet Red Bile Glucose Agar	(M581, HiMedia, Mumbai)
10.	Brilliant Green Bile Broth	(M1211,HiMedia,Mumbai)
11.	Durham Tubes	(GW163,HiMedia, Mumbai)
12.	SIM Medium	(M181, KiMedia,Mumbai)
13.	MacConkey agar	(M008S, HiMedia, Mumbai)
14.	Bile Esculin Azide Agar	(M4931, HiMedia, Mumbai)
15.	DNase Test Agar Base w/Methyl Green	(M1419, HiMedia, Mumbai)
16.	Alkaline Peptone Water	(M618, HiMedia, Mumbai)
17.	Thiosulfate-Citrate-Bile Salt Sucrose Agar	(M189, HiMedia, Mumbai)
18.	Simmons Citrate Agar	(M099, HiM edia, Mumbai)
19.	MR-VP Medium (Glucose-Phosphate Broth)	(M070, HiMedia, Mumbai)
20.	Coagulase Plasma	(FD248, HiMedia, Mumbai)
21.	Tryptone Broth	(M463, HiMedia, Mumbai)
22.	Brain Heart Infusion Broth	(M210, HiMedia, Mumbai)
23.	Rappaport-Vassiliadis medium	(M880, HiMedia, Mumbai)
24.	Mueller Hinton Agar	(M173, HiMedia, Mumbai)

25. McFarland Standard Set (R092, HiMedia, Mumbai)

26. Peptone Diluent (0.5%) (pH 7.0) (Feng et al. 2002)

a. Peptone 0.5 g

b. Distilled water 1000 ml

27. Physiological Saline (0.85%) (Feng et al. 2002)

a. Sodium Chloride 0.85 g

b. Distilled water 1000 ml

28. Butterfield's Phosphate-Buffered Water (pH 7.2) (Feng et al. 2002)

a. Potassium Dihydrogen Phosphate 34 g

b. b. Distilled water 500 ml

29 Lactose Broth (Feng et al. 2002)

a. Beef extract 3 g

b. Peptone 5 g

c. Lactose 5 g

d. Distilled water 1000 ml

30. Gelatin Hydrolysis Test (dela Cruz and Torres 2012)

a. Gelatin 120 g

b. Peptone 5 g

c. Beef extract 3 g

d. Distilled water 1000 ml

31. Urease reaction test (Brink 2010)

Christensen's Urea Agar

a. Urea 20 g

b. Sodium chloride 5 g

c. Monopotassium phosphate 2 g

d. Peptone 1 g

e. Dextrose 1 g

f. Phenol red 0.12 g

g. Agar 15 g

32. Nitrate reduction test (Buxton 2011)

a. Peptone 5.0 g

b. Beef extract 3.0 g

c. Potassium nitrate 1 g

d. Distilled water 1000 ml

e. pH 7

f. Zinc Powder 0.3 g

33. Carbohydrate fermentation test (Reiner 2012)

a. Trypticase or proteose peptone 10

b. Sodium chloride (NaCl) 5 g

c. Beef extract (optional) 1 g

d. Phenol red 0.18 g

(7.2 ml of 0.25% phenol red solution)

e. Distilled and deionized water 1000 ml

f. Carbohydrate 10 g

34. Sugars

a. Arabinose (RM 045, HiMedia, Mumbai)

b. Sorbitol (GRM109, HiMedia, Mumbai)

c. Mannose (RM104, HiMedia, Mumbai)

d. Adonitol (RM096, HiMedia, Mumbai)

e. Ribose (GRM 197, HiMedia, Mumbai)

f. Maltose (RM018, HiMedia, Mumbai)

g. Dextrose (glucose) (RM 077, HiMedia, Mumbai)

h. Lactose (RM 565, HiMedia, Mumbai)

i. Mannitol (PT0604, HiMedia, Mumbai)

j. Raffinose (RM 107, HiMedia, Mumbai)

k. Rhamnose (RM 062, HiMedia, Mumbai)

l. Sucrose (RM201, HiMedia, Mumbai)

m. Trehalose (RM 110, HiMedia, Mumbai)

n. Xylose (RM 111, HiMedia, Mumbai)

REAGENTS USED

1. 3% Hydrogen Peroxide Solution (88597, Merck, New Jersey)

2. Nitrate Reduction Test Reagent (M439S, HiMedia, Mumbai)

3.	Kovacs' Indole Reagent	(R008, HiMedia, Mumbai)
4.	Methyl Red Indicator	(1007, HiMedia, Mumbai)
5.	Barrit Reagent A	
	a. α- Naphthol	5 g
	b. Alcohol (absolute)	100 ml
6.	Barrit Reagent B	
	a. Potassium Hydroxide	40 g
	b. Distilled water	100 ml
7.	Nitrate Reagent A	
	a. Sulphanilic acid	0.8 g
	b. 5 N Acetic acid	100 ml
8.	Nitrate Reagent B	
	a. α - Napthylamine	0.6 g
	b. N Acetic acid	100 ml
9.	Gram's Crystal Violet	(S012, HiMedia, Mumbai)
10.	Iodine solution	(M425, HiMedia, Mumbai)
	a. Iodine	1.0 g
	b. Potassium iodide	2.0 g

c. Distilled water	300 ml
11. Safranin	(RM1315, HiMedia, Mumbai)
a. Safranin	2.5 g
b. 95% ethanol	100 ml
12. Malachite green (5% solution)	(S020, HiMedia, Mumbai)
a. Malachite green 5.0 g	5.0 g
b. Distilled water	100 ml
13. Ethanol	(MB106, HiMedia, Mumbai)
14. Ethidium Bromide	(RM813, HiMedia, Mumbai)
15. 1×TAE buffer	(ML016, HiMedia, Mumbai)
16. Agarose	(V3125, Promega, US)
17. Gel loading dye	(G1881, Promega, US)
18. Nuclease free Water	(P1193, Promega, USA)
19. Sodium Hydroxide Solution	(MF8D, Merck Millipore, US)
20. Gotaq green Master Mix	(M7122, Promega, US)
21. Proteinase K	(V3021, Promega, US)
22. RNAse	(A7973, Promega, US)
23. DNSA	(GRM1582, HiMedia, Mumbai)

24. Sodium acetate (S2889, Merck, US)

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

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

27 Sodium n-Dodecyl Sulfate (20% Solution w/v) (428018, Merck, US)

28. Polyethylene Glycol (MW400) (GRM3662, HiMedia, Mumbai)

29. Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (P3803, Merck, USA)

30. PCR Primers (ILS, Delhi)

31. **KITS USED**

a. NucleoSpin Food DNA Extraction Kit (NC0118566, MACHEREY-NAGEL, Germany)

b. Ridascreen Staphylococcus Enterotoxin Assay Kit (SET Total) (R4105, r-biopharm, Germany)

c. Tecra *Bacillus* Diarrhoeal Enterotoxin Assay Kit (BDEVIA48, 3M, USA)

d. Tecra *Salmonella* Visual Immunoassay (SALVIA96, 3M, USA)

32 **Antibiotics used**

a. Amoxicillin/Clavulanate (30µg) (SD063, HiMedia, Mumbai)

b. Ceftazidime (30µg) (SD062, HiMedia, Mumbai)

c. Cefuroxime (30µg) (SD061, HiMedia, Mumbai)

d. Cefepime (30µg) (SD219, HiMedia, Mumbai)

e. Nalidixic acid (30µg) (SD021, HiMedia, Mumbai)

f. Tobramycin (10µg) (SD044, HiMedia, Mumbai)

g. Ciprofloxacin (5µg) (SD060, HiMedia, Mumbai)

h. Erythromycin (15µg) (SD013, HiMedia, Mumbai)

‘ i. Chloramphenicol (30µg) (SD006, HiMedia, Mumbai)

j. Penicillin G (10µg) (SD028, HiMedia, Mumbai)

k. Ampicillin (10µg) (SD002, HiMedia, Mumbai)

l. Streptomycin (10µg) (SD031, HiMedia, Mumbai)

m. Tetracycline (30µg) (SD133, HiMedia, Mumbai)

n. Gentamicin (10µg) (SD016, HiMedia, Mumbai)

o. Vancomycin (30µg) (SD045, HiMedia, Mumbai)

p. Oxacillin (1µg) (SD088, HiMedia, Mumbai)

q. Rifampicin (5 µg) (SD030, HiMedia, Mumbai)

r. Cefoxitin (30µg) (SD041, HiMedia, Mumbai)

s. Aztreonam (30µg) (SD212, HiMedia, Mumbai)

t. Clindamycin (2µg) (SD164, HiMedia, Mumbai)

u. Co-trimoxazole (25 µg) (SD010, HiMedia, Mumbai)

v. Norfloxacin (10µg) (SD057, HiMedia, Mumbai)

w. Nitrofurantion (200 µg) (SD090, HiMedia, Mumbai)

x. Cefotaxime/Clavulanic acid (30µg) (SD157, HiMedia, Mumbai)

y. Trimethoprim (5µg) (SD039, HiMedia, Mumbai)

z. Ceftriazone (30µg) (SD065, HiMedia, Mumbai)

REFERENCE STRAINS

1. *Salmonella enterica* ser. typhimurium (MTCC 3223) (Microbial Type Culture Collection, CSIR-Institute of Microbial Technology, Chandigarh, India)
2. *Bacillus cereus* (MTCC 1272)
3. *Staphylococcus aureus* (MTCC 740)

INSTRUMENT USED

a. Biological Incubator (Accumax, CIS-24BL, Kolkata)

b. Stomacher (Seward, United Kingdom)

c. Water Distillation unit (Riviera, 72240020, Kolkata)

d. Water Bath (RIME-1322, Remi, Mumbai)

e. Laminar Air Flow (1386, Thermo Scientific, USA)

f. UV-Transilluminator (MD-25/HD-25, Wealtec, USA)

g. Bio Spectrometer (Eppendorf, Germany)

h. Gel Doc Imaging System	(Bio Rad, USA)
i. Freezer (-80^{0}C)	(TSE240A, Thermo fisher, USA)
j. Freezer (-20^{0}C)	(ROFV-170, Remi, Mumbai)
k. Thermal Cyclers	(2720, Applied Biosystems, USA)
l. Electrophoresis Unit	(Bio Rad, USA)
m. Orbital Shaker Incubator	(RSB-12, Remi)
n. Analytical Weighing Balance	(AX 204, Mettler, Kolkata)
o. Microwave Oven	(28L, Samsung, Mumbai)
p. Hot Air Oven	(Instrumentation India, Kolkata)
q. Digital pH meter	(A321, Thermo Scientific, USA)
r. Centrifuge	(CL21, Thermo Scientific, USA)
s. Autoclave	(Instrumentation India, Kolkata)
t. Compound Microscope	(EX1000, Olympus, Japan)
u. Phase contrast microscope	(CKX41, Olympus, Japan
v. Moisture Analyser	(MB120, Ohaus, USA)
w. ABI-DNA- Sequencer	(ABI 35600, HITACHI, Japan)
x. NGC Illumina-Miseq	(Illumina Platform, USA)
y. Nano-DropND-1000	(Nano Drop Technologies, USA)

z. Qubit Fluorimeter (Invitrogen, USA)

a. iMark Microplate Reader (Bio Rad, USA)

SOFTWARE USED

1. Sequence Scanner (Applied Biosystems-V1.0, USA)
2. ChromasPro (Technelysium-V1.34, Australia)
3. MEGA 7 (Pennsylvania State University Vl.7.0.26, USA)
4. PAST (Palaeontological Association-V4.0, Norway)
5. QIIME (University of Colorado- V2-2019.10, USA)
6. PEAR (Dalhousie University, V0.9.5, Canada)
7. PICRUST (Dalhousie University, V2-2019,Canada)
8. GRAPHPAD PRISM (GraphPad software, Inc-V8.0.2, USA)
9. Igraph R packages (GNU GPL-V2.0, USA)
10. STAMP (GNU GPL-V 2.1.3, USA)

METHODOLOGY

SURVEY

A field survey was conducted in different villages and local markets of four districts of Sikkim state in India viz. East Sikkim (Gangtok, Rangpo, Singtam and Rhenock), North Sikkim (Lachung), South Sikkim (Jorethang) and West Sikkim (Geyzing) and sought the information on traditional methods of preparation for productions of different meat and fish products. The documentation was carried during 2015 to 2018. Ethnic people who produce meat and fish products were interviewed and traditional knowledge of preparation of various meat and fish products were sought using questionnaire (Table C).

Table C: Questionnaire for documentation of traditionally processed meat and fish product in Sikkim

I.General information **Date**:

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

II.Information on products:

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

Remarks:

Name and signature of investigator:

SAMPLE COLLECTIONS

A total of 57 different traditionally prepared meat (27) and fish (30) samples were collected from local people of different parts of Sikkim in India, respectively. The various meat products collected were beef *kargyong*, pork *kargyong*, *satchu* and *khyopeh*. Among the fish products, *suka ko maacha*, *sidra* and *sukuti* were collected. Sealed gamma irradiated sterile bags and containers were used for collection of sample and these samples were stored in desiccator at room temperature at 2-4^{o} C for the further analysis.

PHYSICO-CHEMICAL ANALYSIS

The pH was determined directly with a pH Meter. The measurement was taken three times, changing the insertion place of the electrode. Moisture contents of samples were measured by Moisture Analyzer.

CULTURE DEPENDENT ANALYSIS

Food sample preparation

Twenty-five grams of each sample was homogenized for 1 min at medium speed in a Stomacher in 225 ml buffered peptone-water. An aliquot (1 ml) of the homogenate was taken for aerobic plate count and Enterobacteriaceae enumeration (Feng et al. 2002; Hammack 2003).

Enumeration of Microbial Load

Decimal dilutions (0.1 ml) of the sample homogenate in Butterfield's phosphate-buffered water were inoculated in plate count agar, nutrient agar, Baird Parker agar, *Bacillus cereus* agar and Violet Red Bile Glucose agar using the surface spread method

(Feng et al. 2002). The plates were incubated at 35°C for 24 h to 48 h and the colonies were counted and results were expressed as colony-forming units per gram (cfu/g). The 3M^{TM} Petrifilm^{TM} aerobic count plate was also used to enumerate the microbial load from the collected meat and fish sample. This Petrifilm plate is a dry rehydratable film with a sample-ready-culture medium system containing modified standard methods nutrients, a soluble gelling agent and a tetrazolium indicator that facilitates colony enumeration (Nelson et al. 2013).

Enumeration of *Staphylococcus*

The presence or absence of *Staphylococcus* in each sample was assessed according to the Food and Drug Administration (FDA) standard methods (Tallent et al. 2016). Baird–Parker agar plates were supplemented with potassium tellurite and egg yolk emulsion. Plates were incubated at 37 °C for 36– 48 h. Convex, black, shiny colonies with narrow white margin surrounded by clear zone were regarded as *Staphylococcus*.

Enumeration of *Enterococcus*

Ten grams of samples were diluted in 90 mL of peptone water (0.1% w/v) for its homogenization by mechanic stirring. In products with high-fat content, 1% v/v Tween 80 was added. Aliquots of serial decimal dilutions from homogenized food were spread in bile esculin azide (BEA) agar plates and incubated for 24 h at 35ºC. Colonies that showed black pigmentation on the BEA agar were regarded as *Enterococcus* (Delpech et al. 2012).

Enumeration of *Bacillus*

Individual food samples in quantities of 10 g each were blended in 90 ml of 0.85% sterile saline in a Stomacher for 2 min and serially diluted. Aliquots of 0.1 ml of the appropriate dilutions were surface plated in duplicate on pre-poured plates of *B. cereus* agar. Blue colonies with positive precipitation were regarded as *Bacillus* (Rai et al. 2010).

Enumeration of *Vibrio*

Twenty five grams of dried fish samples were placed in 225 ml of sterile 3.5% NaCl solution and homogenized in blenders. 10 ml of each homogenate was inoculated into 100 ml alkaline peptone water (APW) (1% peptone, 1% NaCl, pH 8.0) and incubated at 37 °C for 14 h with shaking. Enrichment broth (1 ml) was properly serial 10-fold diluted and plated onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar and incubated at 37 °C for 20 h. Colonies with typical dark green or yellow, 2 to 4 mm in diameter, humid, and shiny were regarded as *Vibrio* (Kaysner and DePaola 2004).

Enumeration of *Escherichia* and Coliform Bacteria

10 grams of each sample was homogenized for 1 min at medium speed in a Stomacher in 90 ml buffered peptone-water and serial dilutions were prepared. An aliquot (1 ml) of the homogenate was transferred to violet red bile agar plates and incubated at 18-24 h at 35°C. Colonies with purple-red, 0.5 mm or larger and surrounded by zone of precipitated bile acids were regarded as Coliforms. The colonies were further transferred to BGLB broth and incubated at 35°C for 24-48 h for gas production. Pure cultural isolates were inoculated in EMB agar and MacConkey agar for differentiation of enteric bacteria (Feng et al. 2002).

Enumeration of *Salmonella*

The 25 g of sample was blended in 225 ml of sterile lactose broth in a Stomacher for 3 min. Homogenized mixture was transferred into a sterile jar securely capped and kept at room temperature for 1 h. The 0.1 ml mixture was transferred into 10 ml Rappaport-Vassiliadis (RV) medium and incubated at 42 °C for 24 h in a circulating, thermostatically- controlled, water bath. A loopful of cultured broth was streaked onto XLD Agar and incubated at 35 °C for 24 h. Pink colonies with or without black colour was regarded as *Salmonella* (Andrews et al. 2011).

Presumptive Test for Characterization

Gram Stain

All the bacterial isolates from different media were sub-cultured into nutrient agar and the pure cultures were subjected to Gram staining and examined under the microscope. Gram positive bacteria appeared purple and Gram negative bacteria appeared pink short rods (Smith and Hussey 2005).

Spore Stain

The endospores stain was performed for *Bacillus* sp. After growth on nutrient agar at 37 °C for 24 h, the organisms were suspended in a drop of water on a glass slide. The bacteria were heat fixed by passing through the flame and stained with 5% Malachite green for 5 min by placing flame under the slide until it started producing steam. The slide was rinsed with tap water and were counterstained with 0.25% Safranin O for 30 sec. It was then washed, dried and observed under microscope. Endospores appeared bright green and vegetative cells appeared pink (Hussey and Zayaitz 2007).

Motility Tests

Motility test of the bacterial isolates was performed using SIM medium. Pure isolated colony was stabbed in SIM medium using sterile needle within 1 cm of the bottom of the tube and incubated at 37 °C for 18-24 h. A positive motility growth was indicated by a diffuse growth away from the line of inoculation (Shields and Cathcart 2011).

Enzymatic tests

Catalase Test

A loopful of bacterial isolates was placed into the test tube containing 5-6 drops of 3% Hydrogen Peroxide. Tube was placed against a dark background and observed for immediate bubbles formation. A positive reaction was represented by presence of bubbles and no bubble formation represented catalase negative (Reiner 2010).

Urease Test

A heavy inoculum from 24 h pure culture was streaked into the entire slant surface of a tube containing Christensen's Medium and incubated at 35 °C. The slant was observed for a colour change at 6 h, 24 h and every day for up to 6 days. Urease production is indicated by a bright pink colour on the slant that extend into the butt (Brink 2010).

Gelatin Hydrolysis Test

A heavy inoculum of 18-24 h tested bacteria was stab-inoculated into the tubes containing nutrient gelatin. The inoculated tubes and uninoculated control tubes were incubated at 25 for upto 1 week and checked everyday for gelatin liquefaction. Liquefaction due to gelatinase activity was confirmed by immersing tubes in an ice bath for 15- 30 min and then tubes were tilted to observe if gelatin was hydrolysed. The

positive test of hydrolysed gelatin resulted in a liquid medium after exposure to cold temperature, whereas the negative tests remained solid (dela Cruz and Torres 2012).

Nitrate Reduction Test

Bacterial cultures were grown in 5 ml nitrate broth for 12-24 h at 35 °C. One ml of the culture was mixed with 3 drops of reagent (Reagent A and Reagent B) for nitrate reduction test and observed for development of a red/yellow colour, indicating the presence/absence of nitrate. A small amount of zinc dust was added to the tube for 5 days to observe for the development of red colour, indicating the absence of nitrate reduction (Buxton 2011).

Coagulase Test

About 3-4 isolated colonies of suspected *Staphylococcus* was emulsified in 0.3 ml of BHI broth and incubated at 35-37°C for 18-24 h. The 0.5 ml of reconstituted plasma was added into the BHI culture, mixed thoroughly and incubated at 37°C. The culture was observed for clot formation at intervals over next 46 h. Tubes were incubated for overnight at room temperature when no clot was observed by the end of 4 h,. The positive cultures exhibited clotting by the end of 24 h and negative cultures showed absence of clot formation (Bennet et al. 1986).

DNA Hydrolysis Test

The pure culture of suspected *Staphylococcus* was streaked on DNase Agar and incubated at 37°C for 24 h. DNA was hydrolysed and methyl green was released combined with highly polymerised DNA at pH of 7.5 turning the medium colourless

around the test organism indicating positive result. The negative test showed no degradation of DNA and medium remained green (Kateete et al. 2010).

Biochemical Tests

Carbohydrate Fermentations

Aseptically, 2-3 drops of test organism from 18-24 h BHI broth culture was inoculated in a Phenol Red Carbohydrate Broth containing Durham's tube and incubated at 37°C for 24 h. A yellow colour indicates that enough acid products have been produced by fermentation of the sugar to lower the pH to 6.8 or less. A delayed fermentation produces an orange colour and bubble trapped within Durham tube indicated gas production. A reddish or pink colour indicated a negative reaction (Reiner 2012).

IMVic (*Indole, Methyl Red, Voges-Proskauer and Citrate Utilization*) *Tests*

Indole Test

A small amount of pure culture was inoculated in the tube of Tryptone broth and incubated at 37°C for 24-48 h. Five drops of Kovacs reagent was added directly onto the cultured tube. A positive indole test was indicated by the formation of pink to red colour (cherry red ring) in the reagent layer on top of the medium within seconds of adding reagents. A negative test was indicated by yellow colour on top of reagent layer (MacWilliams 2012).

Methyl Red and Voges-Proskauer Test

A fresh tested culture was inoculated in 5 ml MR-VP broth and incubated for 48 h at 35°C. The 2.5 m of culture was transferred into a new sterile tube and 5 drops of methyl red reagent was added. The MR positive test organism showed red coloration of

the medium and negative test organism showed yellow coloration of medium due to low acid production (McDevitt 2009). In the remaining culture grown in MR-VP broth, 0.6 ml of Barritt's reagent A and 0.2 ml of Barritt's reagent B was added. The tubes were then shaken for 30 secs to 1 min and allowed the tube to stand for 30 min to 1 h. VP positive organism showed red coloration on top of culture and VP negative organism showed yellowish colour (McDevitt 2009).

Citrate Test

A Simmon Citrate medium was prepared in a tube slant and the fresh pure test organism was inoculated on the surface of the slant, incubated at 37°C for 18-24 h. A positive organism was represented by growth on the surface and change in colour from original green to blue due to raise in pH of the medium to >7.6 (MacWilliams 2009).

Molecular Identification of Bacterial Isolates

Genomic DNA Extraction

Genomic DNA was extracted from each bacterial isolate by standard phenol/chloroform method of Cheng and Jiang (2006) with little modifications. One 1 ml of overnight grown culture was centrifuged at 8000 rpm for 10 min and pellets were collected, and then centrifuged at 3,000 rpm, suspended in 40 µl 1X TE buffer. Freshly prepared 15 µl of lysozyme and 15 µl of RNAse enzyme were mixed with pellets and incubated at 37^{o} C for 3 h. After incubation, 15 µl of 20% SDS (sodium dodecyl sulphate) and 15 µl of proteinase-K were added and further incubated for 3 h at 55^{o} C. Equal volume of phenol-chloroform-isoamyl solution (25:24:1) was added to the above mixture, centrifuged at 10,000 rpm for 15 min, 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 for 1 h at -20^{0}c. The mixture was again centrifuged at 10,000 rpm for 30 min, the pellets were then 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 1X TE buffer and stored at -20^{o}c for further analysis. The quality of genomic DNA was checked by electrophoresis in 0.8% agarose gel and quantified using Nano-Drop ND-1000 spectrometer (Kumbhare et al. 2015).

PCR amplification

The PCR of 16S rRNA gene from 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. The reaction mixture, conditions and protocol for the polymerase chain reaction amplification was done following the method of Chagnaud et al. (2001). The PCR

amplification was performed in a mixture containing final volume of 50 µl of Go green Taq master mix (1x) (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 and 72°C for 30 sec for 35 cycles; and 72°C for 7 min. The PCR products were detected by electrophoresis using 1 % agarose, the bands were stained with 7 µl/100 mL of ethidium bromide and visualized in UV source Gel-Doc. Standard 100 base pair DNA ladder was used for the verification of amplicon size.

Purification of the PCR amplicons

The amplified PCR products were purified using PEG (polyethylene glycol)-NaCl (sodium chloride) (20%w/v of PEG, 2.5 M NaCl) precipitation method of Schmitz and Riesner (2006) with little modifications. About 0.6 volume of 20% PEG-NaCl was added to final volume of PCR products and incubated for 30 min at 37 ^{o}C. After centrifugation at 12000 rpm for 30 min, aqueous solution was discarded; pellet was washed twice with freshly prepared ethanol (70%) by centrifugation at 12000 rpm for 30 min. The collected pellet was then air-dried over-night and 20 µl of nuclease-free water was added and finally the purified product was loaded in 1% agarose gel.

16S rRNA Gene Sequencing

PCR products were set up in 5 µl volume for a single primer amplification with the same universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') (Lane 1991) for separate reactions of each primer. The PCR reaction was set as follows: denaturation (96 ^{o}C, 10 sec), annealing (50^{o}C, 5 sec), elongation (60^{o}C, 2 min) with a stop reaction at 4^{o}C. The amplicons were

then precipitated with 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, centrifuged at 12,000 rpm for 20 min, further washed with 70% ethanol, air-dried and suspended in 10µl formamide. Sequencing of the amplicons was performed by Sanger Sequencing method (Heather and Chain 2016) which was carried out in an automated DNA Analyzer.

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). Identification 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 using MEGA7.0 software by the neighbor joining method, the phylogenetic tree was constructed (Gascuel and Steel 2006; Kumar et al. 2016). Diversity indices were determined using a PAST (PAleontological STatistics) v.3.25, (Hammer et al. 2001). The Chao 1 value for species richness was calculated (Chao and Chiu 2016).

Data Availability of 16S rRNA Gene Sequencing

Meat Products: The sequences retrieved from the 16S rRNA gene sequencing were deposited at GenBank-National Center for Biotechnology Information (NCBI) with nucleotide accession number: MK774708, MK774756-MK774760, MK775240-MK775245, MK780051, MK780063, MK788132- MK788134, MK791682 and MK791725.

Fish Products: The sequences retrieved from the 16S rRNA gene sequencing were deposited at GenBank-National Center for Biotechnology Information (NCBI) with the nucleotide accession number: MK774706-MK774707, MK774761-MK774768, MK775239, MK775246-MK775248, MK780040-MK780050, MK780052-MK780062 and MK789854- MK789855.

Bacterial Toxins in Meat and Fish Products by Enzyme-Linked Immunosorbent Assay (ELISA)

Extraction of *Bacillus* Diarrhoeal Enterotoxin

To 25g of sample is 50mL of 0.25M Tris buffer (pH 8) was added and blend for 3 minutes at high speed. Slurry was transferred to centrifuge bottle and centrifuged for 10 min at ≥3000 x *g*. Supernatant was then filtered through a prepared syringe and the eluate was adjusted to pH 7.0-8.0. 5ml of eluate was mixed thoroughly with 50 µl of sample additive (Beattie and Williams 2002; Rahmati and Labbe 2008). The sample was then subjected to ELISA test using Tecra *Bacillus* Diarrhoeal Enterotoxin Visual immunoassay according to manufacturer's instructions.

Extraction of *Salmonella* from meat and fish products

To 25g sample, 225 mL of Lactose Broth was added and incubated at 36°C (±1 °C) for 22-26 h. The 0.1 mL of primary enriched sample was transferred into 10 mL of Rappaport Vassiliadis broth and incubated at 42°C (±1 °C) for 18-24 h. One mL of enrichment sample was transferred into 25 µL of Sample Additive in a tube and heated for 15 minutes in a boiling water bath (Perez-Montano et al. 2012). The sample was then subjected to ELISA test using Tecra Salmonella Visual immunoassay according to manufacturer's instructions.

Extraction of Staphylococcal Enterotoxin

To 10 g of sample 15 ml PBS was added (pH 7.4), and homogenized for about 3 min at high speed in blender. Test sample was centrifuged for about 10 min in bench centrifuge at 3500 *g* and 10 °C. The 100 µl of sample was added per well in the assay (Bennett 2005). The sample was then subjected to ELISA test using Ridascreen *Staphylococcus* Enterotoxin Assay (SET Total) according to manufacturer's instructions.

Molecular detection of virulence genes

Twenty-four bacterial isolates were tested for the presence of some virulence genes of *Staphylococcus aureus* (Meat products=4; Fish products=9), *Bacillus cereus* (Meat product=1; Fish products=5) and *Salmonella enterica* (Meat product=1; Fish products=4) by PCR analysis. The target enterotoxin genes, amplification size, primer sequences and reaction conditions used in this experiment are given in Table D.

Table D. Sequences of primers and PCR reaction conditions used in this study

Target Organism	Amplification target	Amplicon size (bp)	Sequence (5'-3')	Reaction conditions	References
Bacillus cereus	*Nhea*	755	F: GTTAGGACAATC ACCGC R: ACGAATGTAATT TGAGT GC	94°C, 2 min → (94°C, 60 sec → 56°C, 60 sec → 70°C, 120 sec) 35 cycles →72°C, 5 min	Guinebretiere and Broussolle (2002)
Bacillus cereus	*Nheb*	743	F:TTTAGTAGTGGTC TGTACGC R:TTAATGTTCGTTA ATCCTGC	94°C, 2 min → (94°C, 60 sec → 54°C, 60 sec → 72°C, 120 sec) 35 cycles →72°C, 5 min	Guinebretiere et al. (2002)
Staphylococcus aureus	*Sea*	180	F:TAAGGAGGTGGT GCCTATGG R: CATCGAAACCAG CCAAAGTT	94°C, 5 min → (94°C, 1 min→56°, 1 min → 68°C 1 min) 30 cycles →72°C, 7 min	Cremonesi et al. (2005)
Salmonella enterica	*InvA*	275	F: TATCGCCACGTT CGGCAA R:TCGCACCGTCAA AGGAACC	95°C, 1 min → (95°C, 20 sec, 55°C, 20 sec→ 72°C, 2 min) 35 cycles → 72°C for 4min.	Nayak et al. (2004)
Salmonella enterica	*Stn*	617	F: TTGTGTCGCTAT CACTGGCAACC R: ATTCGTAACCCG CTCTCGTCC	94°C, 5min → (94°C, 60 sec, 59°C, 60 sec→ 72°C, 1 min) 35 cycles → 72°C for 10 min	Murugkar et al. 2003

Antibiotic Susceptibility Profile of Isolated Bacteria

The antibiotic susceptibility tests was carried out following modified disk agar diffusion procedures of Kirby-Bauer disc diffusion as recommended by the Clinical and Laboratory Standard Institute (Patel et al. 2017). Bacterial isolates were cultured aerobically in 10ml Mueller-Hinton (MH) broth at 37°C for 24 h. Overnight cultures, grown on MH broth (OD adjusted to 0.5 MacFarland unit), was swabbed evenly with sterile non-toxic cotton swab on MH agar plates and left to dry for 2 to 4 min. Then, antimicrobial sensitivity discs were placed on the culture by using a disk dispenser and incubated for 24 h at 37ºC. An appeared zone of inhibition was measured after incubation and compared with the zone diameter interpretative chart to determine susceptibility of the isolates to antibiotics. Zone diameters were interpreted and categorized as susceptible, intermediate or resistant according to CLSI guidelines (Patel et al. 2017). Antibiotics used in this study included amoxicillin/clavulanate (30 μg), ceftazidime (30 μg), cefuroxime (30 μg), cefepime (30 μg), trimethoprim (5 μg), nitrofurantoin (300 μg), nalidixic acid (30 μg), tobramycin (10 μg), ciprofloxacin (5 μg), erythromycin (15 μg), chloramphenicol (30 μg), penicillin G (10 μg), ampicillin (10 μg), streptomycin (10 μg), tetracycline (30 μg), gentamicin (10 μg), vancomycin (30 μg), oxacillin (1 μg), rifampicin (5 μg), cefoxitin (30 μg), aztreonam (30 μg), clindamycin (2 μg), co-trimoxazole (25 μg), norfloxacin (10 μg), cefotaxime/clavulinic acid (30 μg), ceftriazone (30 μg) (Thung et al. 2018; Jamali et al. 2015).

CULTURE INDEPENDENT ANALYSIS

High-Throughput Sequencing in Traditionally Processed Meat and Fish Products of Sikkim

Sample collection

Four samples of traditionally processed meat products viz. one each of beef *kargyong* and pork *kargyong* were collected from local market in Gangtok, and one each of yak *satchu* and *khyopeh* were collected from different households in Lachen regions of North Sikkim. Similarly, 9 samples of traditionally sun-dried fish products: *suka ko maacha* (3 samples)*, sidra* (3) and *sukuti* (3), respectively were collected from different local markets of Sikkim. The products were aseptically collected in sterile poly-bags kept in an icebox carrier, transported to the laboratory and stored at -20 ˚C for further analysis.

Homogenization of sample

10 g of each sample was homogenized in a 90 ml of sterile 0.1 M phosphate buffer saline (pH 6.4) using Stomacher at 200 rpm for 2 min (Keisam et al. 2016). After homogenization, the big debris was allowed to settle down for 5 min and the homogenates was used for DNA extraction.

Metagenomic DNA extraction

Genomic DNA was extracted from each sample using Nucleospin® Food kit according to manufacturer's protocol (Shangpliang et al. 2018). Concentration of genomic DNA of each sample was quantified using Eppendorf BioSpectrometer and the bands were visualized in agarose gel (0.8%) using Gel Doc.

Sequencing using MiSeq Illumina Platform

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

Bioinformatics analysis

Microbiome bioinformatics was performed using Quantitative Insights Into Microbial Ecology (QIIME) 2-2019.10 environment workflow (Bolyen et al. 2019). Firstly, the quality of the raw reads was checked using FastQC (Andrews 2010) and accordingly the adapter sequences were then trimmed by Trim Galore algorithm (Krueger 2017). We have chosen to use an alternative joining method of the raw paired-end sequences using PEAR (PHP Extension and Application Repository) program (Zhang et al. 2013), which was then followed by importing the sequences as QIIME2 artifact into QIIME2 environment as single-end sequences (SingleEndFastqManifestPhred33 format). Quality-filtered joined reads such as chimera, singleton and short sequences were denoised using deblur algorithm (q2-deblur denoise-16S) (Amir et al. 2017) against a positive filter (Greengenes 13_8) and the resulting sub-operational-taxonomic-unit

(sOTUs) were then aligned with multiple alignment using fast Fourier transform (mafft) (Katoh et al. 2002) (via q2-alignment). Approximately-maximum-likelihood phylogenetic tree from alignments of nucleotide sequences was created using fasttree2 (Price et al. 2010) (via q2-phylogeny) for diversity analysis. Taxonomic assignment was achieved using the Greengenes reference databases (13_8_99% OTUs) (McDonald et al. 2012a) via q2-feature-classifier classify-consensus-vsearch (Rognes et al. 2016). The taxonomic BIOM (biological observation matrix) (McDonald et al. 2012b) file was then exported and collapsed at different taxonomic level for further analysis.

Predictive Gene Functionality

The representative sequences along with their frequency tables were clustered in accordance to the Greengenes reference database (13_8_99% OTUs) via q2-vsearch-cluster-features-closed-reference (Rognes et al. 2016). Prediction of functional composition of marker gene (16S rRNA gene), based on the abundances of gene families in the samples, was deduced as per the standard integrated genomes database using PICRUSt2 algorithm (Douglas et al. 2019). Here, the amplicon sequence variants (ASVs) were first aligned by multiple-sequence alignment of 16S sequences with HMMER (Howard Hughes Medical Institute 2018), where the most likely placements of the ASVs in the reference tree with evolutionary placement-ng (EPA-ng) algorithm (Barbera et al. 2019) and Genesis Applications for Phylogenetic Placement Analyses (GAPPA) omics (Czech and Stamatakis 2019) were applied. We applied castor R package (Louca et al. 2018) in PICRUSt2 pipeline for prediction of gene families with the default run (maximum parsimony). Metagenome prediction was run using (metagenome_pipeline.py) (Ye and Doak 2009) and the output features were mapped into KEGG (Kyoto Encyclopaedia of Genes and Genomes) database for systematic

analysis of gene functions (Kanehisa and Goto 2000). The resulting data was then explored using BURRITO software (McNally et al. 2018) for visualizing gene family and their relative abundance. Pathway levels of defined gene functionality (Scala et al. 2019) with high-level function (Level 1) *viz.,* metabolism, environmental information processing, genetic information processing, human diseases, cellular processes, organismal system and drug development were interpreted, which was further divided into lower Level 2 and were mapped with gene copy numbers (reads) using 16S rRNA sequences profile inferred by PICRUSt2 against KEGG database in samples (Kanehisa et al. 2017).

Statistical analysis

Alpha diversity metrices (Observed OTUs, Shannon's diversity index, Faith's Phylogenetic Diversity, Evenness) were studied using q2-core-metrices-phylogenetic (Kim 2017). Beta diversity (Bray-Curtis distance) was estimated using q2-diversity (Astudillo-Melgar et al. 2019). Alpha and beta diversities were conducted using hypothesis testing [ANOSIM (Analysis of Similarities and PERMANOVA (permutational multivariate analysis of variance)] (Anderson 2017) and were checked in QIIME2-2018.10 environment (McMurdie and Holmes 2013). Student's T-test was also applied for alpha diversity metrices. Graphical representation of shared and unique genera (with >1% relative abundance) was also visualized using iGraph R-package. Beta diversity was analysed using Bray-Curtis dissimilarities and PCA (principal component analysis) plot was constructed using PASTv4. Statistical analysis was applied to the predictive functional features using STAMP (statistical analysis of metagenomic profiles) software by ANOVA (analysis of variance) hypothesis testing

method (Parks et al. 2014). Analysis was also checked for significant features among the samples using Welch's test-two-sided properties (Parks et al. 2014).

Data Availability of High-Throughput Sequencing

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

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

RESULTS

DOCUMENTATION OF TRADITIONAL MEAT AND FISH PRODUCTS OF SIKKIM

Extensive field survey was conducted to seek the information on traditional methods of preparation, culinary, mode of consumption and socio-economy of various traditionally processed meat and fish products from different places of Sikkim (Figure 1).

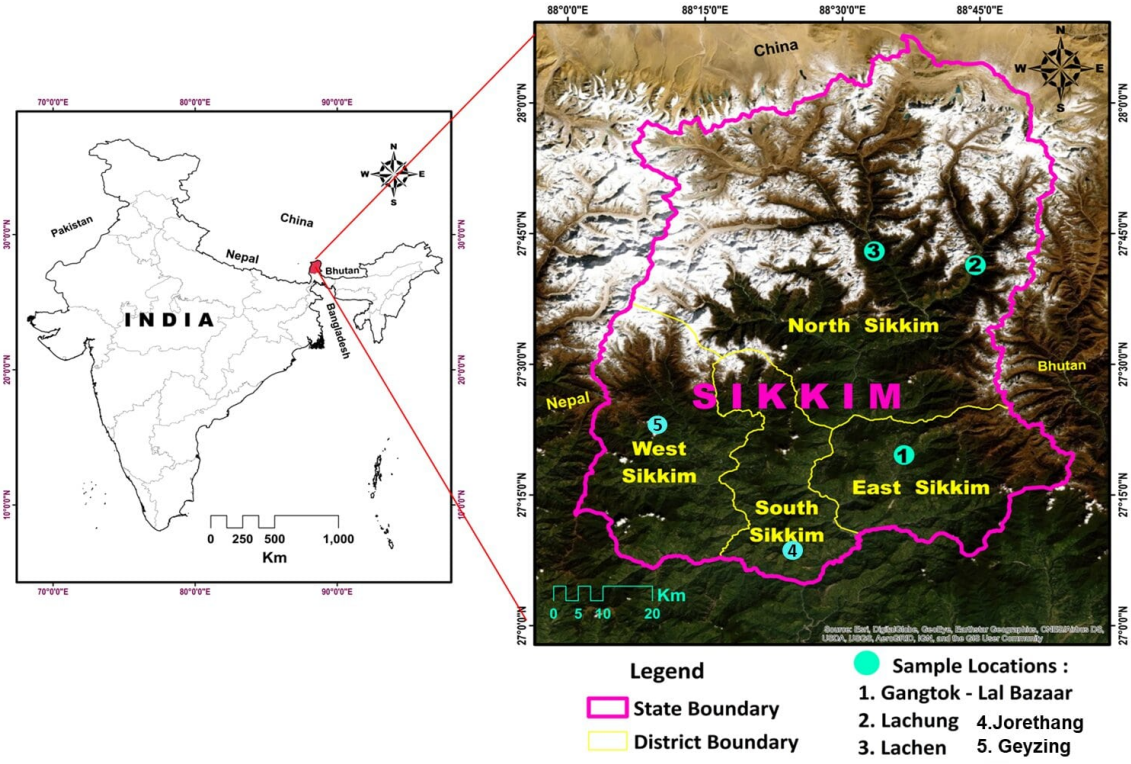


Figure 1: Map showing study sites of sample collection in Sikkim.

Based on personal observation and interviews with the local producers using questionnaire (Table A), four different types of meats are traditionally processed in Sikkim which include a traditional sausage prepare from beef and pork, locally called *lang* (beef) *kargyong* and *faak* (pork) *kargyong*, smoked and sun-dried yak meat product called *satchu*, and fermented yak meat product known as *khyopeh*. Similarly, three different traditionally prepared sundried fish products locally known as *suka ko maacha, sidra* and *sukuti* of Sikkim were documented (Tables 1 and 2).

Table 1: Traditional processed meat products of Sikkim				
Product	**Types**	**Raw materials**	**Nature and Culinary**	**Major consumer**
Beef *kargyong*	Smoked	Intestine (fatty), chopped meat fat of beef, salt, ginger and garlic	Hard/soft texture, brown in colour; sausage-like, curry	Bhutia, Lepcha, Tibetan and Sherpa
Pork *kargyong*	Smoked	Intestine, blood and chopped meat of pork, salt, ginger, garlic	Hard/soft texture, brown in colour; sausage-like, curry	Bhutia, Lepcha, Tibetan and Sherpa
Satchu	Sun-dried/smoked	Long strands-like yak meat	Hard texture, brown in colour; dried meat, curry	Bhutia, Lepcha, Tibetan and Sherpa
Khyopeh	Fermented	Minced meat and fat of yak inside rumen, garlic, ginger, salt	Rough, hard, brownish; curry	Bhutia

Table 2: Traditionally processed fish products of Sikkim				
Product	**Types**	**Substrate**	**Nature and Culinary**	**Major consumer**
Suka ko maacha	Smoked/sun-dried	Fish (*Schizothorax* spp.)	Smoked/sun-dried, side dish, curry	Nepali, Bhutia and Lepcha
Sidra	Sun-dried	Fish (*Puntius sarana*)	Dried, pickle, curry	Nepali, Bhutia and Lepcha
Sukuti	Sun-dried	Fish (*Harpodon nehereus*)	Dried, pickle, curry	Nepali, Bhutia and Lepcha

(1) TRADITIONALLY PROCESSED MEAT PRODUCTS

BEEF *KARGYONG*

Traditional sausage is called *kargyong* in Sikkim which is prepared from minced beef stuffed into animal intestine, and is commonly consumed by the Bhutia, Lepcha,

Tibetan and Sherpa communities of Sikkim. It is soft or hard in texture and brown in colour. The Lepcha prefers the name *tiklee* for *kargyong.*

Traditional method of preparation

Beef flesh with its fat are chopped, and is mixed with crushed garlic, ginger with addition salt and a little amount of water. The mixture is then filled into a beef intestine with 2-4 cm in diameter and 40-50 cm length which is used as natural casings. One end of the casing is tied up with a rope, and other end is covered after filling of minced meat, and then boiled for 20-30 min. Unlike other sausages, no nitrates and nitrides are added during preparation of *kargyong*. Cooked sausages are then taken out and hooked up in the bamboo stripes above the traditional earthen oven and are dried/smoked for 10-15 days to obtain *kargyong* (Figure 2a).

Mode of consumption

Beef *kargyong* is cooked for 10-15 min, dewatered, sliced and fried in edible oil by adding onion, tomato, powdered or ground chilies, and salt and is made into curry. It is also consumed as fried sausage with *raksi*, a traditional distilled alcoholic drink (Tamang 2010).

Socio-economic importance

Beef *kargyong* is also prepared at households for home consumption and also during festivals. Fried *kargyong* is popular side-dish in many local restaurants and hotels in Sikkim. The cost of *kargyong* is approximately Rs. 275/ to 350/kg.

PORK *KARGYONG*

Pork *kargyong* is an indigenous sausage-like meat product prepared from pork in Sikkim and is prepared and eaten by Bhutia, Lepcha, Tibetan and Sherpa ethnic groups.

Traditional method of preparation

Lean pork meats, locally known as *faak*, with its fat are minced, mixed with shredded ginger and garlic, and salt with addition of water. Then meat mixture is filled into the pig-intestine with a size of 2-4 cm in diameter and 40-50 cm length, used as natural casings. One end of intestine is sealed and other end is closed by a rope after filling up the minced meat mixtures which is then cooked for 20-30 min. After cooking, sausages are taken out from the utensil and hung in the bamboo-made stripes and kept above the earthen oven kitchen for smoking/drying for at least 10-15 days (Figure 2b). No nitrates and nitrides are added during preparation of pork *kargyong*.

Mode of consumption

Pork *kargyong* is eaten as a curry fried in oil with green chili, garlic, tomato and salt.

Socioeconomic importance

Pork *kargyon*g is sold in local market, costing approximately Rs.300/- to Rs. 350/- per kg. It is commonly prepared during festivals and social-functions in Sikkim.

SATCHU

Traditional dried or smoked meat is known as *satchu* in Sikkim with thread or strand like appearance, and is commonly eaten by Bhutia, Lepcha, Tibetan and Sherpa communities.

Traditional method of preparation

Preferably red meat of beef or yak is sliced into long threads of about 60-100 cm and is garnished with turmeric powder, edible oil or butter and salt. The meat strands are then hung in the bamboo-made stripes or wooden stick and are smoked above the earthen oven kitchen for 7-10 days (Figure 2c). *Satchu* may be preserved at room temperature for several weeks.

Mode of consumption

Curry of *satchu* is made by frying in butter, locally called *gheu* or *maa* or edible oil with chopped garlic, ginger, chilli and a desirable amount of salt. It is consumed by the Bhutia, Tibetan, Lepcha and Sherpa communities of Sikkim. Fried *satchu* is a popular side-dish or snack which is served with traditional alcoholic beverages or drink.

Socio-economic importance

Satchu is available in the local restaurants and food stalls in Sikkim and it costs approximately Rs. 400-500/kg.

KHYOPEH

Khyopeh is a unique naturally fermented meat product, prepared in the North district of Sikkim by the *Lachungpa* community. Texture of *khyopeh* is hard and brown in colour (Figure 2d).

Traditional method of preparation

The preparation of *khyopeh* is seasonal and is usually prepared during Buddhist festivals generally be held in the month of December where yaks are slaughtered for

the festivals. After slaughter, parts of liver, lungs, fats, intestines and innards of dead yaks are collected for preparation of *khyopeh*. Chopped meats and innards of yak are mixed with the required amount of salt, and the mixtures are stuffed into the rumen (stomach), which was previously removed from slaughtered yak, tied up with a twine and hung into a bamboo stripes for natural fermentation for 4-6 months above earthen oven (Figure 2d).

Mode of consumption

It is eaten as raw or cooked with nettle leaves, locally called *sishnu* (*Urtica dioica*) in the main meal with boiled rice in North Sikkim.

Socio-economic importance

The practice of preparing *khyopeh* is quite rare and confined to Lachung village of north Sikkim which are quite far from the urban localities. Hence, it is not found in the local markets of Sikkim. It is usually prepared for home consumption and festivals. It is believed by the villagers that yak meat products have an immense medicinal potential. Yaks graze on herbs especially *Cordyceps sinensis***,** locally called as *yarsagumba***,** which is found only in high altitude of mountains and is believed to be an excellent potent for strengthening the immune system.

Beef meat and ingredients
↓ ←Add water
Stuff into the intestine
↓
Tie both ends
↓
Boil till the inner content is cooked
↓
Air dry/smoke dry for 10-15 days
↓
Beef *kargyong*

(a)

Mixture of pork meat and ingredients
↓ ←Add water
Stuff into the intestine
↓
Tie both ends
↓
Air dry/ smoke for 10-15 days
↓
Pork *kargyong*

(b)

Red meat of yak
↓
Cut into strands
↓
Mixed with turmeric powder, mustard oil and salt
↓
Smoke or air dry for 7-10 days
↓
Yak *satchu*

(c)

Innards of yak chopped
↓ ←Add water
Chopped meat stuffed inside the removed rumen previously from dead yak
↓
Open portion of rumen closed and tied with rope
↓
Hung in a bamboo stripes for drying
↓
Ferment for 4-6 months
↓
Khyopeh

(d)

Figure 2: Traditional method of preparation of various meat products of Sikkim. (a) Beef *kargyong* (b) Pork *kargyong* (c) *Satchu* and (d) *Khyopeh*.

(1) TRADITIONALLY PROCESSED FISH PRODUCTS

SUKA KO MAACHA

Traditional smoked fish is popularly known as *suka ko maacha* in Sikkim.

Traditional method of preparation

Two types of fishes are preferred for the preparation of *suka ko maacha* by the people of Sikkim residing near streams or rivers. Edible river fishes are locally known as '*dothay asala'* (*Schizothorax richardsoni* Gray) and '*chuchay asala'* (*Schizothorax progastus* McClelland). The fishes are collected in a bamboo basket locally called 'bhukh' from the rivers, and caught fishes are degutted, cleaned thoroughly, rubbed with salt and turmeric powder, hooked in a bamboo-made strings and are hung above the earthen-oven in kitchen for smoking for 7-10 days (Figure 3a). *Suka ko maacha* may be kept in dry container at room temperature for 3-4 months.

Mode of consumption

Suka ko maacha curry is delicacy which is cooked with tomato, vegetable, chili and salt and is served with cooked rice in main meal.

Socio-economic importance

It is commonly sold in the local markets in Sikkim at the price of Rs. 150-200/kg.

SIDRA

Sidra is a sun-dried fish product commonly consumed by the Gorkha/Nepali in Sikkim.

Traditional method of preparation

During its preparation, the whole fish (*Puntius sarana* Hamilton) is cleaned and kept outside in direct sun for dehydration for 4-7 days (Figure 3b). *Sidra* is stored in dry container at room temperature for 3-4 months.

Mode of consumption

Sidra is consumed as pickle. During pickle making, *sidra* is roasted and is mixed with dried chili, boiled tomato and salt to make a thick pickle paste. *Sidra ko achar* (pickle) served with cooked rice and 'khalo dal' (black gram soup) present typical gastronomy of Gorkha/Nepali cuisine in Sikkim.

Socio-economic importance

Sidra is sold in local markets which costs approximately Rs. 200-250/kg.

SUKUTI

Sukuti is also very popular sun-dried fish product in Sikkim.

Traditional method of preparation

During preparation of *sukuti,* fish (*Harpodon nehereus* Hamilton) is collected, washed, and mixed with salt, and then dried in the sun for 4-7 days (Figure 3c). Shelf-life of *sukuti* is 4-6 months at moist-free room temperature.

Mode of consumption

Sukuti is consumed as pickle, soup and curry. During curry preparation, *sukuti* is fried, mixed with dry chili, onion and salt to make a pickle. It is usually eaten with boiled rice and black gram soup.

Socio-economic importance

It is commonly sold at local markets in Sikkim at the rate of approximately Rs. 200-250/kg.

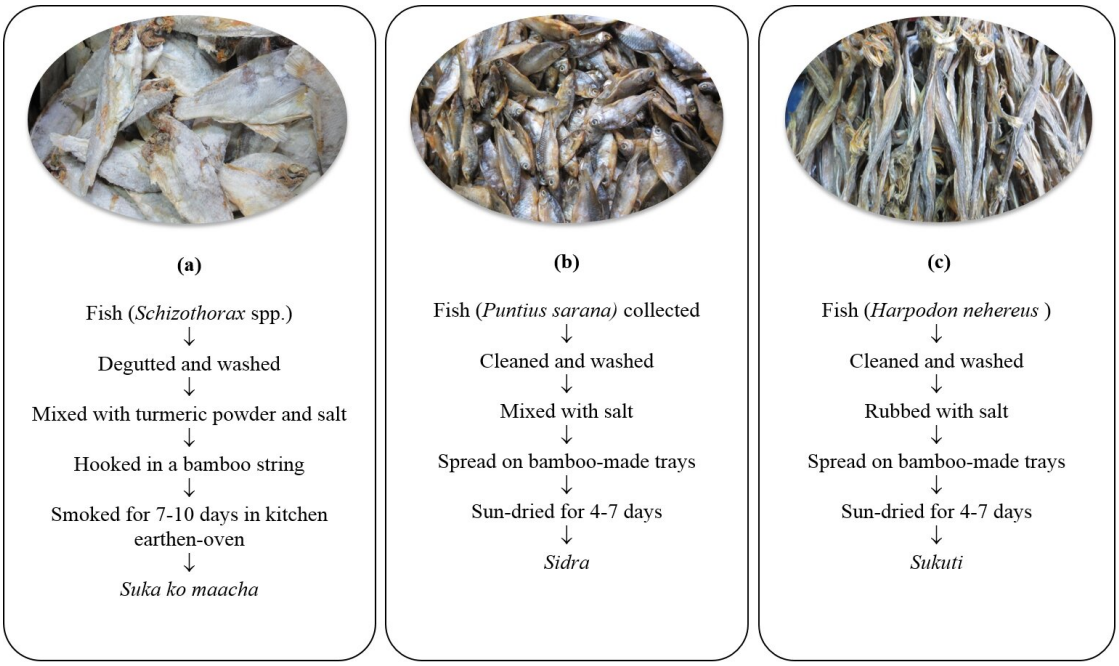


Figure 3: Traditional method of preparation of various fish products of Sikkim. a) *Suka ko maacha*, (b) *Sidra* and (c) *Sukuti*.

CULTURE DEPENDENT ANALYSIS

Physico-chemical analysis

A total of 27 meat products and 30 fish products were collected for analysis. The average pH value of beef *kargyong* was 5.8 (5.7-5.9), pork *kargyong* was 5.5 (5.3-5.8), *satchu* was 5.4 (5.3-5.7), and *khyopeh* was 5.9 (5.8-6.1). The average moisture content in beef *kargyong* was 14.1% (10.8%-18%), pork *kargyong* was 11.2% (8.8%-14.8%), *satchu* was 8.5% (7.3%-10.7%), and *khyopeh* was 2.5% (1.5%-3.5%) (Table 3). Similarly, the average pH value of *suka ko maacha* was 6.7 (6.7-7.0), *sidra* was 6.7 (6.6-6.8) and *sukuti* was 6.7 (6.7-6.8). The average moisture content in *suka ko maacha* was 10.7% (9.6%-13.3%), *sidra* was 10.9% (10.6%-11.9%), and *sukuti* was 9.1% (7.6%-11.3%) (Table 4).

Table 3: Collection sites, altitude, moisture, pH and bacterial load of traditionally prepared meat products of Sikkim

Sample	District (number of samples)	Collection Site	Altitude (Meter)	Moisture content (%)	pH	Bacterial load (cfu/g)			
						Aerobic bacteria (10^6)	*Staphylococcus* (10^5)	*Bacillus* (10^5)	Total Coliform (10^6)
Beef *kargyong*	East Sikkim (4)	Lalbazaar, Gangtok	1639	18.0 (16.3-19.7)	5.8 (5.5-6.1)	24.0 (23.0-25.0)	25.0 (23-27)	2.2 (2.1-2.3)	0.82 (0.25-1.4)
		Martam	1652	15.1 (14.7-15.5)	5.8 (5.8-5.9)	12.3 (2.4-13)	2.0 (1.8-2.2)	1.9 (1.8-2.1)	1.4 (0.21-2.6)
	West Sikkim (2)	Geyzing	1443	12.8 (12.4-13.3)	5.9 (5.8-6.1)	0.22 (0.18-0.27)	2.0 (2.0-2.1)	2.1 (1.6-2.7)	0.27 (0.26-0.29)
	South Sikkim (2)	Kitam	535	10.8 (10.2-11.4)	5.7 (5.6-5.8)	1.0 (0.23-1.9)	1.9 (1.7-2.1)	10.5 (2.1-19)	0.9 (0.21-1.7)
Pork *kargyong*	East Sikkim (4)	Lalbazaar, Gangtok	1639	14.8 (8.6-16.5)	5.3 (5.2-5.4)	0.74 (0.28-1.2)	23.0 (22.0-24.0)	11.5 (2.0-21)	0.22 (0.21–2.3)
		Baluakhani Rd	1722	8.8 (8.6-9.1)	5.4 (5.4-5.5)	0.22 (0.21-0.23)	15.0 (15.0-16.0)	2.4 (2.3-2.6)	2.2 (2.1-2.3)
	West Sikkim (2)	Geyzing	1443	11.8 (11.3-12.4)	5.8 (5.8-5.9)	1.2 (0.1-2.2)	0.74 (0.18-1.3)	1 (0.2-1.8)	0.15 (0.13.-0.18)
	South Sikkim (2)	Kitam	535	9.4 (9.1-9.7)	5.8 (5.8-5.9)	1.8 (1.7-1.9)	2.2 (1.9-2.6)	1.2 (0.15-2.3)	1.0 (0.2-1.8)
Satchu	East Sikkim (4)	Lalbazaar, Gangtok	1639	7.3 (7.1-7.6)	5.3 5.3-5.4	1.2 (0.21-2.2)	10.9 (1.8-20)	1.7 (1.5-1.9)	34.0 (23-45)
		Martam	1652	7.6 (7.2-8.1)	5.3 5.3-5.4	2.8 (2.7-2.9)	19.5 (16.0-23.0)	4.6 (4.5-4.7)	1.5 (0.27-2.8)
	West Sikkim (2)	Geyzing	1443	10.7 (6.8-7.1)	5.7 (5.7-5.8)	1.4 (1.3-1.6)	2.2 (2.1-2.3)	0.38 (0.17-0.21)	0.16 (0.12-0.20)
Khyopeh	North Sikkim (5)	Lachung	2625	2.5 (1.5-3.5)	5.9 (5.8-6.1)	1.2 (0.15-2.3)	0.01 (0.002-0.02)	absent	00021 0.00012-0.00031

cfu/g = colony forming units per gram

Table 4: Bacterial load of traditionally prepared fish products from different places of Sikkim

Sample	District (number of samples)	Collection Site	Altitude (Metre)	Moisture content (%)	pH	Bacterial load (cfu/g)			
						Aerobic bacteria (10^6)	*Staphylococcus* (10^5)	*Bacillus* (10^5)	Total Coliform (10^6)
Suka ko maacha	East Sikkim (8)	Lalbazaar, Gangtok	1639	9.6 (9.1-15.0)	7.0 6.9-7.1	2.3 (2.2-2.4)	2.1 (1.9-2.3)	0.02 (0.20-0.03)	2.3 (2.2-2.5)
		Rangpo	305	10.2 (10.2-10.3)	6.7 (6.7-6.8)	0.2 (0.21-0.22)	2.5 (2.5-2.6)	17 (16-18)	2.0 (1.8-2.3)
		Singtam	360	9.85 (9.6-10.1)	6.7 (6.7-6.8)	2.3 (2.1-2.6)	2.4 (2.1-2.7)	1 (1.8-0.20)	0.63 (0.16-1.1)
		Rhenock	915	13.3 (12.3-14.4)	6.7 (6.7-6.8)	1.9 (1.6-2.2)	1.9 (1.5-2.3)	1.9 (1.5-2.3)	0.21 (0.19-0.23)
	South Sikkim (2)	Jorethang	337	10.7 (10.3-11.2)	6.7 (6.6-6.8)	2.5 (0.24-2.3)	2.2 (2.2-2.3)	0.19 (0.18-0.2)	1.9 (1.8-2.0)
Sidra	East Sikkim (8)	Lalbazaar, Gangtok	1639	11 (7.8-14.2)	6.8 (6.8-6.9)	2.2 (1.9-2.5)	18.5 18-19	24 (23-25)	2.3 (1.9-2.8)
		Rangpo	305	10.6 (10.2-11.1)	6.8 (6.7-6.9)	12.3 (2.6-22)	12.5 0.16-25	22 (18-26)	26 (25-28)
		Singtam	360	10.6 (10.5-10.7)	6.7 (6.6-6.8)	2.0 (1.7-2.3)	2.0 1.8-2.2	1.9 (1.8-2.1)	2.3 (2.1-2.6)
		Rhenock	915	11.9 (11.8-12.1)	6.6 (6.6-6.7)	2.3 (2.2-2.4)	2.1 2.1-2.2	2.5 (1.6-2.7)	1.4 (0.29-2.7)
	South Sikkim (2)	Jorethang	337	10.8 (10.2- 11.4)	6.6 (6.5-6.7)	21.0 19.0-23.0	1.9 1.7-2.1	2.0 1.9-2.1	17.5 (17-18)
Sukuti	East Sikkim (8)	Lalbazaar, Gangtok	1639	11.3 (10.2-12.4)	6.7 (6.7-6.8)	1.9 (1.8-2.0)	25.0 (2.7-23.0)	0.86 (0.23-1.5)	12 (1.3-23)
		Rangpo	305	9.6 (9.2-10.1)	6.8 (6.7-6.9)	1.2 (0.23-2.2)	2.3 (2.1-2.5)	40.5 (25-28)	0.12 (0.22-023)
		Singtam	360	7.6 (7.2-8.1)	6.8 (6.7-6.8)	1.2 (0.23-2.2)	2.2 (2.1-2.3)	1.9 (1.9-2.0)	12.5 (2.0-23)
		Rhenock	915	9.6 (9.1-10.2)	6.7 (6.6-6.7)	1.7 (1.7-1.8)	2.1 (2.1-2.2)	0.92 (0.14-1.7)	0.24 (0.23-0.26)
	South Sikkim (2)	Jorethang	337	7.6 (7.2-8.0)	6.7 (6.7-6.8)	2.3 (1.8-2.8)	2.2 (1.9-2.5)	1.9 (18-2.0)	12.5 (0.02-25)

cfu/g = colony forming units per gram

Phenotypic characterisation

The samples were further processed for aerobic bacterial count (Figure 4). In meat products the average aerobic bacterial population observed in beef *kargyong* was 9.3 x 10^6 (2.2 x 10^5 – 2.4 x 10^7) cfu/g, pork *kargyong* was 1.0 x 10^5 (2.2 x 10^5 – 1.8 x 10^6) cfu/g, *satchu* was 1.8 x 10^6 cfu/g (1.2 – 2.8 x 10^6) cfu/g and *khyopeh* was 1.2 x 10^6 (1.5 x 10^5 – 2.3 x 10^6) cfu/g, respectively. The average staphylococcal count was found highest in *satchu* with 1.8 x 10^6 (2.2 x 10^5 – 2.0 x 10^6) cfu/g followed by pork *kargyong* with 1.2 x 10^6 (7.4 x 10^4 – 2.3 x 10^6) cfu/g, beef *kargyong* with 7.7 x 10^5 (1.9 x 10^5 – 2.5 x 10^6) cfu/g and least in *khyopeh* with 1.0 x 10^3 (2.0 x 10^2 – 2.0 x 10^3) cfu/g. *Bacillus* count in beef *kargyong* varied from 1.9x 10^5 – 1.1 x 10^6 cfu/g with average population of 4.1 x 10^5 cfu/g, pork *kargyong* with average count of 4.0 x 10^5 (1.0 x 10^5 – 1.2 x 10^6) cfu/g, *satchu* with 2.2 x 10^5 (3.8 x 10^4 – 4.6 x 10^5) cfu/g. No growth of *Bacillus* was observed in *khyopeh.* The average count of total coliform in beef *kargyong* was 0.8 x 10^6 (2.7 x 10^5–1.4 x 10^6) cfu/g, pork *kargyong* was 8.9 x 10^5 (1.5 x 10^4–2.2 x 10^6) cfu/g, *satchu* was 1.2 x 10^7 (1.0 x 10^6– 3.4 x 10^7) cfu/g, and *khyopeh* was 2.1 x 10^2 (1.2 x 10^2– 3.1 x 10^2) cfu/g (Table 3).

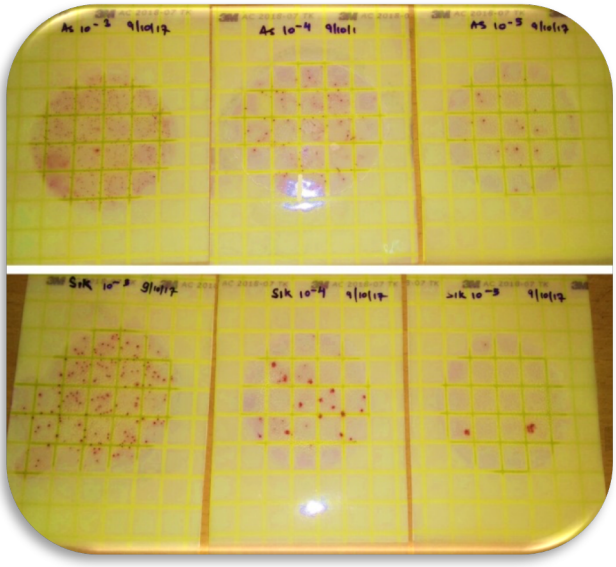


Figure 4: 3M Petrifilm Aerobic Count Plate showing bacterial colonies.

In fish products, the aerobic bacterial count varied from 2.0 x 10^5 cfu/g to 2.1 x 10^7 cfu/g. The average population of aerobic bacteria in *suka ko maacha* was 1.8 x 10^6 (2.0 x 10^5– 2.5 x 10^6) cfu/g, *sidra* was 7.9 x 10^6 (2.0 x 10^6–2.1 x 10^7) cfu/g and *sukuti* was 1.6 x 10^6 (1.2–2.3 x10^6) cfu/g. The average staphylococcal count observed in *suka ko maacha* was 2.2 x 10^5 (1.9–2.5 x 10^5) cfu/g, *sidra* was 7.4 x 10^5 (1.9 x 10^5–1.9 x 10^6) cfu/g and *sukuti* was 6.7 x10^5 (2.1 x 10^5–2.5 x 10^6) cfu/g. Average *Bacillus* count in *suka ko maacha* was 4.0 x 10^5 (2.0 x 10^3–1.7 x 10^6) cfu/g, *sidra* was 1.1 x 10^6 (1.9 x 10^5–2.4 x 10^6) cfu/g and *sukuti* was 9.2 x 10^5 (8.6 x 10^4–4.1 x 10^6) cfu/g. The average population of total coliform found in *suka ko maacha* was 1.4 x 10^6 (6.3 x 10^5–2.3 x 10^6) cfu/g, *sidra* was 9.9 x 10^6 (1.4 x x 10^6 –2.6 x 10^7) cfu/g and *sukuti* was 7.4 x 10^6 (1.2 x 10^5–1.3 x 10^7) (Table 4).

A total of 388 bacterial isolates were isolated from traditional meat products (128) and fish products (283) of Sikkim, respectively. Phenotypic characterization of bacterial isolates was performed on the basis of cultural characteristics, cell morphology, carbohydrate fermentation and enzymatic tests (Table 5 and 6). Selective media were used to isolate some food-borne bacterial pathogens and spoilage bacteria. Gram stains were performed to differentiate between Gram-positive and Gram-negative bacteria (Figure 5). The IMVic (Indole, Methyl Red, Voges-Proskauer and Citrate Utilization) tests were mainly performed to characterize the bacteria belonging to Enterobacteriaceae family. Coagulase reaction and DNAse tests were performed to identify *Staphylococcus.* Other carbohydrate fermentation and enzymatic tests were also performed for preliminary identification of bacteria using taxonomical key (Holt et al. 1994). The bacteria isolated from samples of beef *kargyong*, pork *kargyong, satchu* and *khyopeh* were tentatively identified as *Enterobacter*, *Klebsiella*, *Escherichia, Salmonella*, *Enterococcus*, *Bacillus, Staphylococcus*, *Citrobacter* and *Pseudomonas*

(Table 5). Similarly, bacteria isolated from samples of *suka ko maacha*, *sidra* and *sukuti* were tentatively identified as *Enterobacter*, *Klebsiella*, *Escherichia, Salmonella*, *Vibrio, Bacillus, Staphylococcus*, *Citrobacter* and *Pseudomonas* (Table 6).

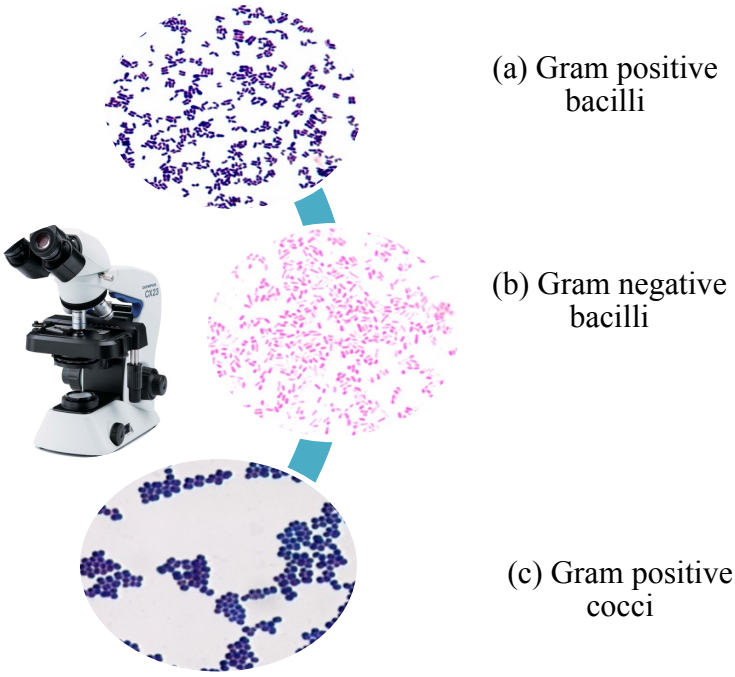


Figure 5: Gram stain of isolated bacteria viewed under microscope (a) Gram-positive baciili, (b) Gram-negative bacilli and (c) Gram-positive cocci.

All bacterial isolates from respective sample were grouped on the basis of phenotypic characteristics, and representative isolates were selected from each grouped strains having similar morphology, sugar fermentation, enzymatic activities and IMViC tests. A total of 19 representative isolates were selected from meat samples and 38 bacterial strains were selected from fish samples which were further carried out for DNA extraction and molecular identification (Table 7-8).

Table 5: Phenotypic and biochemical characterizations of bacteria isolated from traditionally processed meat products of Sikkim

Colony morphology	Media	Gram reaction	Motility	Enzymatic reactions						Sugar fermentation															IMViC test			Tentative identification (Total number of isolates)
				Catalase	Coagulase Reaction	Urease reaction	DNase	Nitrate reduction	Gelatin hydrolysis	Lactose	Sorbitol	Arabinose	Glucose	Mannitol	Mannose	Trehalose	Raffinose	Maltose	Xylose	Rhamnose	Sucrose	Ribose	Adonitol	Indole	Methyl red	Voges-proskauer	Citrate	
Pink without sheen	EMB	-	+	+	-	-(6) v(4)	-	+	-	+(5) v(5)	+	+	+	+	+	+	+(4) -(6)	+	+	+	+	+	-(7) v(3)	-(8) v(2)	-	+	+(6) v(4)	*Enterobacter* (10)
Pink, mucoidy	EMB	-	-	+	-	+	+	+	+	+(8) -(7)	+	+	+	+	+	+	+	+	+	+(10) v(5)	-(6) v(9)	+	+	-	+(12) -(3)	+(13) -(2)	+(11) -(4)	*Klebsiella* (15)
Purple with metallic sheen	EMB	-	+	+	-	-	-	+	-	+(10) v(2)	+(10) -(2)	+	+(8) v(4)	+(9) -(3)	-	+	+	-	+	+(9) v(3)	-(8) v(4)	-	-	+(7) v(5)	+	-	-(5) v(7)	*Escherichia* (12)
Red colonies with black centres	XLD	-	+	+	-	-	-	+	-	-(5) v(3)	+	+	+	+	+ -	+	-	+	+	+(4) -(4)	-(3) +(5)	-	-	-	+	-	+(3) -(5)	*Salmonella* (8)
Blackening of media	BEA	+	-	-	-	-	-	+	-	+	+	-	+	+	+	+	-	+	-	-(10) v(6)	+	+	+	-	-	+	-	*Enterococcus* (16)
Blue	BCA	+	+	+	-	+	-	v(3) +(9)	-	-	-	-(3) +(9)	-	+	-	+	+ -	+	-	-	+	+	-	-	-	-(3) +(9)	+	*Bacillus* (12)
Grey- black	BPA	+	+	+	+(15) -(5)	+(12) -(8)	+	+(9) -(11)	+	+	+(3) v(17)	- (12) v(8)	+	+	+	+	- (19) v(1)	+	-	+	+	+	-	-	-	+(11) -(5) v(4)	-	*Staphylococcus* (20)
Purple- blue black	EMB	-	-	+	-	v	-	+	-	v	+	+	+	-	+	+	-	v	+	+	-	-	+(17) -(8)	+(16) -(9)	+	-	+	*Citrobacter* (25)
Pale-Green	NA	-	+	+	-	-(8) +(2)	-	+(7) -(3)	+	-	-	+(8) -(2)	+	+(9) -(1)	-	-	-	-	-	+(3) -(7)	-	-	-	-	-	-	+	*Pseudomonas* (10)

IMViC test, Indole, Methyl Red, Voges-Proskauer, and Citrate tests; EMB, Eosin methylene blue; XLD, xylose lysine deoxycholate; BEA, Bile esculin agar; BCA, *Bacillus cereus* agar; NA: Nutrient agar;, Negative; +, Positive; v, variable; Number in parenthesis indicates number of isolates

Table 6: Phenotypic and biochemical characterizations of bacteria isolated from traditionally processed fish products of Sikkim

Colony morphology	Media	Gram reaction	Enzymatic reactions								Sugar fermentation															IMViC test			
			Motility	Catalase	Coagulase Reaction	Urease reaction	DNase	Nitrate reduction	Gelatin hydrolysis	Lactose	Sorbitol	Arabinose	Glucose	Mannitol	Mannose	Trehalose	Raffinose	Maltose	Xylose	Rhamnose	Sucrose	Ribose	Adonitol	Indole	Methyl red	Voges-proskauer	Citrate	Tentative identification (Total number of isolates)
Pink without sheen	EMB	-	+	+	-	-(27) v(16)	-	+	-	+(32) v(11)	+	+	+	+	+	+	+(28) -(15)	+	+	+	+	+	-	-(26) v(17)	-	+	+(30) v(13)	*Enterobacter* (43)
Pink, mucoid	EMB	-	-	+	-	+	+	+	+	+(18) -(17)	+	+	+	+	+	+	+	+	+	+(27) v(8)	-(26) v(9)	+	+	-	+(11) -(9) v(15)	+(13) -(10) v(12)	+(17) -(12) v(6)	*Klebsiella* (35)
Purple with metallic sheen	EMB	-	+	+	-	-	-	+	-	+(15) v(7)	+(13) -(9)	+	+	+(12) -(10)	-	+	+	-	+	+(16) v(6)	-(18) v(4)	-	-	+(17) v(5)	+	-	-(15) v(7)	*Escherichia* (22)
Red colonies with black centres	XLD	-	+	+	-	-	-	+	-	-(40) v(10)	+	+	+	+	+	+	-	+	+	+(40) -(10)	-(38) +(12)	-	-	-	+	-	+(32) -(18)	*Salmonella* (50)
Blue	TCBS	-	+	+	-	-	-	+	+	-(10) v(5)	-	-(7) v(8)	+	-	+	+	-	+	-	-	-	-	-	+	-	-	+	*Vibrio* (15)
Blue	BCA	+	+	+	-	+	-	v(23) +(22)	-	-	-	-(33) +(12)	-	+	-	+	+ -	+	-	-	+	+	-	-	-	-(33) +(12)	+	*Bacillus* (45)
Grey- black	BPA	+	+	+	+(15) -(28)	+(23) -(20)	+	+(19) -(24)	+	+	+(33) v(10)	-(12) v(31)	+	+	+	+	-(29) v(14)	+	-	+	+	+	-	-	-	-	+(21) -(15) v(7)	-	*Staphylococc-us* (43)
Purple- blue black	EMB	-	-	+	-	V	-	+	-	v	+	+	+	-	+	+	-	v	+	+	-	-	+	+(6) -(4)	+	-	+	*Citrobacter* (10)
Pale-Green	NA	-	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	*Pseudomonas* (20)

IMViC test, Indole, Methyl Red, Voges-Proskauer, and Citrate tests; EMB, Eosin methylene blue; XLD, xylose lysine deoxycholate; BEA, Bile esculin agar; BCA, *Bacillus cereus* agar; NA: Nutrient agar;, Negative; +, Positive; v, variable; Number in parenthesis indicates number of isolates

Table 7: Grouping of bacterial isolates from traditionally processed meat products of Sikkim on the basis of similar phenotypic characters

Samples	Place of collection	Representative strains		
		Isolate Code	Number of isolates	Total Representative Strains
Beef *Kargyong*	Lal Market, Gangtok	BSLST:44, BULST:54	2	19
		BSMB:16	1	
		BSE:32	1	
		BSE:41	1	
		BSE:27	1	
		BSE:17	1	
Pork *Kargyong*	Retail Shop, Gangtok	PSST:49, PSST:53	2	
		PSKE:30	1	
		PSE:39	1	
		PSE:31	1	
Satchu	Martam, East Sikkim	SMX:21	1	
		SME:36, SME:26	2	
		SME:33	1	
Khyopeh	Lachung, North Sikkim	KHST:43	1	
		KHE:57	1	
		KHE:40	1	

Table 8: Grouping of bacterial isolates from traditionally processed fish products of Sikkim on the basis of similar phenotypic characters

Samples	Place of collection	Representative strains		
		Isolate Code	Number of isolates	Total Representative Strains
Suka ko maacha	Lal Market, Gangtok	ASLST:52 ASE:42 ASE:34	1 1 1	38
Sidra	Lal market, Gangtok	SILST:51 SILX:19 SILT:11	1 1 1	
	Singtam, East Sikkim	SISB:23 SISX:4 SISX:20	1 1 1	
	Rangpo, East Sikkim	SIRST:50, SIRST:56 SIRX:22 SIRE:29 SIRT:12, SIRT:3	2 1 1 2	
	Rhenock, Gangtok	SIRHX:24 SIRHB:9 SIRHE:25	1 1 1	
	Jorethang, South Sikkim	SIJST:46 SIJX:8 SIJB:13	1 1 1	
Sukuti	Rhenock, Gangtok	SKRHX:2 SURHE:38 SURHB:14	1 1 1	
	Lal Market, Gangtok	SULST:47 SULT:15 SUE:25, SUE:28	1 1 2	
	Jorethang, South Sikkim	SUE:37 SUJX:5, SUJX:18	1 2	
	Rangpo, East Sikkim	SURX10 SURST:55, SURST45 SKRT:1	1 2 1	
	Singtam, Gangtok	SUSST:48 SUSB:7 SUE:6	1 1 1	

Genotypic characterization based on 16S rRNA gene sequences

DNA extraction and PCR amplification targeting the 16S rRNA gene sequences of representative bacterial strains were performed for molecular identification of bacteria at the species level. The PCR products were purified and run in the 0.8-1% Agarose Gel (Figure 6). The purified PCR products were further analyzed for DNA sequencing by Sanger Sequencing method. The sequenced bacterial isolates were identified based on BLAST from NCBI database and the sequences were submitted at the GenBank. A total of seventeen bacterial strains were identified from traditionally processed meat (Table 9) and seventeen bacterial strains from traditionally processed fish products of Sikkim (Table 10). Overall, the Gram-negative bacteria were found to be highest with 63% followed by Gram-positive cocci (27%) and Gam-positive bacilli (10%).

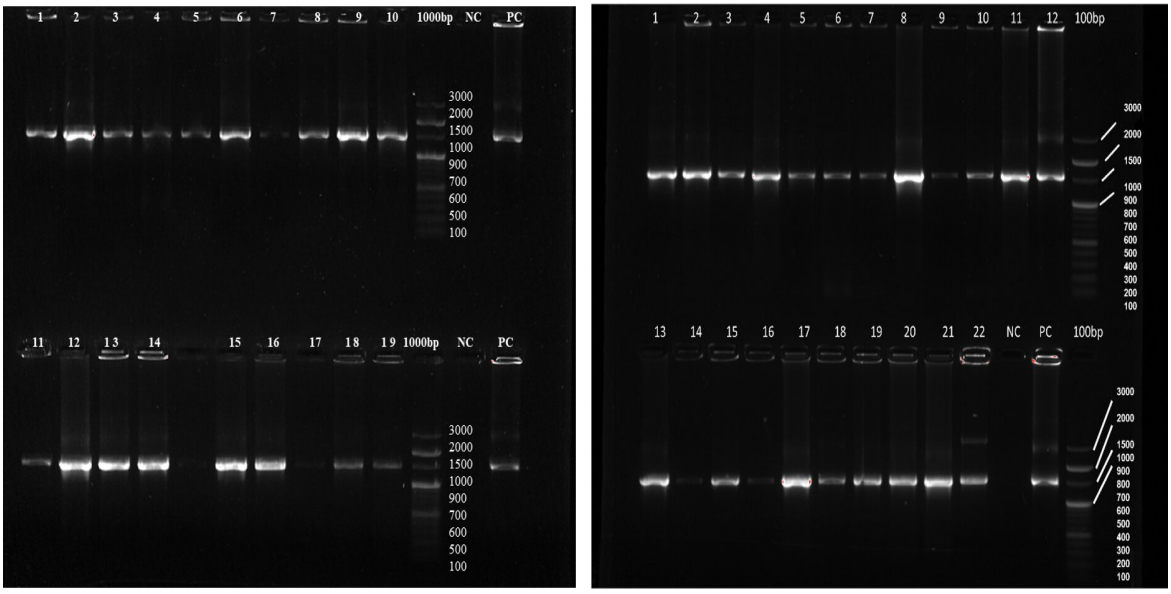


Figure 6: Agarose gel electrophoresis showing purified PCR amplification products of bacterial DNA targeting 16SrRNA region: purified DNA bands of (a) isolates from meat products and (b) isolates from fish products.

Table 9: Identification of bacterial strains isolated from traditionally processed meat products of Sikkim by 16S rRNA gene sequence based on Basic Local Alignment Search Tool (BLAST)

Product	Isolate code	Identity	Type Species (% Similarity)	GenBank Accession Number	Size (base pair)
Beef *Kargyong*	BSE32	*Shigella sonnei*	Shigella sonnei CECT 4887 (93.09%)	MK791725	1073
	BSE27	*Klebsiella pneumonia*	*Klebsiella pneumoniae* subsp. *Rhinoscleromatis* R-70s (98.55%)	MK775240	1442
	BSE41	*Citrobacter freundii*	*Citrobacter freundii* ATCC 8090 (99.16%)	MK775241	1420
	BSE17	*Citrobacter europaeus*	*Citrobacte europaeus* 97/79 (97.23%)	MK774708	1037
	BSLST44	*Staphylococcus piscifermentans*	*Staphylococcus piscifermentans* CIP103958 (98%)	MK788134	1124
	BULST54	*Staphylococcus piscifermentans*	*Staphylococcus piscifermentans* CIP103958 (97.89%)	MK774756	1088
	BSMB16	*Bacillus cereus*	*Bacillus cereus* ATCC 14579 (97%)	MK780063	1040
Pork *Kargyong*	PSE31	*Pseudocitrobacter anthropi*	*Pseudocitrobacter anthropi* C138 (98.24%)	MK775242	1420
	PSE39	*Citrobacter werkmanii*	*Citrobacter werkmanii* CDC 0876-58 (97.96%)	MK775243	1415
	PSKE30	*Burkholderia cepacia*	*Burkholderia cepacia* ATCC 25416 (98.36%)	MK775244	1460
	PSST49	*Staphylococcus saprophyticus*	*Staphylococcus saprophyticus subsp. saprophyticus* ATCC 15305 (98.81%)	MK774760	1424
	PSST53	*Staphylococcus aureus*	*Staphylococcus aureus ATCC 12600*	MK775245	1433
Satchu	SME36	*Klebsiella grimontii*	*Klebsiella grimontii* SB73 (92.47%)	MK791682	1126
	SME26	*Klebsiella aerogenes*	*Klebsiella aerogenes* NCTC10006 (98.28%)	MK788132	1418
	SMX21	*Salmonella enterica*	*Salmonella enterica* subsp. *arizonae* DSM 9386 (97%)	MK780051	1432
	SME33	*Citrobacter freundi*	*Citrobacter freundi* ATCC 8090	MK788133	1423
Khyopeh	KHE40	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469 (99.58%)	MK774757	1415
	KHST43	*Macrococcus caseolyticus*	*Macrococcus caseolyticus* subsp. *hominis* CCM 7927 (99.16%)	MK774759	1434
	KHE57	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433 (98.26%)	MK774758	1433

ATCC: American Type Culture Collection, DSM: Deutsche Sammlung von Mikroorganismen, CCM: Czech Collection of Microorganisms, CECT: Colección Española de Cultivos Tipo, CDC: Centers for Disease Control and Prevention.

Table 10: Molecular identification of bacterial strains isolated from traditionally processed fish products of Sikkim by 16S rRNA gene sequence based on Basic Local Alignment Search Tool (BLAST)

Product	Isolate code	Identity	Type Species (%Similarity)	GenBank Accession Number	Size (base pair)
Suka ko maacha	ASE34	*Enterobacter hormaechei*	*Enterobacter hormaechei* subsp. *xiangfangensis* 10-17 (99%)	MK774706	1420
	ASE42	*Escherichia coli*	*Escherichia coli* U 5/41 (99.02%)	MK775239	1434
	ASLST52	*Staphylococcus sciuri*	*Staphylococcus sciuri* DSM 20345 (99.79%)	MK774707	1432
Sidra	SIRX22	*Enterobacter cloacae*	*Enterobacter cloacae* subsp. *dissolvens* LMG 2683 (99.43%)	MK774764	1407
	SISX4	*Enterobacter cloacae*	*Enterobacter cloacae* subsp. *dissolvens* LMG 2683 (98.45%)	MK789855	1420
	SISX20	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. *rhinoscleromatis* R-70 (99.51%)	MK780048	1420
	SILX19	*Klebsiella pneumoniae*	*Klebsiella pneumonia* DSM 30104 (98.24%)	MK789854	1411
	SIJX8	*Salmonella enterica*	*Salmonella enterica* subsp. *salamae* DSM 9220 (97.23%)	MK775248	1400
	SIRHE35	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469 (99.29%)	MK774763	1421
	SIRE29	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469 (96.04%)	MK774762	1101
	SIRHX24	*Escherichia coli*	*Escherichia coli* NBRC 102203 (98.23%)	MK780042	1427
	SILT11	*Providencia stuartii*	*Providencia stuartii* DSM 4539 (98.45%)	MK780040	1427
	SIRT12	*Providencia vermicola*	*Providencia vermicola* OP1 (99.72%)	MK780045	1420
	SIRT3	*Providencia rettgeri*	*Providencia rettgeri NCTC 11801 (99.23%)*	MK780046	1424
	SIRHB9	*Bacillus cereus*	*Bacillus cereus* ATCC 14579 (98.60%)	MK780041	1436
	SIJB13	*Bacillus cereus*	*Bacillus cereus* ATCC 14579 (99.79%)	MK775246	1433
	SISB23	*Bacillus cereus*	*Bacillus cereus* ATCC 14579 (100%)	MK780047	1457
	SIJST46	*Staphylococcus edaphicus*	*Staphylococcus edaphicus* CCM 8730 (98.61%)	MK775247	1441
	SIRST50	*Staphylococcus sciuri*	*Staphylococcus sciuri* DSM 20345 (98.18%)	MK780043	1193
	SIRST56	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 12600 (97.24%)	MK780044	1448

	SILST51	*Staphylococcus nepalensis*	*Staphylococcus nepalensis* CW1 (99.23%)	MK774761	1437
Sukuti	SUE28	*Enterobacter hormaechei*	*Enterobacter hormaechei* subsp. *xiangfangensis* 10-17 (98.37%)	MK774766	1417
	SUE25	*Enterobacter hormaechei*	*Enterobacter hormaechei* subsp. *xiangfangensis* 10-17 (99.79%)	MK780052	1418
	SUE6	*Enterobacter hormaechei*	*Enterobacter hormaechei* subsp. xiangfangensis 10-17 (99.64%)	MK774765	1419
	SUE37	*Enterobacter cancerogenus*	*Enterobacter cancerogenus* LMG 2693 (98.34%)	MK780053	1460
	SURHE38	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* DSM 30104 (99.65%)	MK780058	1425
	SUJX18	*Salmonella enterica*	*Salmonella enterica* subsp. *salamae* DSM 9220 (98.24%)	MK774767	1420
	SUJX5	*Salmonella enterica*	*Salmonella enterica* subsp. *enterica* LT2 (98.57%)	MK780054	1411
	SURX10	*Salmonella enterica*	*Salmonella enterica* subsp. *enterica* LT2 (98.29%)	MK780060	1458
	SKRHX2	*Acinetobacter radioresistens*	*Acinetobacter radioresistens* NBRC 102413 (99.44%)	MK780049	1431
	SULT15	*Pseudomonas plecoglossicida*	*Pseudomonas plecoglossicida* NBRC 103162 (99.23%)	MK780056	1430
	SKRT1	*Providencia vermicola*	*Providencia vermicola* OP1 (99.72%)	MK780050	1418
	SULST47	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 12600 (97.69%)	MK780055	1427
	SUSST48	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 12600 (97.20%)	MK780062	1440
	SURST55	*Staphylococcus vitulinus*	*Staphylococcus vitulinus* ATCC 51145(99.93%)	MK774768	1439
	SURST45	*Staphylococcus sciuri*	*Staphylococcus sciuri* subsp. *rodentium* GTC 844 (98.05%)	MK780059	1442
	SURHB14	*Bacillus cereus*	*Bacillus cereus* ATCC 14579 (98.83%)	MK780057	1447
	SUSB7	*Bacillus cereus*	*Bacillus cereus* ATCC 14579 (99.79%)	MK780061	1436

ATCC: American Type Culture Collection, LMG: Laboratorium voor Microbiologie, DSM: Deutsche Sammlung von Mikroorganismen**,** ***NBRC:*** NITE Biological Resource Center***, NCTC:*** National Collection of Type Cultures, CCM: Czech Collection of Microorganisms.

Bacterial species in traditional meat products

In traditionally processed meat products *Staphylococcus piscifermentans* showed the highest abundances of bacteria with 12% followed by *Citrobacter freundii* (10%), *Enterococcus faecalis* (5%), *Salmonella enterica* (5%), *Staphylococcus aureus, Citrobacter werkamanii* (5%), *Klebsiella pneumonia* (5%), *Macrococcus caseolyticus* (5%), *Klebsiella aerogenes* (5%), *Staphylococcus saprophyticus* (5%), *Pseudocitrobacter anthropi* (5%), *Citrobacter europaeus* (5%), *Shigella sonnei* (5%), *Escherichia fergusonii* (5%), *Klebsiella grimontii* (5%), *Burkholderia cepacia* (5%) and *Bacillus cereus* (5%) (Figure 7).

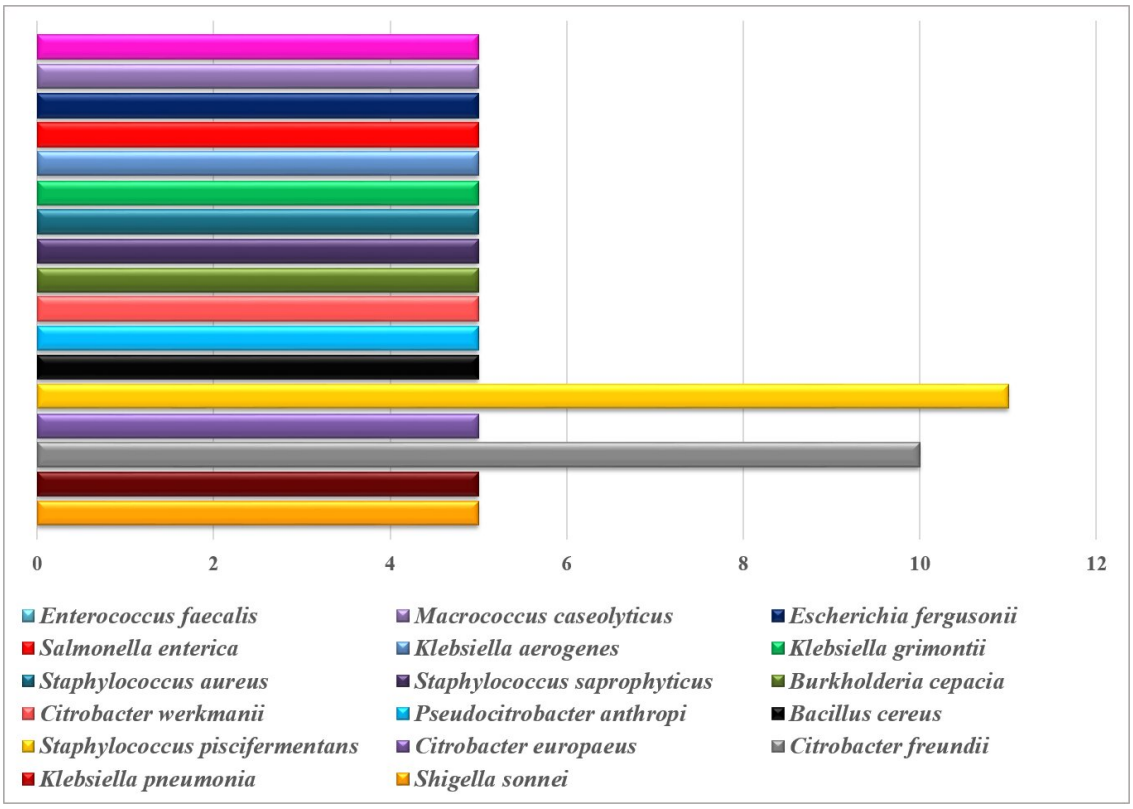


Figure 7: Percentile distribution of different bacterial species present in processed meat products of Sikkim identified by Sanger Sequencing Method.

Beef *Kargyong*

A total of six different bacterial species were identified in beef *kargyong*. They are Gram-positive bacteria: *Staphylococcus piscifermentans* (29%) and *Bacillus cereus*

(14%), and Gram-negative bacteria: *Shigella sonnei* (15%), *Klebsiella pneumoniae* (14%), *Citrobacter europaeus* (14%) and *Citrobacter freundii* (14%) (Figure 8a).

Pork *Kargyong*

In pork *kargyong*, five bacterial species were identified viz. Gram-positive bacteria: *Staphylococcus aureus* (20%), *Staphylococcus saprophyticus* (20%); Gram-negative bacteria: *Burkholderia cepacia* (20%), *Citrobacter werkamanii* (20%) and *Pseudocitrobacter anthropic* (20%) (Figure 8b).

Satchu

Four different bacterial species were identified in *satchu* and they belong to Gram-negative bacteria: *Citrobacter freundii* (25%), *Klebsiella grimontii* (25%), *Salmonella enterica* (25%) and *Klebsiella aerogens* (25%) (Figure 8c).

Khyopeh

Three bacterial species were found in *khyopeh* viz. Gram-positive bacteria: *Enterococcus faecalis* (33%); Gram-negative bacteria: *Escherichia fergusonii* (33%) and *Macrococcus caseolyticus* (33%) (Figure 8d).

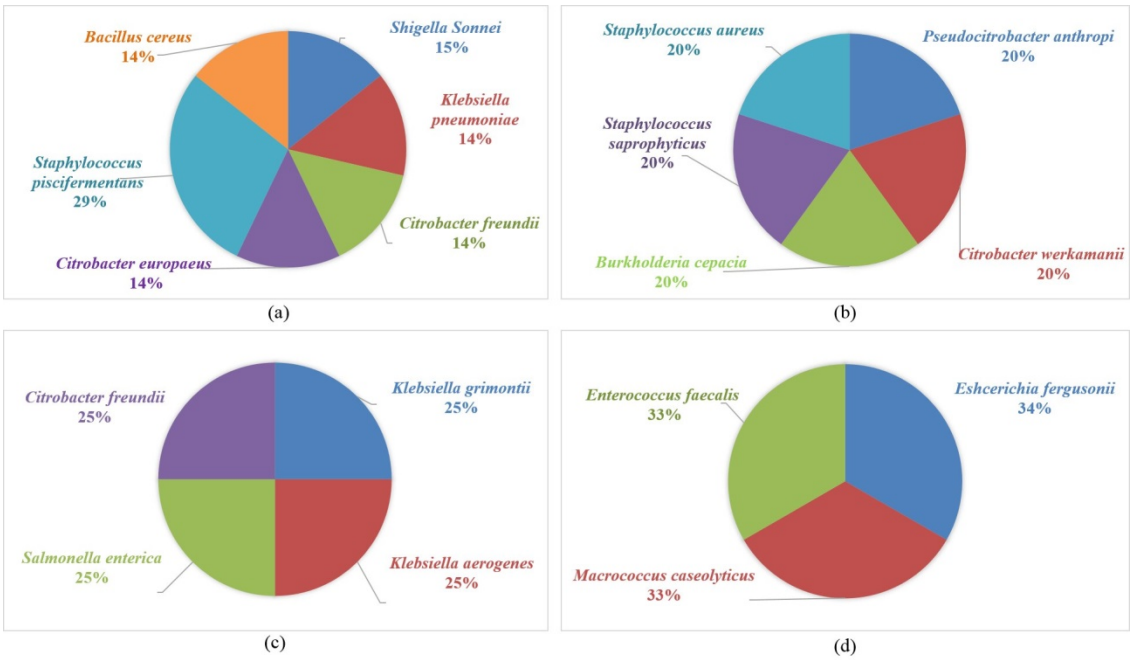


Figure 8: Percentile distributions of different bacterial species present among various meat products (a) Beef *kargyog*, (b) Pork *kargyong*, (c) *Satchu* and (d) *Khyopeh*.

Alpha diversity Indices

The alpha diversity indices of traditionally processed meat products were observed with highest Chao1 index of 15 in pork *kargyong* followed by Chao1 index of 11 in beef *kargyong*, 10 in *satchu* and 6 in *khyopeh*, respectively (Table 11). Shannon-H index was recorded highest in beef *kargyong* with 1.748, pork *kargyong* 1.609, *satchu* 1.386 and lowest in *khyopeh* with 1.009 (Table 11). Simpson index value in beef *kargyong* and pork *kargyong* was 0.8 followed by *satchu* (0.75) and *khyopeh* (0.66), respectively (Table 11).

Table 11: Diversity indices of traditionally processed meat products of Sikkim

Products	Diversity Indices			
	Chao1	Dominance-D	Shannon _H	Simpson_1-D
Beef *kargyong*	11	0.1837	1.748	0.8163
Pork kargyong	15	0.2	1.609	0.8
Satchu	10	0.25	1.386	0.75
Khyopeh	6	0.333	1.099	0.667

Bacterial species in traditional fish products

In traditionally processed fish products of Sikkim, *Bacillus cereus* showed the highest abundances of bacteria with 13% followed by *Salmonella enterica* (11%), *Staphylococcus sciuri* (9%), *Enterobacter hormaechei* (8%), *Staphylococcus aureus* (8%), *Enterobacter cloacae* (8%), *Klebsiella pneumonia* (8%), *Escherichia coli* (6%), *Providencia vermicola* (6%), *Escherichia fergusonii* (5%), *Providencia stuartii* (3%), *Providencia rettgeri* (3%) *Staphylococcus edaphicus* (3%), *Staphylococcus nepalensis* (3%), *Enterobacter cancerogenus* (3%), *Acinetobacter radioresistens* (3%), *Pseudomonas plecoglossicida* (3%) and *Staphylococcus vitulinus* (3%) (Figure 9).

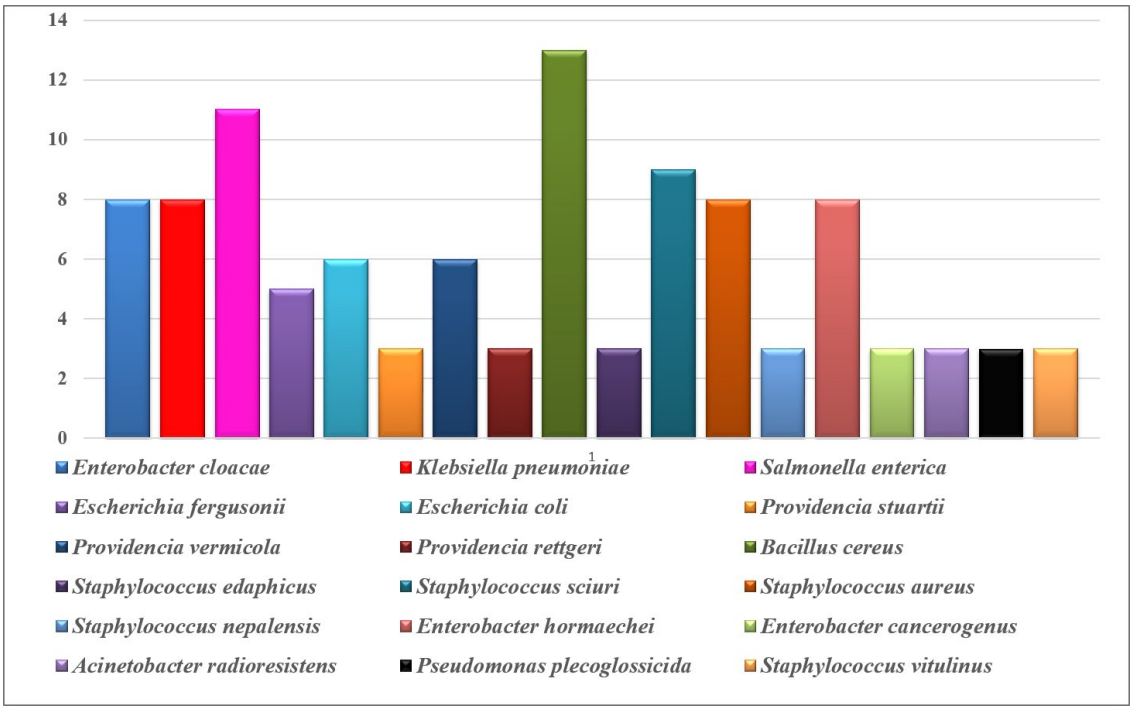


Figure 9: Percentile distribution of different bacterial species present in processed fish products of Sikkim identified by Sanger Sequencing Method.

Suka ko maacha

In *suka ko maacha*, three species of bacteria were identified viz. Gram-positive bacteria: *Staphylococcus sciuri* (33%); Gram-negative bacteria: *Enterobacter hormaechei* (34%), *Escherichia coli* (33%) and (Figure 10a).

Sidra

A total of thirteen different bacterial species were found in *sidra* out of which Gram-positive bacteria were *Bacillus cereus* (17%), *Staphylococcus nepalensis* (6%), *Staphylococcus aureus* (6%), *Staphylococcus edaphicus* (6%), *Staphylococcus sciuri*; and Gram-negative bacteria included *Klebsiella pneumoniae* (11%), *Enterobacter clocae* (11%), *Escherichia fergusonii* (11%), (6%), *Providencia rettgeri* (6%), *Salmonella enterica* (5%), *Providencia vermicola* (5%), *Providencia stuartii* (5%) and *Escherichia coli* (5%) (Figure 10b).

Sukuti

In *sukuti* eleven bacterial species were found. Gram-positive bacteria were represented by *Bacillus cereus* (12%), *Staphylococcus aureus* (12%), *Staphylococcus vitulinus* (6%) and *Staphylococcus sciuri* (6%). Gram-negative bacteria included *Enterobacter hormaechei* (17%), *Salmonella enterica* (17%), followed by *Enterobacter cancerogenus* (6%), *Providencia vermicola* (6%), *Pseudomonas plecoglossicida* (6%), *Acinetobacter radioresistens* (6%), *Klebsiella pneumoniae* (6%) (Figure 10c).

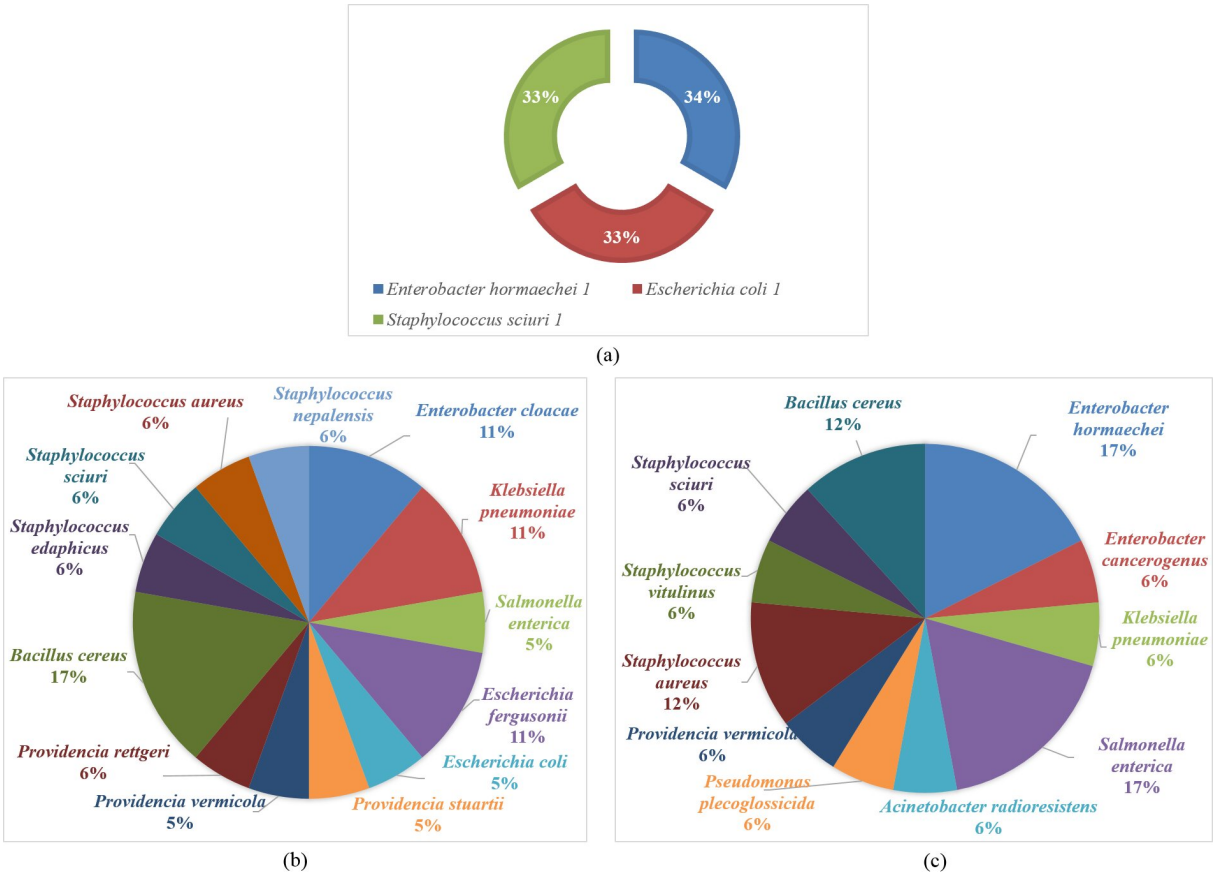


Figure 10: Percentile distribution of different bacterial species present among various fish products (a) *Suka ko maacha*, (b) *Sidra* and (c) *Sukuti*.

Alpha Diversity Indices

The alpha diversity indices of traditionally processed fish products showed Shannon-H index value of 2.476 in *sidra* followed by sukuti (2.282) and *suka ko maacha* (1.099), respectively (Table 12). Chao1 index value in *sidra* was 22 followed by 18 in *sukuti* and 6 in *suka ko maacha*, respectively (Table 12). Simpson index value observed in *sidra* was 0.9, *sukuti* (0.8) and *suka ko maacha* (0.6), respectively (Table 12).

Table 12: Diversity indices of traditionally prepared fish products of Sikkim				
Products	**Diversity Indices**			
	Chao1	**Dominance-D**	**Shannon _H**	**Simpson_1-D**
Suka ko maacha	6	0.333	1.099	0.667
Sidra	22	0.9529	2.476	0.9074
Sukuti	18	0.1142	2.282	0.8858

Molecular Phylogeny

Molecular phylogenetic analysis of bacterial isolates from meat (19 representative isolates) (Fig. 11) and fish products (38 representative isolates) (Fig. 12) based on 16S rRNA region sequencing were constructed using MEGA 7 software. The bootstrap consensus tree derived with 1000 replicates to Neighbour-joining method and Kimura 2-parameter with *Halobacterium noricence* AJ548827 as the out group. Numbers on branches depicted the percent occurrence of a given branch during 1000 replicates (Figure 11-12). Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

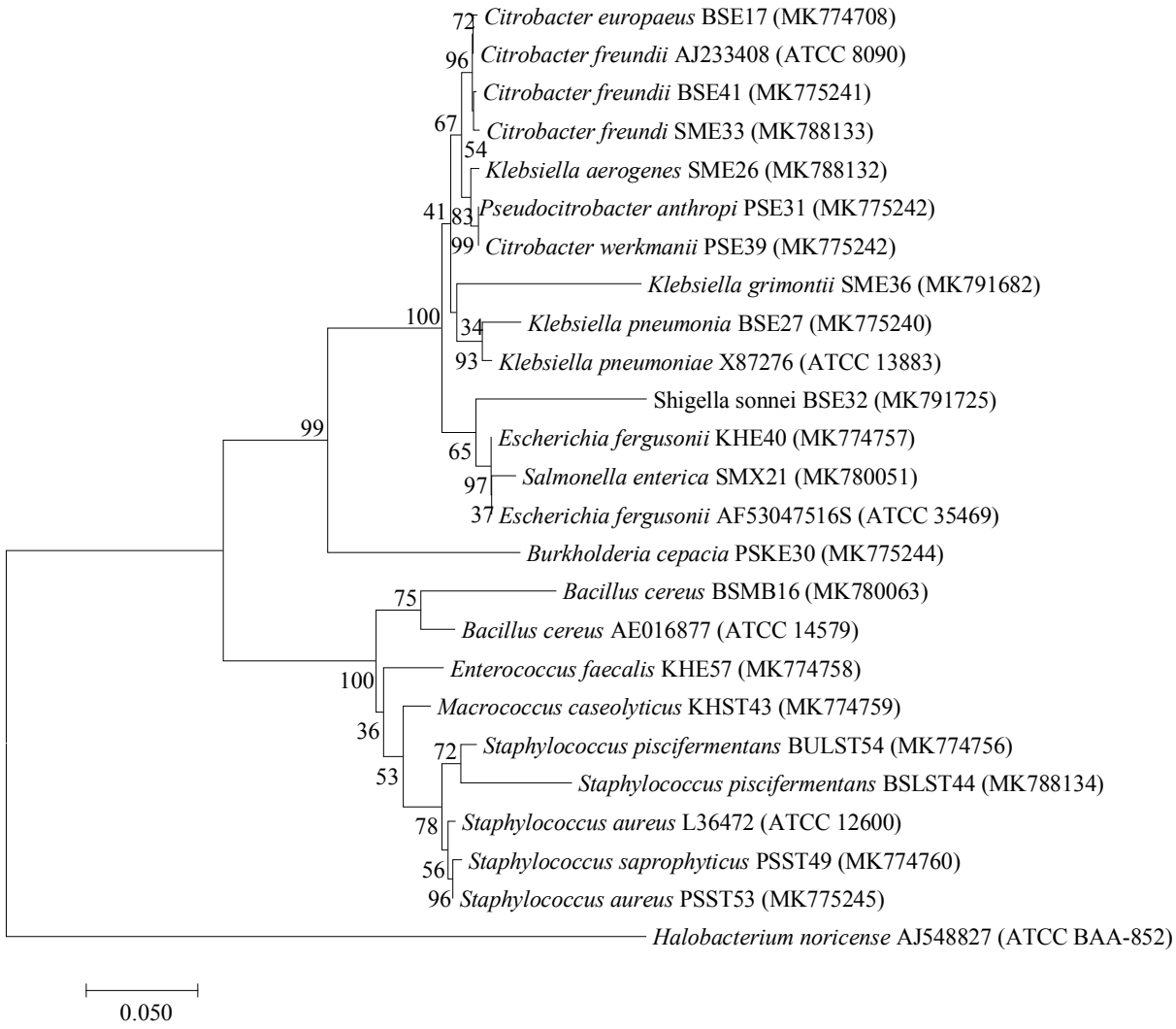


Figure 11: Molecular phylogenetic analysis of 19 bacterial isolates recovered from traditional processed meat products based on 16S rRNA region sequencing. Neighbouring-joining phylogenetic tree representation by MEGA 7 with *Halobacterium noricence* AJ548827 as the out group with the evolutionary history (Saitou and Nei 1987). The optimal tree with a sum of branch length=1.13658194 and the percentage of replicate trees in which the clustered associated taxa in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were calculated by the Kimura 2-parameter method (Tamura et al. 2004) and are expressed in the units of the number of base substitutions per site. Analysis involved 25 nucleotide sequences with a total of 569 positions in the final dataset. Evolutionary analyses were conducted in the MEGA7 software (Kumar et al. 2016).

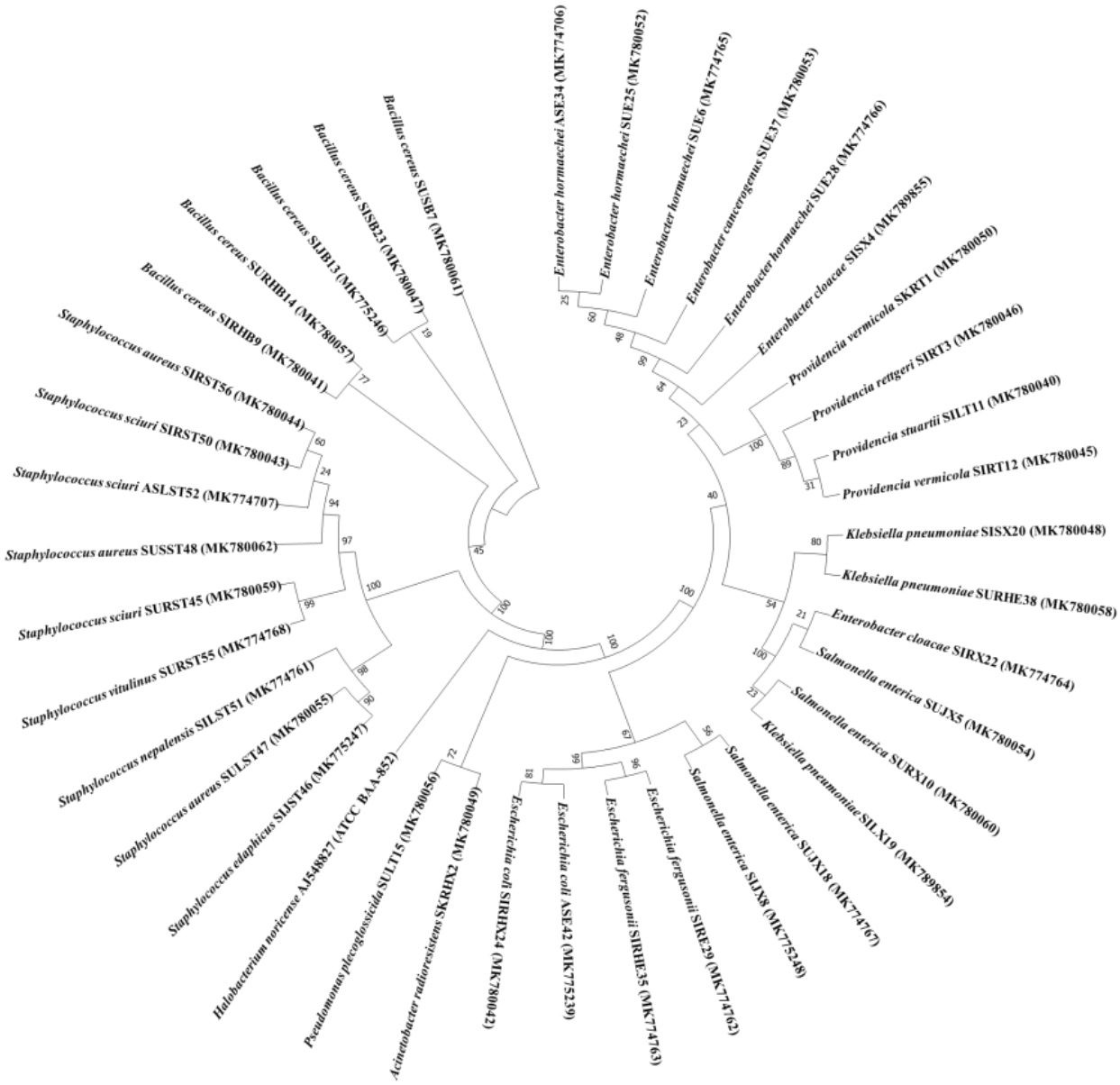


Figure 12: Molecular phylogenetic analysis of 38 bacterial isolates recovered from traditional processed fish products based on 16S rRNA region sequencing. Neighboring-joining phylogenetic tree representation by MEGA 7 with *Halobacterium noricence* AJ548827 as the out group with the evolutionary history (Saitou and Nei 1987). The optimal tree with a sum of branch length=1.13658194 and the percentage of replicate trees in which the clustered associated taxa in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were calculated by the Kimura 2-parameter method (Tamura et al. 2004) and are expressed in the units of the number of base substitutions per site. Analysis involved 39 nucleotide sequences with a

total of 913 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

ENZYME LINKED IMMUNO SORBENT (ELISA) ASSAY

The ELISA tests were done for three different bacterial toxins which include Tecra *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (Figure 13a), Tecra *Salmonella* Visual Immunoassay (Figure13b) and Ridascreen *Staphylococcus* Enterotoxin Assay (Figure 13c). For each sample, the assay was performed in duplicates. A total of 56 samples were tested for ELISA i.e. 26 samples of traditional meat products and 30 traditional fish products.

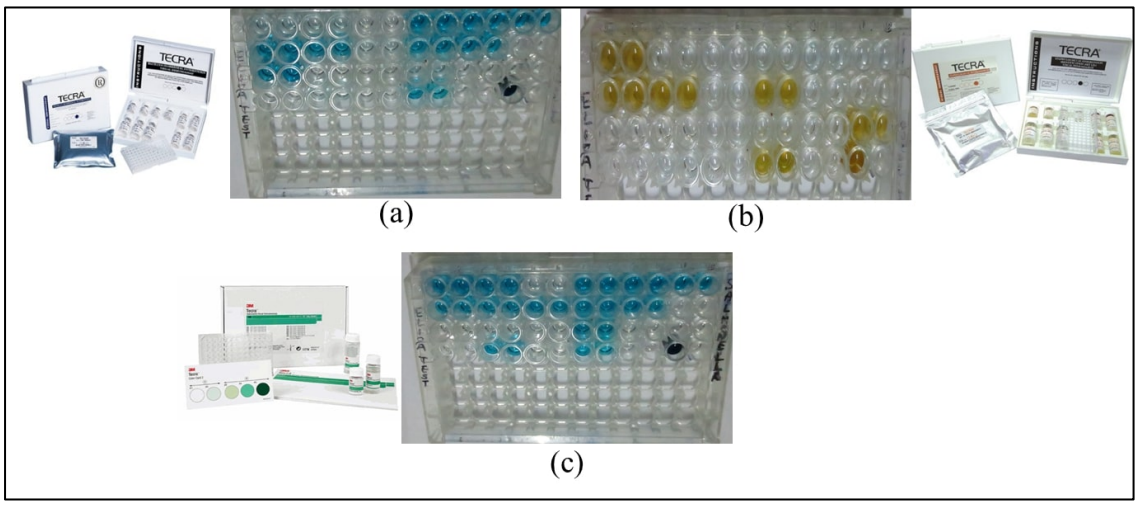


Figure 13: Enzyme Linked Immuno-Sorbent Assay for detection of various enterotoxins from meat and fish products of Sikkim (a) *Salmonella* enterotoxin, (b) Staphylococcal enterotoxin and (c) *Bacillus cereus* enterotoxin.

(1) Assessment of *Bacillus* Diarrhoeal Enterotoxin, *Salmonella* and Staphylococcal Enterotoxins (A-E) from traditionally processed meat products

Out of twenty-six meat products, only four samples viz. beef *kargyong* (n=2) and *satchu* (n=2) purchased from Martam, East Sikkim were tested positive for *Bacillus* Diarrhoeal Enterotoxin (Table 13). *Salmonella* was found positive in *Satchu* of Martam (n=2) and pork *kargyong* of Burtuk, East Sikkim (n=2) (Table 14). Staphylococcal

enterotoxins tested positive for beef *kargyong* of Martam (n=2) and pork *kargyong* of Lalbazaar, East Sikkim (n=2) (Table 15). Interestingly, only *khyopeh* of North Sikkim tested negative for all the three bacterial enterotoxins. Percentile and location wise distribution of bacterial toxins present in various products of Sikkim are shown in Figure 14-15.

Table 13: ELISA Tests for traditionally processed meat products of Sikkim for *Bacillus* Diarrhoeal enterotoxin

Products	Place	Colour reactions	Results
Beef *kargyong* (n=2)	Martam, East Sikkim	Green	+
Satchu (n=2)		Green	+
Beef *Kargyong* (n=2)	Lalbazaar, Gangtok	Colourless	-
Pork *Kargyong* (n=2)		Colourless	-
Satchu (n=2)		Colourless	-
Beef *Kargyong* (n=2)	Kitam, South Sikkim	Colourless	-
Pork *kargyong* (n=2)		Colourless	-
Beef *Kargyong* (n=2)	Geyzing, West Sikkim	Colourless	-
Pork *kargyong* (n=2)		Colorless	-
Satchu (n=2)		Colourless	-
Pork *kargyong* (n=2)	Burtuk, Gangtok	Colourless	-
Yak *Kargyong* (n=2)	Lachung, North Sikkim	Colourless	-
Khyopeh (n=2)		Colourless	-
Positive control		Green	+
Negative control		Colourless	-

n= number of samples tested; –, indicates the absence of toxins; +, indicates the presence of toxins

Table 14: ELISA Tests for traditionally processed meat products of Sikkim for *Salmonella* enterotoxin			
Products	**Place**	**Colour reactions**	**Results**
Beef *kargyong* (n=2)	Martam, East Sikkim	Colourless	-
Satchu (n=2)		Green	+
Beef *Kargyong* (n=2)	Lalbazaar, Gangtok	Colourless	-
Pork *Kargyong* (n=2)		Colourless	-
Satchu (n=2)		Colourless	-
Beef *Kargyong* (n=2)	Kitam, South Sikkim	Colourless	-
Pork *kargyong* (n=2)		Colourless	-
Beef *Kargyong* (n=2)	Geyzing, West Sikkim	Colourless	-
Pork *kargyong* (n=2)		Colourless	-
Satchu (n=2)		Colourless	-
Pork *kargyong* (n=2)	Burtuk, Gangtok	Green	+
Khyopeh (n=2)	Lachung, North Sikkim	Colourless	-
Yak *kargyong* (n=2)		Colourless	-
Positive control		Green	+
Negative control		Colourless	-

n= number of samples tested; –, indicates the absence of toxins; +, indicates the presence of toxins

Table 15: ELISA Tests for traditionally processed meat products of Sikkim Staphylococcal enterotoxin			
Products	**Place**	**Colour reactions**	**Results**
Beef *kargyong* (n=2)	Martam, East Sikkim	Yellow	+
Satchu (n=2)		Colourless	-
Beef *kargyong* (n=2)	Lalbazaar, Gangtok	Colourless	-
Pork *Kargyong* (n=2)		Yellow	+
Satchu (n=2)		Colourless	-
Beef *kargyong* (n=2)	Kitam, South Sikkim	Colourless	-
Pork *kargyong* (n=2)		Colourless	-
Beef *kargyong* (n=2)	Geyzing, West Sikkim	Colourless	-
Pork *kargyong* (n=2)		Colorless	-
Satchu (n=2)		Colourless	-
Pork *kargyong* (n=2)	Burtuk, Gangtok	Colourless	-
Yak kargyong (n=2)	Lachung, North Sikkim	Colourless	-
Khyopeh (n=2)		Colourless	-
Positive control		Yellow	+
Negative control		Colourless	-

n= number of samples tested; –, indicates the absence of toxins; +, indicates the presence of toxins

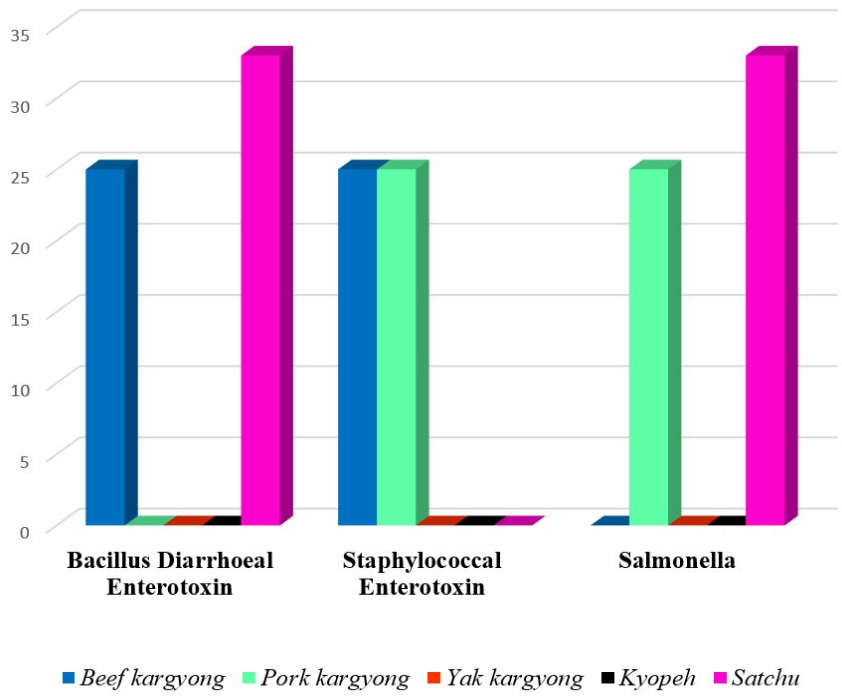


Figure 14: Percentile distribution of different bacterial enterotoxins present among various meat products.

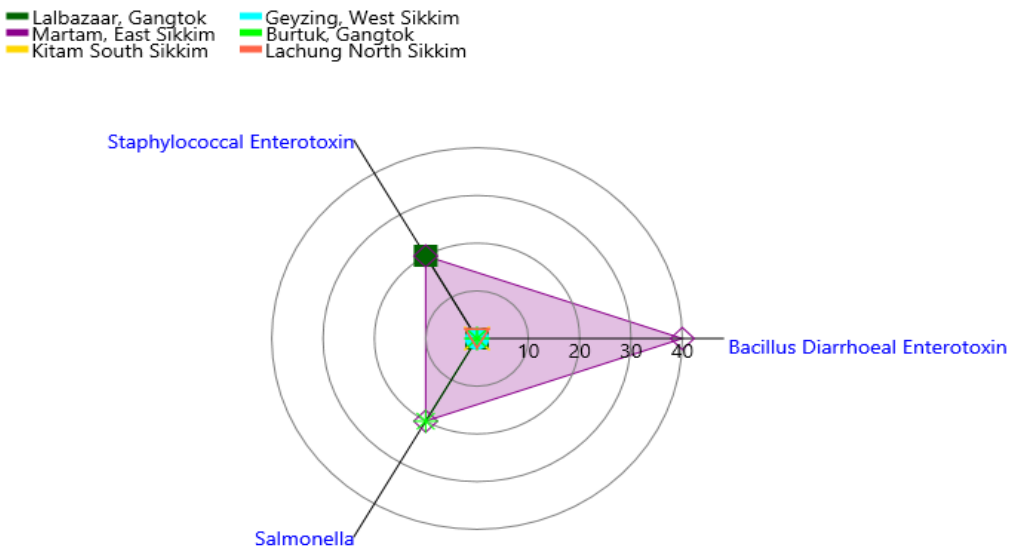


Figure 15: Location-wise distribution of bacterial enterotoxins present in meat products.

(2) Assessment of *Bacillus* Diarrhoeal Enterotoxin, *Salmonella* and Staphylococcal Enterotoxins (A-E) from traditionally processed fish products

A total of thirty fish products were analysed for the detection of bacterial enterotoxins by ELISA technique. Among the fish products, *suka ko maacha*, *sidra* and *sukuti* purchased from Rangpo, East Sikkim (n=6), *sidra* and *sukuti* of Rhenock (n=4), *sidra* and *sukuti* of Jorethang, South Sikkim (n=4) and *sidra* of Singtam (n=2) tested positive for *Bacillus* Diarrhoeal Enterotoxin (Table 16). *Salmonella* was detected in all the three fish products of Rangpo and Jorethang viz. *suka ko maacha*, *sidra* and *sukuti* (n=12), *sidra* and *sukuti* of Rhenock and Singtam (n= 8) (Table 17). Staphylococcal enterotoxins was detected in *suka ko maacha* and *sidra* of Jorethang (n=4), *sukuti* of Lalbazaar and Rangpo (n=4) (Table 18). Percentile and location wise distribution of bacterial toxins present in various fish products of Sikkim are shown in Figure 16-17.

Table 16: ELISA Tests for traditionally processed fish products of Sikkim for *Bacillus* Diarrhoeal enterotoxin			
Products	**Place**	**Color reactions**	**Results**
Suka ko maacha (n=2)	Lalbazaar, Gangtok	Colourless	-
Sidra (n=2)		Colourless	-
Sukuti (n=2)		Colourless	-
Suka ko maacha (n=2)	Rhenock, East Sikkim	Colourless	-
Sidra (n=2)		Green	+
Sukuti (n=2)		Green	+
Suka ko maacha (n=2)	Rangpo, East Sikkim	Green	+
Sidra (n=2)		Green	+
Sukuti (n=2)		Green	+
Suka ko maacha (n=2)	Jorethang, South Sikkim	Colourless	-
Sidra (n=2)		Green	+
Sukuti (n=2)		Green	+
Suka ko maacha (n=2)	Singtam, South Sikkim	Colourless	-
Sidra (n=2)		Green	+
Sukuti (n=2)		Colourless	-
Positive control		Green	+
Negative control		Colourless	-

n= number of samples tested; –, indicates the absence of toxins; +, indicates the presence of toxins

Table 17: ELISA Tests for traditionally processed fish products of Sikkim for *Salmonella* enterotoxin			
Products	**Place**	**Colour reactions**	**Results**
Suka ko maacha (n=2)	Lalbazaar, Gangtok	Colourless	-
Sidra (n=2)		Colourless	-
Sukuti (n=2)		Colourless	-
Suka ko maacha (n=2)	Rhenock, East Sikkim	Colourless	-
Sidra (n=2)		Green	+
Sukuti (n=2)		Green	+
Suka ko maacha (n=2)	Rangpo, East Sikkim	Green	+
Sidra (n=2)		Green	+
Sukuti (n=2)		Green	+
Suka ko maacha (n=2)	Jorethang, South Sikkim	Green	+
Sidra (n=2)		Green	+
Sukuti (n=2)		Green	+
Suka ko maacha (n=2)	Singtam, South Sikkim	Colourless	-
Sidra (n=1)		Green	+
Sukuti (n=1)		Green	+
Positive control		Green	+
Negative control		Colourless	-

n= number of samples tested; –, indicates the absence of toxins; +, indicates the presence of toxins

Table 18: ELISA Tests for traditionally processed fish products of Sikkim for Staphylococcal enterotoxin			
Products	**Place**	**Colour**	**Results**
Suka ko maacha (n=2)	Lalbazaar, Gangtok	Colourless	-
Sidra (n=2)		Colourless	-
Sukuti (n=2)		Yellow	+
Suka ko maacha (n=2)	Singtam, East Sikkim	Colourless	-
Sidra (n=2)		Colourless	-
Sukuti (n=2)		Colourless	-
Suka ko maacha (n=2)	Jorethang, South Sikkim	Yellow	+
Sidra (n=2)		Yellow	+
Sukuti (n=2)		Colourless	-
Suka ko maacha (n=2)	Rangpo, East Sikkim	Colourless	-
Sidra (n=2)		Colourless	-
Sukuti (n=2)		Yellow	+
Suka ko maacha (n=2)	Rhenock, East Sikkim	Colourless	-
Sidra (n=2)		Colourless	-
Sukuti (n=2)		Colourless	-
Positive control		Yellow	+
Negative control		Colourless	-

n= number of samples tested; –, indicates the absence of toxins; +, indicates the presence of toxins

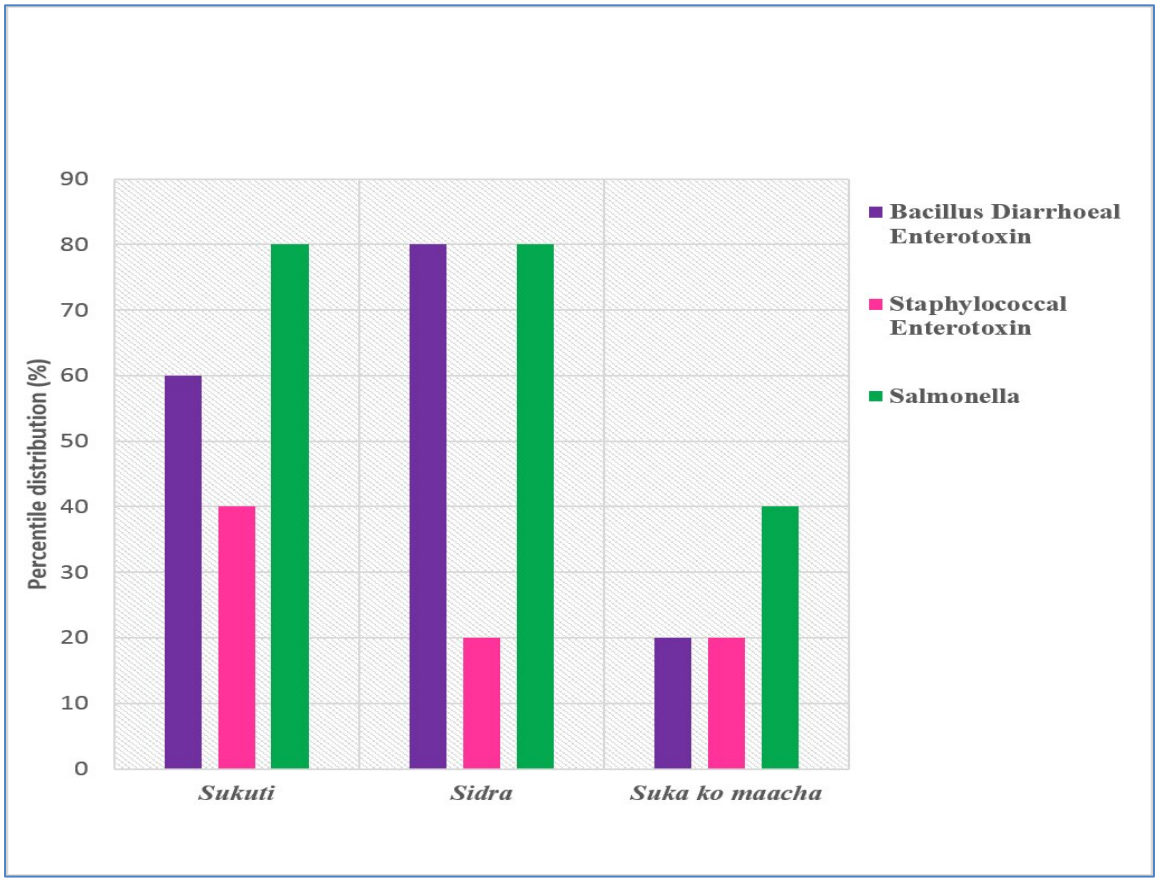


Figure 16: Percentile distribution of different bacterial bacterial enterotoxins present in fish products.

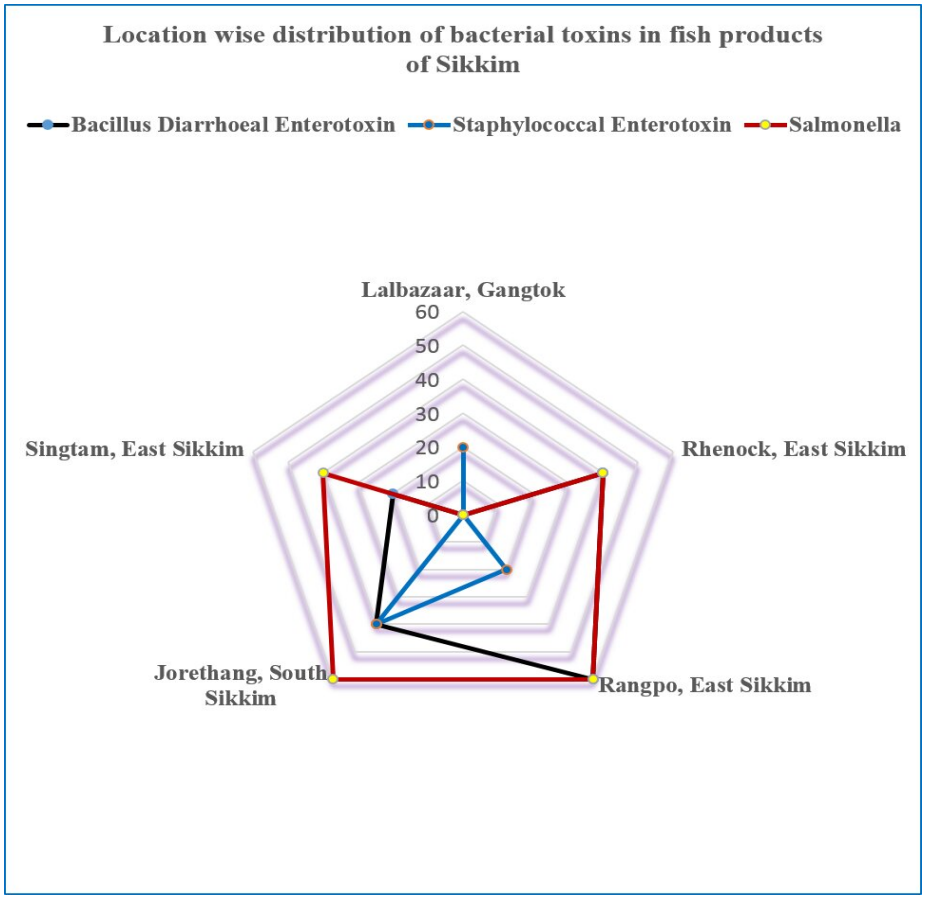


Figure 17: Location-wise distribution of bacterial enterotoxins present in fish products.

DETECTION OF VIRULENCE ENTEROTOXIN GENES

After screening of isolates for toxin production by ELISA technique and molecular identification of representative bacteria, 24 bacterial isolates were tested for the presence of some virulent genes of *Bacillus cereus* in beef *kargyong* (n=1), *sidra* (n=3), *sukuti* (n=2), *Staphylococcus aureus* in beef kargyong (n=2), pork *kargyong* (n=2), *khyopeh* (n=1), *suka ko maacha* (n=1), *sidra* (n=4), *sukuti* (n=3) and *Salmonella enterica* in *satchu* (n=1), *sidra* (n=1) and *sukuti* (n=3) by PCR analysis (Table 19). *Bacillius cereus* isolated from beef *kargyong* (BSMB16) was found negative for *nhea* and *nheb* genes (Figure 18a) whereas all the 5 isolates from fish products viz. *sidra* (SIJB13, SIRHB9, SISB23) and *sukuti* (SUSB7, SURHB14) tested positive for both

nhea and *nheb* enterotoxin genes (Figure 19a,b). Bacterial isolates were also tested for the presence of virulent genes *inv* and *stn* in *Salmonella*. All 5 isolates from meat and fish products were tested negative for *Salmonella* enterotoxin gene (Figure 18b, Figure 19c,d). All strains of *Staphylococcus* were tested for the presence of virulent genes (*sea*) and among the meat products, 3 out of 5 bacterial isolates were found positive for the same. Beef *kargyong* (BSLST44, BULST54) and pork *kargyong* (PSST53) identified as *Staphylococcus piscifermentans* and *Staphylococcus aureus* were found positive for *sea* virulent gene (Figure 18c). *Sea* gene was not found in isolates from *khyopeh* sample. Among the fish products out of 8 isolates, 5 isolates were tested negative for staphylococcal *sea* enterotoxin gene and 3 isolates viz. *sidra* identified as *Staphylococcus aureus* (SULST47, SIRST56) and *Staphylococcus edaphicus* (SIJST46) were found positive for staphylococcal enterotoxin gene (Figure 19e). The overall prevalence of various bacterial enterotoxin genes in fish products are shown in Figure 20.

Table 19: Prevalence of virulent genes of bacterial pathogens isolated from meat and fish products of Sikkim				
Sample	**Identified bacteria**	**Virulent genes**	**Isolates**	**Results**
Beef *Kargyong*	*Bacillus cereus*	*nhea* *nheb*	BSMB16	-
Sidra			SIJB13 SIRHB9 SISB23 SUSB7	+ + + +
Sukuti			SURHB14	+
Negative control			MK775239	-
Positive control			MTCC 1272	+
Beef *Kargyong*	*Staphylococcus*	*Sea*	BSLST44 BULST54	+ +
Pork *Kargyong*			PSST49 PSST53	- +
Khyopeh			KH1	-
Suka ko maacha			ASLST52	-
Sidra			SIRST56 SILST51 SIRST50 SIJST46	+ - - +
Sukuti			SUSST48 SURST45 SULST47	- - +
Negative control			MK775239	-
Positive control			MTCC 740	+
Satchu	*Salmonella*	*inv* *stn*	SMX21	-
Sidra			SIJX8	-
Sukuti			SUJX18 SUJX5 SURX10	- - -
Negative control			MK774707	-
Positive control			MTCC 3223	+

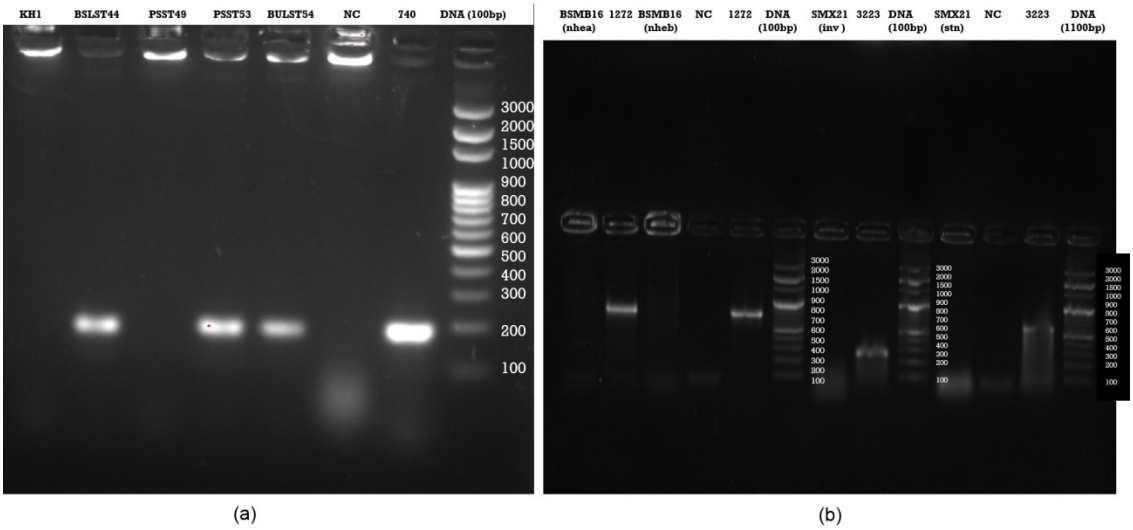


Figure 18: Agarose gel electrophoresis showing PCR amplification products for (a) *Staphylococcus* enterotoxin gene (sea), (b) *Bacillus cereus* enterotoxin genes (nhea, nheab) and (b) *Salmonella* (inv, stn) enterotoxin genes isolated from meat products of Sikkim.

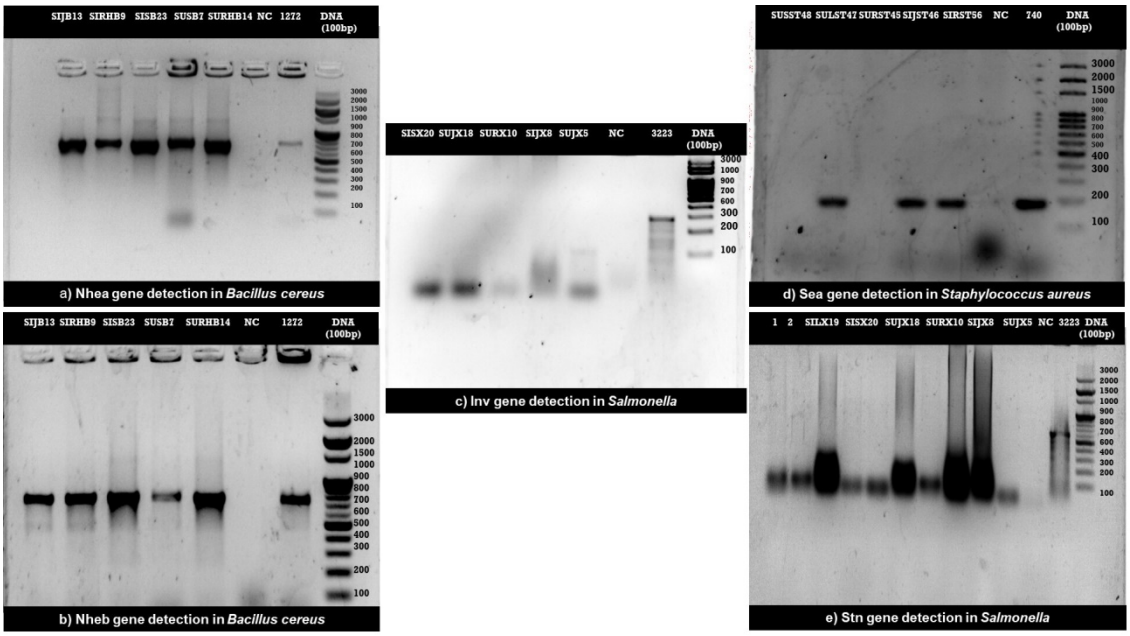


Figure 19: Agarose gel electrophoresis showing PCR amplification products. (a) Nhea gene detection in *Bacillus cereus*, (b) Nheb gene detection in *Bacillus cereus,* (c)Inv gene detection in *Salmonella* (d) *Staphylococcus* enterotoxin gene (sea) detection, and (e) Stn enterotoxin gene detection in *Salmonella* isolated from fish products of Sikkim.

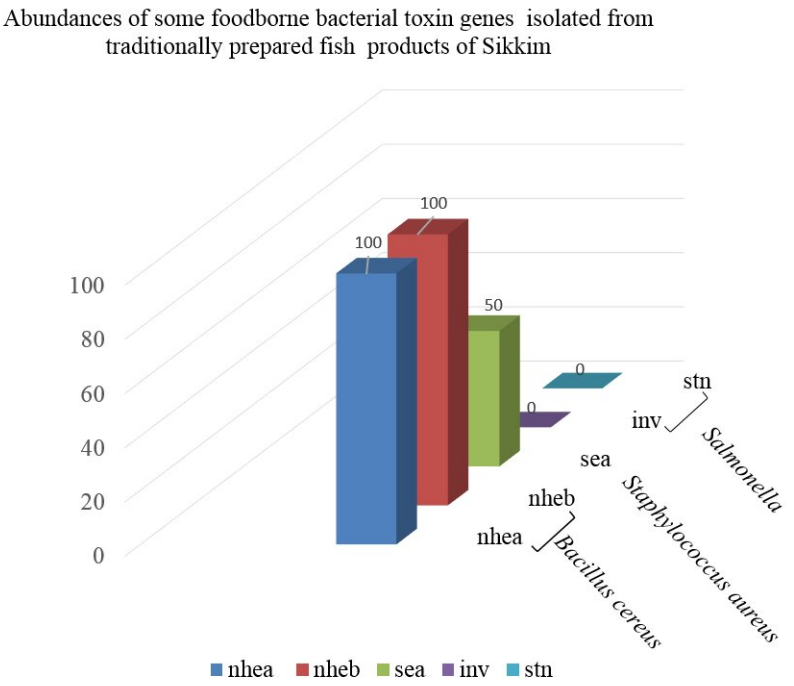


Figure 20: Prevalence of various bacterial enterotoxin genes in fish products of Sikkim.

ANTIBIOTIC SUSCEPTIBILITY TESTS

The antibiotic susceptibility tests were performed for 57 bacterial isolates using various classes of antibiotics (Table 20-30). Although many isolated strains were susceptible to various antibiotics, however some bacteria exhibited characteristics resistance to certain tested antibiotics (Figure 21). Among the strains isolated from traditional meat sample, all the 19 isolates were found to be sensitive towards gentamicin, cotrimoxazole, norfloxacin and trimethoprim but highest level of resistance was observed in amoxicillin-clavulanate (58%) followed by ampicillin (27%). Antibiotic sensitivity was shown by all Gram-positive bacteria against clindamycin and erythromycin, however, 50% of resistance pattern was observed in oxacillin followed by penicillin (33%) (Figure 22).

Overall, the bacterial isolates from fish products showed higher sensitivity patterns (>90%) towards norfloxacin, gentamicin, streptomycin, ciprofloxacin, tobramycin and trimethoprim. About 53% of the bacterial strains showed resistant patterns towards amoxycillin-clavulanate followed by nitrofurantoin (45%), ampicillin (37%), aztreonam (30%) and cefoxitin (26%). All Gram-positive bacterial strains were found to be resistant to oxacillin followed by penicillin which showed 65% of resistant patterns. All the bacterial strains belonging to genera *Staphylococcus*, *Enterococcus* and *Bacillus* were found sensitive towards clindamycin (100%) followed by erythromycin which showed 79% of sensitivity patterns (Figure 23).

Table 20: Antibiotic susceptibility test of Gram-negative bacteria isolated from meat products of Sikkim

Antimicrobial agents	Disk content (mcg)	Interpretative Criteria as per CLSI guidelines (mm)			Isolates with Zone of Inhibition (mm)													
					BSE32		SME36		PSE39		BSE17		BSE41		SME33		PSKE30	
		S	I	R														
Ampicillin (AMP)	10	≥17	14-16	≤13	18	S	14	R	18	S	15	I	13	R	16	I	22	S
Gentamicin (GEN)	10	≥15	13-14	≤12	18	S	18	S	17	S	17	S	16	S	17	S	21	S
Streptomycin (S)	10	≥15	12-14	≤11	14	I	17	S	16	S	16	S	14	I	16	S	20	S
Tetracycline (TE)	30	≥15	12-14	≤11	16	S	21	S	18	S	19	S	19	S	19	S	21	S
Chloramphenicol (C)	30	≥18	13-17	≤12	23	S	24	S	20	S	21	S	22	S	22	S	25	S
Cotrimoxazole (COT)	25	≥16	11-15	≤10	24	S	23	S	23	S	22	S	24	S	24	S	22	S
Nitrofurantoin (NIT)	300	≥17	15-16	≤14	18	S	19	S	16	S	16	I	18	S	17	S	16	I
Cefuroxime (CXM)	30	≥18	15-17	≤14	18	S	22	S	19	S	19	S	20	S	22	S	21	S
Cefoxitin (CX)	30	≥18	15-17	≤14	19	S	20	S	17	I	NZ	R	NZ	R	NZ	R	23	S
Ciprofloxacin (CIP)	5	≥21	16-20	≤15	27	S	27	S	25	S	25	S	25	S	30	S	25	S
Norfloxacin (NX)	10	≥17	13-16	≤12	27	S	24	S	26	S	26	S	23	S	29	S	27	S
Nalidixic acid (NA)	30	≥19	14-18	≤13	24	S	20	S	18	I	16	I	17	I	20	S	16	I
Tobramycin (TOB)	10	≥15	13-14	≤12	15	S	16	S	16	S	16	S	15	S	16	S	21	S
Ceftraizone (CTR)	30	≥23	20-22	≤19	25	S	25	S	25	S	24	S	26	S	27	S	26	S
Cefotaxime/Clavulinic acid (CEC)	30	≥18	15-17	≤14	26	S	24	S	26	S	24	S	28	S	28	S	32	S
Amoxycillin/Clavulanate (AMC)	30	≥18	14-17	≤13	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	14	I
Aztreonam (AT)	30	≥21	18-20	≤17	24	S	26	S	23	S	21	S	27	S	27	S	33	S
Cefepime (CPM)	30	≥25	19-24	≤18	25	S	28	S	25	S	22	I	25	S	30	S	25	S
Ceftaxidime (CAZ)	30	≥21	18-20	≤17	23	S	23	S	22	S	20	I	22	S	25	S	23	S
Trimethoprim (TR)	5	≥16	11-15	≤10	20	S	18	S	20	S	19	S	18	S	17	S	20	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

Table 21: Antibiotic susceptibility test of Gram-negative bacteria isolated from meat products of Sikkim																
Antimicrobial agents	**Disk content (mcg)**	**Interpretative Criteria as per CLSI guidelines (mm)**			**Isolates with Zone of Inhibition (mm)**											
					SME26		**BSE27**		**SMX21**		**KHE40**		**KHE57**		**PSE31**	
		S	**I**	**R**												
Ampicillin (AMP)	10	≥17	14-16	≤13	17	S	10	R	5	R	22	S	26	S	19	S
Gentamicin (GEN)	10	≥15	13-14	≤12	16	S	18	S	17	S	17	S	16	S	18	S
Streptomycin (S)	10	≥15	12-14	≤11	16	S	17	S	15	S	19	S	16	S	17	S
Tetracycline (TE)	30	≥15	12-14	≤11	16	S	18	S	NZ	R	22	S	26	S	21	S
Chloramphenicol (C)	30	≥18	13-17	≤12	19	S	23	S	22	S	26	S	21	S	24	S
Cotrimoxazole (COT)	25	≥16	11-15	≤10	21	S	20	S	20	S	25	S	NZ	R	23	S
Nitrofurantoin (NIT)	300	≥17	15-16	≤14	18	S	12	R	17	S	19	S	18	S	19	S
Cefuroxime (CXM)	30	≥18	15-17	≤14	20	S	19	S	21	S	23	S	25	S	22	S
Cefoxitin (CX)	30	≥18	15-17	≤14	19	S	18	S	18	S	22	S	17	I	20	S
Ciprofloxacin (CIP)	5	≥21	16-20	≤15	25	S	22	S	29	S	30	S	16	I	27	S
Norfloxacin (NX)	10	≥17	13-16	≤12	24	S	22	S	28	S	26	S	17	S	24	S
Nalidixic acid (NA)	30	≥19	14-18	≤13	21	S	18	I	22	S	23	S	22	S	20	S
Tobramycin (TOB)	10	≥15	13-14	≤12	17	S	16	S	17	S	17	S	16	S	16	S
Ceftraizone (CTR)	30	≥23	20-22	≤19	24	S	16	S	28	S	28	S	26	S	25	S
Cefotaxime/Clavulinic acid (CEC)	30	≥18	15-17	≤14	27	S	24	S	25	S	24	S	25	S	24	S
Amoxycillin/Clavulanate (AMC)	30	≥18	14-17	≤13	NZ	R	NZ	R	NZ	R	NZ	R	19	S	NZ	R
Aztreonam (AT)	30	≥21	18-20	≤17	22	S	29	S	22	S	24	S	23	S	26	S
Cefepime (CPM)	30	≥25	19-24	≤18	26	S	22	I	25	S	30	S	26	S	28	S
Ceftaxidime (CAZ)	30	≥21	18-20	≤17	23	S	21	S	26	S	26	S	23	S	23	S
Trimethoprim (TR)	5	≥16	11-15	≤10	21	S	23	S	26	S	19	S	24	S	15	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

Table 22: Antibiotic susceptibility test of Gram-positive bacteria isolated from meat products of Sikkim																
Antimicrobial agents	**Disk content (mcg)**	**Interpretative Criteria as per CLSI guidelines (mm)**			**Isolates with Zone of Inhibition (mm)**											
					KHST43		**BULST54**		**PSST49**		**PSST53**		**BSLST44**		**BSMB16**	
		S	**I**	**R**												
Ampicillin (AMP)	10	≥17	14-16	≤13	32	S	27	S	25	S	18	S	17	S	11	R
Gentamicin (GEN)	10	≥15	13-14	≤12	20	S	20	S	26	S	15	S	16	S	22	S
Streptomycin (S)	10	≥15	12-14	≤11	22	S	17	S	18	S	17	S	15	S	20	S
Tetracycline (TE)	30	≥15	12-14	≤11	26	S	21	S	18	S	17	S	18	S	23	S
Chloramphenicol (C)	30	≥18	13-17	≤12	22	S	20	S	19	S	21	S	20	S	19	S
Cotrimoxazole (COT)	25	≥16	11-15	≤10	24	S	23	S	27	S	18	S	21	S	10	R
Nitrofurantoin (NIT)	300	≥17	15-16	≤14	18	S	19	S	24	S	22	S	21	S	20	S
Cefuroxime (CXM)	30	≥18	15-17	≤14	27	S	21	S	25	S	20	S	28	S	12	R
Cefoxitin (CX)	30	≥18	15-17	≤14	22	S	26	S	24	S	19	S	22	S	20	S
Ciprofloxacin (CIP)	5	≥21	16-20	≤15	28	S	25	S	26	S	22	S	23	S	24	S
Norfloxacin (NX)	10	≥17	13-16	≤12	26	S	23	S	27	S	25	S	23	S	28	S
Nalidixic acid (NA)	30	≥19	14-18	≤13	20	S	20	S	19	S	21	S	24	S	19	S
Tobramycin (TOB)	10	≥15	13-14	≤12	15	S	16	S	17	S	25	S	16	S	12	R
Ceftraizone (CTR)	30	≥23	20-22	≤19	24	S	24	S	23	S	25	S	26	S	25	S
Cefotaxime/Clavulinic acid (CEC)	30	≥18	15-17	≤14	26	S	24	S	23	S	24	S	25	S	16	R
Amoxycillin/Clavulanate (AMC)	30	≥18	14-17	≤13	22	S	20	S	22	S	21	S	20	S	19	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

Table 23: Antibiotic susceptibility test of Gram-positive bacteria isolated from meat products of Sikkim																
Antimicrobial agents	**Disk content (mcg)**	**Interpretative Criteria as per CLSI guidelines (mm)**			**Isolates with Zone of Inhibition (mm)**											
					KHST43		**BULST54**		**PSST49**		**PSST53**		**BSLST44**		**BSMB16**	
		S	**I**	**R**												
Aztreonam (AT)	30	≥21	18-20	≤17	23	S	22	S	23	S	21	S	23	S	15	R
Cefepime (CPM)	30	≥25	19-24	≤18	26	S	27	S	28	S	27	S	26	S	11	R
Ceftaxidime (CAZ)	30	≥21	18-20	≤17	22	S	23	S	21	S	22	S	23	S	16	R
Trimethoprim (TR)	5	≥16	11-15	≤10	23	S	21	S	24	S	21	S	23	S	25	S
Oxacillin (OX)	1	≥18	-	≤17	14	R	14	R	23	S	19	S	14	I	10	R
Rifampicin (RIF)	5	≥26	23-25	≤23	26	S	24	I	25	I	26	S	27	S	15	R
Penicillin (P)	10	29	-	28	29	S	28	I	24	R	29	S	32	S	NZ	R
Vancomycin (VA)	30	≥17	15-16	≤14	16	I	15	I	15	I	18	S	18	S	18	S
Clindamycin (CD)	2	≥21	15-20	≤14	24	S	22	S	21	S	24	S	23	S	21	S
Erythromycin (ERY)	15	≥23	14-22	≤13	24	S	25	S	24	S	23	S	24	S	25	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

Table 24: Antibiotic susceptibility test of Gram-negative bacteria isolated from fish products of Sikkim																			
Antimicrobial agents	**Disk content (mcg)**	**Interpretative Criteria as per CLSI guidelines (mm)**			**Isolates with Zone of Inhibition (mm)**														
					SIRHE35		**SUE25**		**SURX10**		**SUJX5**		**SIRHX24**		**SISX20**		**SUJX18**		**SIRX22**	
		S	**I**	**R**															
Ampicillin (AMP)	10	≥17	14-16	≤13	21	S	13	R	10	R	8	R	NZ	R	11	R	NZ	R	27	S
Gentamicin (GEN)	10	≥15	13-14	≤12	19	S	16	S	17	S	17	S	18	S	17	S	16	S	21	S
Streptomycin (S)	10	≥15	12-14	≤11	17	S	19	S	15	S	15	S	16	S	16	S	15	S	22	S
Tetracycline (TE)	30	≥15	12-14	≤11	20	S	17	S	15	S	16	S	14	I	16	S	NZ	R	NZ	R
Chloramphenicol (C)	30	≥18	13-17	≤12	25	S	21	S	22	S	23	S	20	S	17	I	21	S	22	S
Cotrimoxazole (COT)	25	≥16	11-15	≤10	24	S	11	I	19	S	23	S	21	S	21	S	24	S	19	S
Nitrofurantoin (NIT)	300	≥17	15-16	≤14	20	S	12	R	13	R	13	R	13	R	13	R	14	R	10	R
Cefuroxime (CXM)	30	≥18	15-17	≤14	22	S	18	S	19	S	18	S	19	S	21	S	NZ	R	26	S
Cefoxitin (CX)	30	≥18	15-17	≤14	21	S	NZ	R	NZ	R	NZ	R	NZ	S	20	S	21	S	21	S
Ciprofloxacin (CIP)	5	≥21	16-20	≤15	27	S	30	S	24	S	28	S	22	S	24	S	28	S	24	S
Norfloxacin (NX)	10	≥17	13-16	≤12	28	S	26	S	23	S	28	S	21	S	21	S	30	S	28	S
Nalidixic acid (NA)	30	≥19	14-18	≤13	22	S	21	S	21	S	19	S	20	S	18	I	15	I	22	S
Tobramycin (TOB)	10	≥15	13-14	≤12	16	S	15	S	16	S	11	R	23	S	16	S	11	R	20	S
Ceftraizone (CTR)	30	≥23	20-22	≤19	26	S	26	S	24	S	22	I	25	S	25	S	21	S	26	S
Cefotaxime/Clavulinic acid (CEC)	30	≥18	15-17	≤14	27	S	25	S	24	S	29	S	24	S	23	S	21	S	22	S
Amoxycillin/Clavulanate (AMC)	30	≥18	14-17	≤13	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	12	R
Aztreonam (AT)	30	≥21	18-20	≤17	26	S	22	S	23	S	24	S	20	I	NZ	R	20	I	26	S
Cefepime (CPM)	30	≥25	19-24	≤18	28	S	28	S	24	I	29	S	26	S	25	S	24	S	28	S
Ceftaxidime (CAZ)	30	≥21	18-20	≤17	25	S	21	S	20	I	24	S	22	S	22	S	22	S	24	S
Trimethoprim (TR)	5	≥16	11-15	≤10	24	S	23	S	25	S	24	S	23	S	21	S	NZ	R	19	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

Table 25: Antibiotic susceptibility test of Gram-negative bacteria isolated from fish products of Sikkim																				
Antimicrobial agents	**Disk content (mcg)**	**Interpretative Criteria as per CLSI guidelines (mm)**			**Isolates with Zone of Inhibition (mm)**															
					SKRH2		**SIJX8**		**SKRT1**		**SIRT3**		**SILT11**		**SIRT12**		**SUE6**		**SURHE 38**	
		S	**I**	**R**																
Ampicillin (AMP)	10	≥17	14-16	≤13	16	I	18	S	33	S	22	S	17	S	22	S	14	I	29	R
Gentamicin (GEN)	10	≥15	13-14	≤12	21	S	17	S	20	S	18	S	18	S	18	S	12	R	20	S
Streptomycin (S)	10	≥15	12-14	≤11	25	S	15	S	16	S	17	S	19	S	17	S	17	S	16	S
Tetracycline (TE)	30	≥15	12-14	≤11	24	S	23	S	26	S	NZ	R	NZ	R	NZ	R	16	S	13	R
Chloramphenicol (C)	30	≥18	13-17	≤12	16	I	25	S	21	S	19	S	21	S	19	S	23	S	20	S
Cotrimoxazole (COT)	25	≥16	11-15	≤10	24	S	30	S	23	S	19	S	20	S	19	S	20	S	28	S
Nitrofurantoin (NIT)	300	≥17	15-16	≤14	10	R	21	S	16	S	9	R	10	R	10	R	17	S	10	R
Cefuroxime (CXM)	30	≥18	15-17	≤14	19	S	19	S	19	S	26	S	26	S	26	S	18	S	19	S
Cefoxitin (CX)	30	≥18	15-17	≤14	18	S	26	S	22	S	21	S	24	S	21	S	NZ	R	18	S
Ciprofloxacin (CIP)	5	≥21	16-20	≤15	24	S	28	S	20	I	26	S	28	S	26	S	27	S	24	S
Norfloxacin (NX)	10	≥17	13-16	≤12	22	S	22	S	24	S	28	S	26	S	28	S	25	S	21	S
Nalidixic acid (NA)	30	≥19	14-18	≤13	22	S	27	S	19	S	18	S	19	S	19	S	24	S	20	S
Tobramycin (TOB)	10	≥15	13-14	≤12	21	S	17	S	20	S	17	S	18	S	17	S	10	R	16	S
Ceftraizone (CTR)	30	≥23	20-22	≤19	19	R	26	S	21	S	28	S	30	S	28	S	22	I	24	S
Cefotaxime/Clavulinic acid (CEC)	30	≥18	15-17	≤14	21	S	22	S	18	S	27	S	23	S	27	S	24	S	26	S
Amoxycillin/Clavulanate (AMC)	30	≥18	14-17	≤13	NZ	R	NZ	R	24	S	9	R	19	S	9	R	NZ	R	NZ	R
Aztreonam (AT)	30	≥21	18-20	≤17	17	R	22	S	NZ	R	29	S	30	S	29	S	25	S	24	S
Cefepime (CPM)	30	≥25	19-24	≤18	24	I	26	S	22	I	26	S	28	S	26	S	22	I	26	S
Ceftaxidime (CAZ)	30	≥21	18-20	≤17	18	I	18	I	16	R	25	S	28	S	25	S	18	I	25	S
Trimethoprim (TR)	5	≥16	11-15	≤10	12	I	24	S	28	S	15	I	17	S	15	I	19	S	17	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

Table 26: Antibiotic susceptibility test of Gram-negative bacteria isolated from fish products of Sikkim																			
Antimicrobial agents	**Disk content (mcg)**	**Interpretative Criteria as per CLSI guidelines (mm)**			**Isolates with Zone of Inhibition (mm)**														
					SIRE29		**SUE37**		**ASE42**		**SUE28**		**ASE34**		**SULT15**		**SISX4**		**SILX19**	
		S	**I**	**R**															
Ampicillin (AMP)	10	≥17	14-16	≤13	18	S	10	R	18	S	16	I	15	I	NZ	R	NZ	R	1	R
Gentamicin (GEN)	10	≥15	13-14	≤12	16	S	17	S	16	S	17	S	17	S	15	S	18	S	19	S
Streptomycin (S)	10	≥15	12-14	≤11	14	I	18	S	17	S	19	S	16	S	15	S	18	S	14	I
Tetracycline (TE)	30	≥15	12-14	≤11	17	S	24	S	21	S	22	S	17	S	10	R	17	S	16	S
Chloramphenicol (C)	30	≥18	13-17	≤12	23	S	20	S	23	S	24	S	19	S	NZ	R	22	S	19	S
Cotrimoxazole (COT)	25	≥16	11-15	≤10	21	S	21	S	19	S	20	S	18	S	NZ	R	19	S	23	S
Nitrofurantoin (NIT)	300	≥17	15-16	≤14	20	S	18	R	21	S	13	R	19	R	NZ	R	11	R	12	R
Cefuroxime (CXM)	30	≥18	15-17	≤14	19	S	25	S	19	S	17	I	20	S	NZ	R	16	I	19	S
Cefoxitin (CX)	30	≥18	15-17	≤14	22	S	20	R	22	S	12	R	23	R	NZ	R	NZ	R	NZ	R
Ciprofloxacin (CIP)	5	≥21	16-20	≤15	25	S	25	S	23	S	23	S	26	S	17	I	26	S	26	S
Norfloxacin (NX)	10	≥17	13-16	≤12	21	S	23	S	19	S	21	S	18	S	18	S	27	S	27	S
Nalidixic acid (NA)	30	≥19	14-18	≤13	18	I	20	S	20	S	24	S	23	S	NZ	R	19	S	19	S
Tobramycin (TOB)	10	≥15	13-14	≤12	17	S	16	S	17	S	17	S	11	R	NZ	R	17	S	16	S
Ceftraizone (CTR)	30	≥23	20-22	≤19	27	S	24	S	24	S	20	I	20	I	16	R	18	R	25	S
Cefotaxime/Clavulinic acid (CEC)	30	≥18	15-17	≤14	18	S	20	S	22	S	23	S	22	S	19	S	21	S	26	S
Amoxycillin/Clavulanate (AMC)	30	≥18	14-17	≤13	10	R	5	R	19	S	3	R	10	R	NZ	R	NZ	R	NZ	R
Aztreonam (AT)	30	≥21	18-20	≤17	23	S	22	S	24	S	25	S	26	S	16	R	22	S	1	R
Cefepime (CPM)	30	≥25	19-24	≤18	27	S	23	S	23	I	22	I	19	I	18	R	21	I	26	S
Ceftaxidime (CAZ)	30	≥21	18-20	≤17	22	S	22	S	22	S	24	S	16	R	19	I	18	I	22	S
Trimethoprim (TR)	5	≥16	11-15	≤10	25	S	19	S	18	S	18	S	23	S	NZ	R	24	S	23	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

Table 27: Antibiotic susceptibility test of Gram-positive bacteria isolated from fish products of Sikkim																				
Antimicrobial agents	**Disk content (mcg)**	**Interpretative Criteria as per CLSI guidelines (mm)**			**Isolates with Zone of Inhibition (mm)**															
					SURST45		**ASLST52**		**SULST47**		**SIRST56**		**SIJST46**		**SUSST48**		**SIRST50**		**SILST51**	
		S	**I**	**R**																
Ampicillin (AMP)	10	≥17	14-16	≤13	18	S	19	S	18	S	17	S	21	S	20	S	18	S	18	S
Gentamicin (GEN)	10	≥15	13-14	≤12	17	S	16	S	16	S	15	S	17	S	16	S	15	S	16	S
Streptomycin (S)	10	≥15	12-14	≤11	16	S	17	S	19	S	17	S	17	S	16	S	16	S	17	S
Tetracycline (TE)	30	≥15	12-14	≤11	16	S	16	S	20	S	20	S	18	S	19	S	21	S	20	S
Chloramphenicol (C)	30	≥18	13-17	≤12	19	S	23	S	24	S	23	S	21	S	25	S	27	S	24	S
Cotrimoxazole (COT)	25	≥16	11-15	≤10	17	S	19	S	19	S	18	S	19	S	21	S	19	S	23	S
Nitrofurantoin (NIT)	300	≥17	15-16	≤14	20	S	18	S	18	S	21	S	23	S	18	S	17	S	18	S
Cefuroxime (CXM)	30	≥18	15-17	≤14	19	S	22	S	23	S	21	S	23	S	22	S	21	S	24	S
Cefoxitin (CX)	30	≥18	15-17	≤14	19	S	27	S	25	S	20	S	19	S	21	S	18	S	22	S
Ciprofloxacin (CIP)	5	≥21	16-20	≤15	23	S	25	S	24	S	24	S	21	S	26	S	25	S	23	S
Norfloxacin (NX)	10	≥17	13-16	≤12	18	S	27	S	19	S	21	S	20	S	18	S	19	S	20	S
Nalidixic acid (NA)	30	≥19	14-18	≤13	22	S	13	R	21	S	21	S	23	S	20	S	19	S	22	S
Tobramycin (TOB)	10	≥15	13-14	≤12	16	S	16	S	18	S	16	S	17	S	16	S	15	S	16	S
Ceftraizone (CTR)	30	≥23	20-22	≤19	24	S	20	I	25	S	25	S	23	S	26	S	24	S	25	S
Cefotaxime/Clavulinic acid (CEC)	30	≥18	15-17	≤14	26	S	23	S	25	S	23	S	21	S	20	S	21	S	20	S
Amoxycillin/Clavulanate (AMC)	30	≥18	14-17	≤13	23	S	21	S	21	S	19	S	21	S	19	S	23	S	24	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

Table 28: Antibiotic susceptibility test of Gram-positive bacteria isolated from fish products of Sikkim																				
Antimicrobial agents	**Disk content (mcg)**	**Interpretative Criteria as per CLSI guidelines (mm)**			**Isolates with Zone of Inhibition (mm)**															
					SURST45		**ASLST52**		**SULST47**		**SIRST56**		**SIJST46**		**SUSST48**		**SIRST50**		**SILST51**	
		S	**I**	**R**																
Aztreonam (AT)	30	≥21	18-20	≤17	22	S	23	S	22	S	22	S	225	S	24	S	25	S	23	S
Cefepime (CPM)	30	≥25	19-24	≤18	27	S	26	S	28	S	27	S	27	S	28	S	26	S	27	S
Ceftaxidime (CAZ)	30	≥21	18-20	≤17	23	S	22	S	25	S	22	S	21	S	27	S	23	S	22	S
Trimethoprim (TR)	5	≥16	11-15	≤10	24	S	17	S	19	S	17	S	17	S	16	S	17	S	18	S
Oxacillin (OX)	1	≥18	-	≤17	12	R	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R
Rifampicin (RIF)	5	≥26	23-25	≤23	27	S	27	S	15	R	23	I	27	S	26	S	23	I	15	R
Penicillin (P)	10	29	-	28	29	S	25	R	NZ	R	24	R	30	S	31	S	NZ	R	29	S
Vancomycin (VA)	30	≥17	15-16	≤14	9	R	18	S	16	I	10	R	19	S	16	I	11	R	12	R
Clindamycin (CD)	2	≥21	15-20	≤14	25	S	25	S	23	S	24	S	21	S	23	S	27	S	25	S
Erythromycin (ERY)	15	≥23	14-22	≤13	27	S	11	R	24	S	23	S	25	S	24	S	26	S	24	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

Table 29: Antibiotic susceptibility test of Gram-positive bacteria isolated from fish products of Sikkim																
Antimicrobial agents	**Disk content (mcg)**	**Interpretative Criteria as per CLSI guidelines (mm)**			**Isolates with Zone of Inhibition (mm)**											
					SURST55		**SIJB13**		**SIRHB9**		**SURHB14**		**SISB23**		**SUSB7**	
		S	**I**	**R**												
Ampicillin (AMP)	10	≥17	14-16	≤13	18	S	11	R	12	R	NZ	R	9	R	10	R
Gentamicin (GEN)	10	≥15	13-14	≤12	17	S	16	S	17	S	16	S	16	S	17	S
Streptomycin (S)	10	≥15	12-14	≤11	16	S	18	S	16	S	15	S	16	S	22	S
Tetracycline (TE)	30	≥15	12-14	≤11	20	S	17	S	17	S	21	S	22	S	16	S
Chloramphenicol (C)	30	≥18	13-17	≤12	23	S	21	S	15	I	23	S	24	S	18	S
Cotrimoxazole (COT)	25	≥16	11-15	≤10	19	S	19	R	10	R	8	R	10	R	8	R
Nitrofurantoin (NIT)	300	≥17	15-16	≤14	21	S	18	S	19	S	19	S	21	S	20	S
Cefuroxime (CXM)	30	≥18	15-17	≤14	19	S	13	R	12	R	12	R	10	R	11	R
Cefoxitin (CX)	30	≥18	15-17	≤14	20	S	11	R	12	R	13	R	12	R	NZ	R
Ciprofloxacin (CIP)	5	≥21	16-20	≤15	23	S	22	S	22	S	23	S	22	S	21	S
Norfloxacin (NX)	10	≥17	13-16	≤12	18	S	19	S	19	S	21	S	23	S	20	S
Nalidixic acid (NA)	30	≥19	14-18	≤13	21	S	21	S	17	I	15	I	14	I	20	S
Tobramycin (TOB)	10	≥15	13-14	≤12	16	S	11	R	12	R	NZ	R	NZ	R	10	R
Ceftraizone (CTR)	30	≥23	20-22	≤19	25	S	23	S	24	S	24	S	25	S	25	S
Cefotaxime/Clavulinic acid (CEC)	30	≥18	15-17	≤14	21	S	17	I	11	R	16	R	12	R	13	R
Amoxycillin/Clavulanate (AMC)	30	≥18	14-17	≤13	20	S	21	S	20	S	22	S	21	S	23	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

Table 30: Antibiotic susceptibility test of Gram-positive bacteria isolated from fish products of Sikkim																
Antimicrobial agents	**Disk content (mcg)**	**Interpretative Criteria as per CLSI guidelines (mm)**			**Isolates with Zone of Inhibition (mm)**											
					SURST55		**SIJB13**		**SIRHB9**		**SURHB4**		**SISB23**		**SUSB7**	
		S	**I**	**R**												
Aztreonam (AT)	30	≥21	18-20	≤17	22	S	12	R	12	R	15	R	16	R	15	R
Cefepime (CPM)	30	≥25	19-24	≤18	27	S	15	R	13	R	16	R	11	R	16	R
Ceftaxidime (CAZ)	30	≥21	18-20	≤17	25	S	16	R	15	R	16	R	11	R	12	R
Trimethoprim (TR)	5	≥16	11-15	≤10	21	S	20	S	18	S	19	S	18	S	20	S
Oxacillin (OX)	1	≥18	-	≤17	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R
Rifampicin (RIF)	5	≥26	23-25	≤23	23	I	17	R	15	R	11	R	14	R	19	R
Penicillin (P)	10	29	-	28	30	S	24	R	12	R	NZ	R	NZ	R	NZ	R
Vancomycin (VA)	30	≥17	15-16	≤14	11	R	18	S	16	I	18	S	19	S	18	S
Clindamycin (CD)	2	≥21	15-20	≤14	23	S	22	S	24	S	23	S	25	S	23	S
Erythromycin (ERY)	15	≥23	14-22	≤13	24	S	25	S	18	I	11	R	26	S	25	S

CLSI=Clinical and Laboratory Standards Institute, NZ= No zone, S= sensitive, I= intermediate, R= resistance

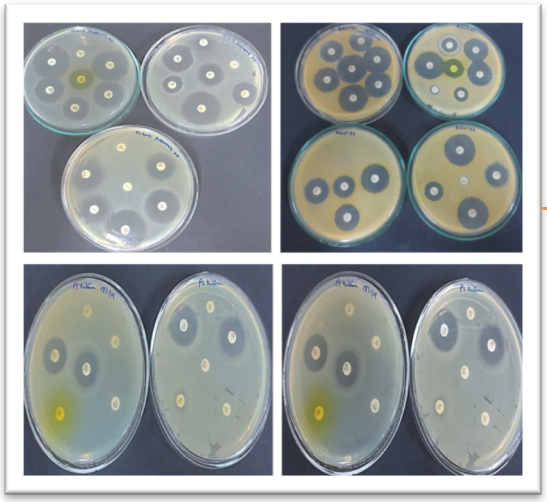


Figure 21: Mueller Hinton agar plates showing the antibiotic sensitivity tests of bacterial strains isolated from meat and fish products of Sikkim by Kirby-Bauer disk diffusion method.

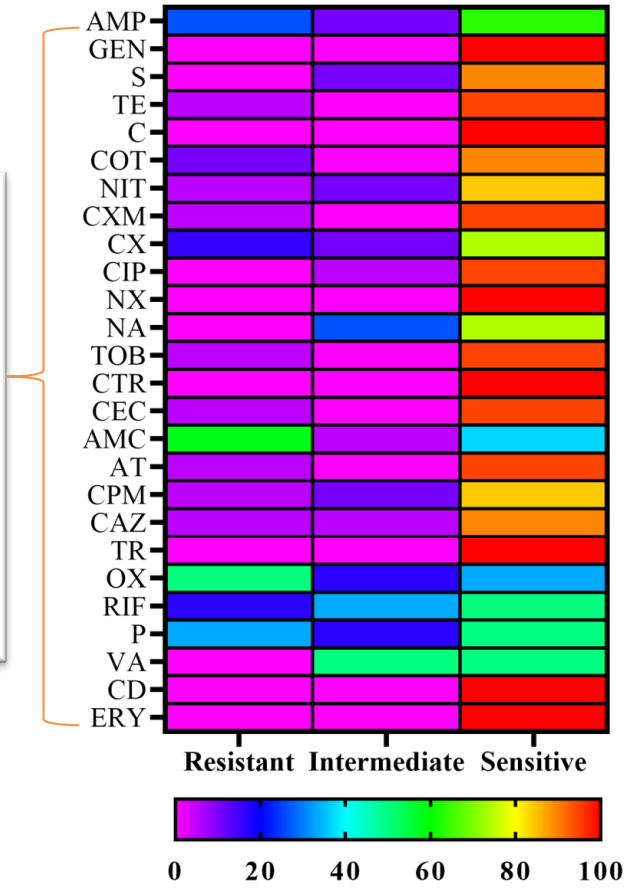


Figure 22: Percentile distribution of bacterial strains isolated from meat products showing resistant, intermediate and sensitive patterns towards various classes of antibiotics.

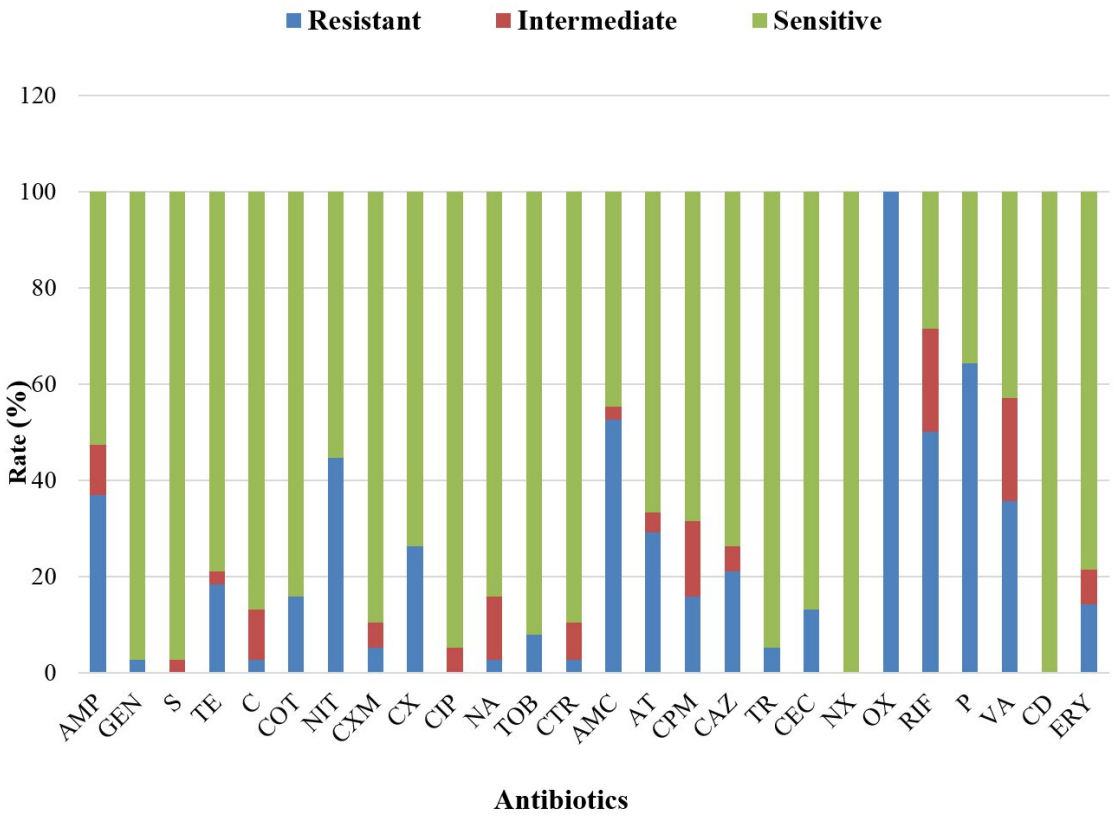


Figure 23: Percentile distribution of bacterial strains isolated from fish products showing resistant, intermediate and sensitive patterns towards various classes of antibiotics.

NEXT GENERATION SEQUENCING ANALYSIS (High-throughput Sequencing)

Although the main objective of present studies was to assess the microbiological safety of traditionally processed meat and fish products of Sikkim, we attempted to study the metataxonomic of meat products (beef *kargyong,* pork *kargyong, satchu*, *khyopeh*) and fish products (*suka ko maacha, sidra, sukuti*) to profile the complete bacterial communities by High-throughput sequencing technique and also to infer their predictive gene functionalities using bioinformatics for various metabolic pathways and other health-promoting benefits.

BACTERIAL DIVERSITY IN TRADITIONAL MEAT PRODUCTS

Overall Bacterial Communities

Genomic DNA extracted from four samples of beef *kargyong,* pork *kargyong, satchu* and *khyopeh* was analyzed by high-throughput sequencing method. A total of 170,114 raw reads were obtained from sequencing. After joining through PEAR software by default parameters, a total number of 81,538 quality-checked reads with 350 bp uniform length were further analyzed. Overall bacterial diversity based on >1% relative abundance showed that Firmicutes (50.11%) was the most dominant phylum followed by Proteobacteria (43.99%), Bacteroidetes (5.72%), and other phyla in including both classified and unclassified with <1% relative abundance (0.31%) (Figure 24a). At the family level, Moraxellaceae (21.83%) was the dominant family followed by Enterococcaceae (19.77%), Lactobacillaceae (15.32%), Weeksellaceae (13.18%), Staphylococcaceae (8.94%), Xanthomonadaceae (3.38%), Leuconostocaceae (3.05%), Carnobacteriaceae (2.79%), Pseudomonadaceae (1.74%), Rhizobiaceae (1.35%), Enterobacteriaceae (1.27%), Sphingobacteriaceae (1.21%), Rhodobacteraceae (1.13%), Bdellovibrionaceae (1.12%), and Clostridiaceae (1.12%) (Figure 24b). Families with <1% abundance were grouped as others (2.8%). *Psychrobacter* (21.93%) was the most abundant genus followed by *Enterococcus* (19.67%), *Lactobacillus* (15.46%), *Lactococcus* (6.77%), *Ignatzschineria* (5.36%), *Staphylococcus* (3.94%), *Chryseobacterium* (3.82%), *Carnobacterium* (2.79%), *Pseudomonas* (2.09%),

Bdellovibrio (1.79%), *Clostridium* (1.79%), *Novosphingobium* (1.79%), *Leuconostoc* (1.72%), *Weissella* (1.37%), *Agrobacterium* (1.32%), *Acinetobacter* (1.28%) and other genera with <1% abundance (7.13%) (Figure 24c). Species present in the samples were *Lactobacillus sakei* (26.38%), *Psychrobacter pulmonis* (24.87%), *Carnobacterium divergens* (11.44%), *Staphylococcus equorum* (5.03%), *Staphylococcus succinus* (4.81%), *Bdellovibrio bacteriovorus* (4.76%), *Pseudomonas veronii* (2.64%), *Ketogulonicigenium vulgare* (2.38%), *Pseudomonas stutzeri* (2.38%), *Lactococcus garvieae* (2.34%), *Kurthia zopfii* (1.78%), *Psychrobacter sanguinis* (1.30%), *Weissella cibaria* (1.17%), *Staphylococcus sciuri* (1.06%) and other species with <1% abundance (2.89%) (Figure 24d).

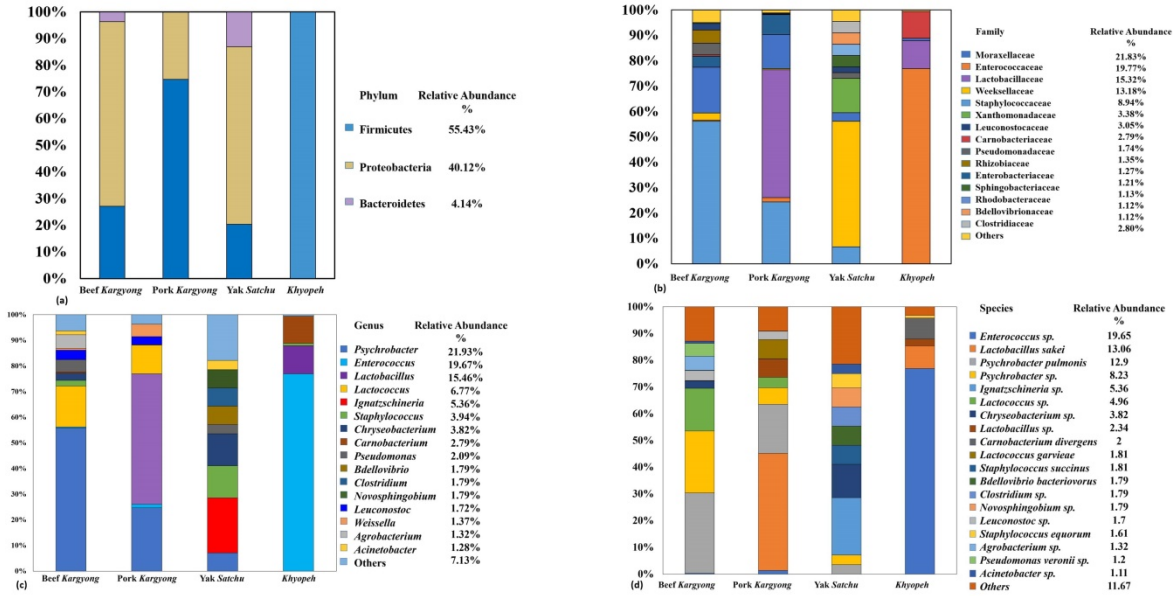


Figure 24: Bar-plot showing the metataxonomic distribution of bacterial diversity in meat products of Sikkim at (a) Phylum, (b) Family, (c) Genus and (d) Species level by High-throughput sequencing method.

Bacterial diversity in beef *kargyong*

The phylum Proteobacteria (68.64%) was dominant in beef *kargyong* which was followed by the phylum Firmicutes (26.84%) and Bacteroidetes (3.56%) and Actinobacteria (0.95%) (Figure 25a). At the family level, the bacterial distribution was shared by Moraxellaceae (56.14%), Staphylococcaceae (18.06%), Rhizobiaceae (5.15%), Pseudomonadaceae (4.69%), Leuconostocaceae (4.30%), Planococcaceae (3.16%), Weeksellaceae (2.73%), Enterobacteriaceae (2.55%) and other families with <1% relative abundance belonged to Micrococcaceae, Enterococcaceae, Carnobacteriaceae, Flavobacteriaceae, Sphingobacteriaceae, Listeriaceae, Neisseriaceae, and Peptostreptococcaceae (Figure 25a). *Psychrobacter* was the most dominant genus with a relative abundance of 55.9% and was shared by *Lactococcus* (15.93%), *Agrobacterium* (5.27%), *Pseudomonas* (4.80%), *Leuconostoc* (3.70%), *Kurthia* (3.23%), *Chryseobacterium* (2.79%), *Staphylococcus* (2.18%), *Acinetobacter*

(1.51%) and few other genera >1% belong to *Arthrobacter*, *Weissella*, *Carnobacterium*, *Myroides*, *Enterococcus*, *Sphingobacterium*, *Erwinia*, *Macrococcus*, *Brochothrix*, *Vagococcus*, *Vitreoscilla*, *Streptococcus* and *Paraclostridium* (Figure 25a). At the species level, *Psychrobacter pulmonis* (30.09%) was the dominant species followed by *Psychrobacter* spp. (23.17%), *Lactococcus* spp. (15.93%), *Agrobacterium* spp. (5.27%), *Pseudomonas veronii* (4.80%), *Leuconostoc* spp. (3.70%), *Kurthia zopfii* (3.23%), *Chryseobacterium* spp. (2.79%), *Psychrobacter sanguinis* (2.28%) and *Staphylococcus sciuri* (1.89%). We have also detected few bacterial species with <1% relative abundance which comprised of *Myroides odoratimimus*, *Weissella ceti*, *Sphingobacterium faecium*, *Weissella viridescens*, *Psychrobacter arenosus*, *Macrococcus caseolyticus*, *Acinetobacter johnsonii*, *Acinetobacter guillouiae*, *Staphylococcus equorum*, *Acinetobacter lwoffii*, *Staphylococcus succinus*, *Carnobacterium divergens*, *Enterococcus casseliflavus*, *Psychrobacter pacificensis*, *Enterococcus* spp., *Arthrobacter* spp., *Acinetobacter* spp., *Carnobacterium* spp., *Erwinia* spp., *Brochothrix* spp., *Vagococcus* spp., *Vitreoscilla* spp., *Streptococcus* spp., *Staphylococcus* spp., *Myroides* spp., and *Paraclostridium* spp.,

Bacterial diversity in pork *kargyong*

In contrast to beef *kargyong*, Firmicutes (74.52%) was found dominant phylum in pork *kargyong* followed by Proteobacteria (25.18%) (Figure 25b) and an insignificant presence of Bacteroidetes and Actinobacteria was recorded. Lactobacillaceae was the dominant family with 50.32% relative abundance and was shared by Moraxellaceae (24.43%), Staphylococcaceae (13.36%), Leuconostocaceae (7.90%), Enterococcaceae (1.64%) and others families with <1% abundance (2.35%) (Figure 25b) were Listeriaceae, Weeksellaceae, Enterobacteriaceae, Rhizobiaceae, Corynebacteriaceae,

Micrococcaceae, Carnobacteriaceae, Flavobacteriaceae, Xanthomonadaceae and Rhodobacteraceae. *Lactobacillus* (50.88%) was the dominant genus followed by other genera- *Psychrobacter* (24.67%), *Lactococcus* (11.15%), *Weissella* (4.80%), *Leuconostoc* (3.19%), *Macrococcus* (2.23%), *Enterococcus* (1.41%) and few other genera <1% relative abundance were *Brochothrix*, *Vagococcus*, *Corynebacterium*, *Staphylococcus*, *Carnobacterium*, *Myroides*, *Micrococcus*, *Acinetobacter*, *Anaerospora*, *Citrobacter*, *Rothia* and *Stenotrophomonas* (Figure 25b). At the species-level, *Lactobacillus sakei* (43.78%) was found to be the dominant species followed by *Psychrobacter pulmonis* (18.33%), *Lactococcus garvieae* (7.23%), *Lactobacillus* spp. (6.87%), *Psychrobacter* spp. (6.18%), *Lactococcus* spp. (3.91%), *Weissella cibaria* (3.62%), *Leuconostoc* spp. (3.08%), *Macrococcus caseolyticus* (2.26%), *Enterococcus* sp. (1.38%) and *Weissella ceti* (1.17%). Other few lesser occurred species with less than 1% relative abundance were *Lactobacillus brevis*, *Corynebacterium variabile*, *Psychrobacter sanguinis*, *Leuconostoc fallax*, *Carnobacterium divergens*, *Staphylococcus sciuri*, *Myroides odoratimimus*, *Micrococcus luteus*, *Enterococcus casseliflavus*, *Rothia nasimurium*, *Brochothrix* spp., *Vagococcus* spp., *Staphylococcus* spp., *Acinetobacter* spp., *Anaerospora* spp., *Citrobacter* spp. and *Stenotrophomonas* spp.

Bacterial diversity in yak *satchu*

Proteobacteria (66.67%) was found most abundant phylum in yak *satchu* followed by Firmicutes (20.37%) and Bacteroidetes (12.96%) (Figure 25c). Here, a different pattern of abundance at family level was observed where Weeksellaceae (49.44%) was dominant family followed by Xanthomonadaceae (13.48%), Moraxellaceae (6.74%), Sphingobacteriaceae (4.49%), Rhodobacteraceae (4.49%), Bdellovibrionaceae (4.49%),

Clostridiaceae (4.49%), Staphylococcaceae (3.37%), Pseudomonadaceae (2.25%), Enterobacteriaceae (2.25%), Caulobacteraceae (2.25%) and Verrucomicrobiaceae (2.25%) (Figure 25c). *Ignatzschineria* (21.43%) was the dominant genus followed by *Chryseobacterium* (12.50%), *Staphylococcus* (12.50%), *Psychrobacter* (7.14%), *Bdellovibrio* (7.14%), *Clostridium* (7.14%), *Novosphingobium* (7.14%), *Pseudomonas* (3.57%), *Acinetobacter* (3.57%), *Anaerospora* (3.57%), *Brevundimonas* (3.57%), *Enterobacter* (3.57%), *Ketogulonicigenium* (3.57%) and *Luteolibacter* (3.57%) (Figure 25c). Species level distributions in yak *satchu* showed *Ignatzschineria* spp. (21.43%), *Chryseobacterium* spp. (12.50%), *Staphylococcus succinus* (7.14%), *Bdellovibrio bacteriovorus* (7.14%), *Clostridium* spp. (7.14%), *Novosphingobium* spp. (7.14%), *Staphylococcus equorum* (5.36%), *Psychrobacter pulmonis* (3.57%), *Psychrobacter* spp. (3.57%), *Acinetobacter* spp. (3.57%), *Anaerospora* spp. (3.57%), *Brevundimonas* spp. (3.57%), *Enterobacter* spp. (3.57%), *Ketogulonicigenium vulgare* (3.57%), *Luteolibacter* spp. (3.57%) and *Pseudomonas stutzeri* (3.57%).

Bacterial diversity in *khyopeh*

Khyopeh was represented only by the phylum Firmicutes (100%) and was comprised of families Enterococcaceae (76.92%), Lactobacillaceae (10.96%), Carnobacteriaceae (10.58%), Staphylococcaceae (0.96%) and Listeriaceae (0.58%) (Figure 25d). *Enterococcus* (76.92%) was dominant genus followed by *Lactobacillus* (10.96%), *Carnobacterium* (10.58%), *Staphylococcus* (0.96%), and *Brochothrix* (0.58%) (Figure 25d). Species level distribution showed *Enterococcus* spp. (76.92%), *Lactobacillus sakei* (8.46%), *Carnobacterium divergens* (7.88%), *Carnobacterium* spp. (2.69%), *Lactobacillus* spp. (2.50), *Staphylococcus equorum* (0.96), and *Brochothrix* spp. (0.58%) (Figure 25d).

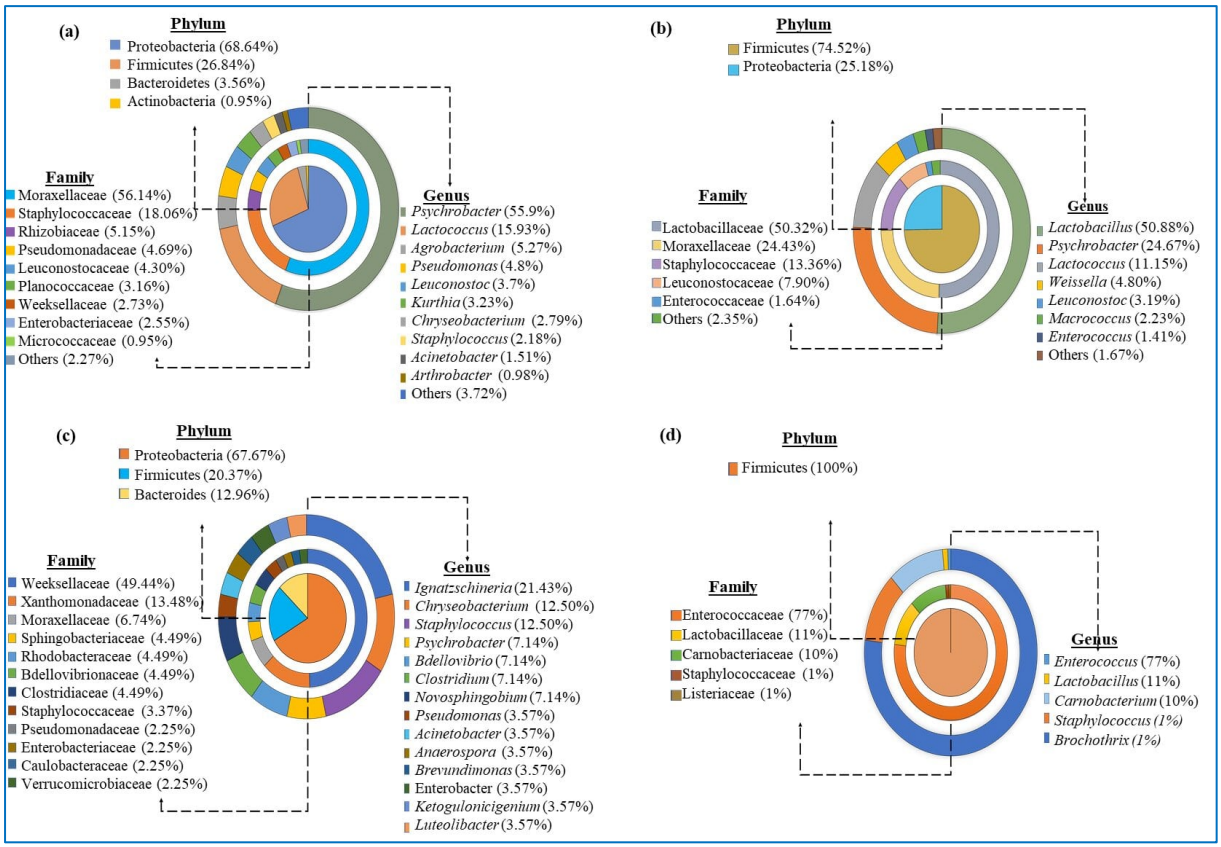


Figure 25: Doughnut-chart showing bacterial composition of meat products of Sikkim (a) Beef *kargyong* (b) Pork *kargyong* (c) Yak *Satchu* and (d) *Khyopeh.*

Unique species

We observed several genera as unique/rare to each product based on analysis of shared OTUs contents among the samples. *Streptococcus*, *Arthrobacter*, *Kurthia*, *Agrobacterium*, *Vitreoscilla*, *Erwinia*, *Sphingobacterium* and *Paraclostridium* were unique to beef *kargyong* (Figure 4). Similarly, *Rothia, Micrococcus, Stenotrophomonas, Corynebacterium* and *Citrobacter* were unique genera in pork *kargyong*. *Novosphingobium, Bdellovibrio, Brevundimonas, Clostridium, Ketogulonicigenium, Ignatzschineria, Enterobacter* and *Luteolibacter* were unique genera in yak *satchu*. However, no unique genus was observed in *khyopeh*. Interestingly, *Staphylococcus* was the most common genus which was observed in all meat products (Figure 26).

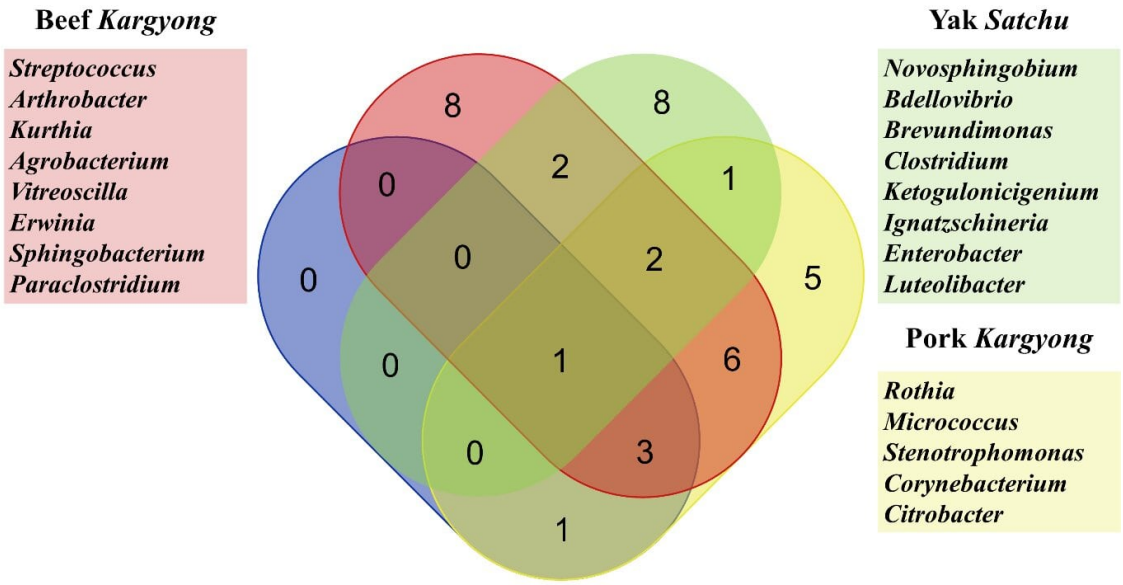


Figure 26: Venn diagram showing the core microbiota and the unique genera present in beef *kargyong,* pork *kargyong* and yak *satchu*. Numbers denote the number of genera present in samples; some shared and some unique to particular sample. No unique genus was observed in *khyopeh*. *Staphylococcus* was the only genus to be shared among the traditionally processed meat products of Sikkim.

Alpha Diversity Indices

Bacterial FastQC showed 170,114 total raw reads generated by HTS. Deblur denoising and filtering algorithms resulted 81,538 filtered reads and 413 observed OTUs. All sequence reads were rarefied at sampling depth of 3875 for alpha diversity calculation (Figure 27). Goods coverage (Table 31) recorded 0.99 to 1.0 which reflected that sequencing captures the maximum of the bacterial diversity in the samples. Alpha diversity metrics showed a maximum bacterial diversity in beef *kargyong* and lowest in *yak satchu* (Table 31).

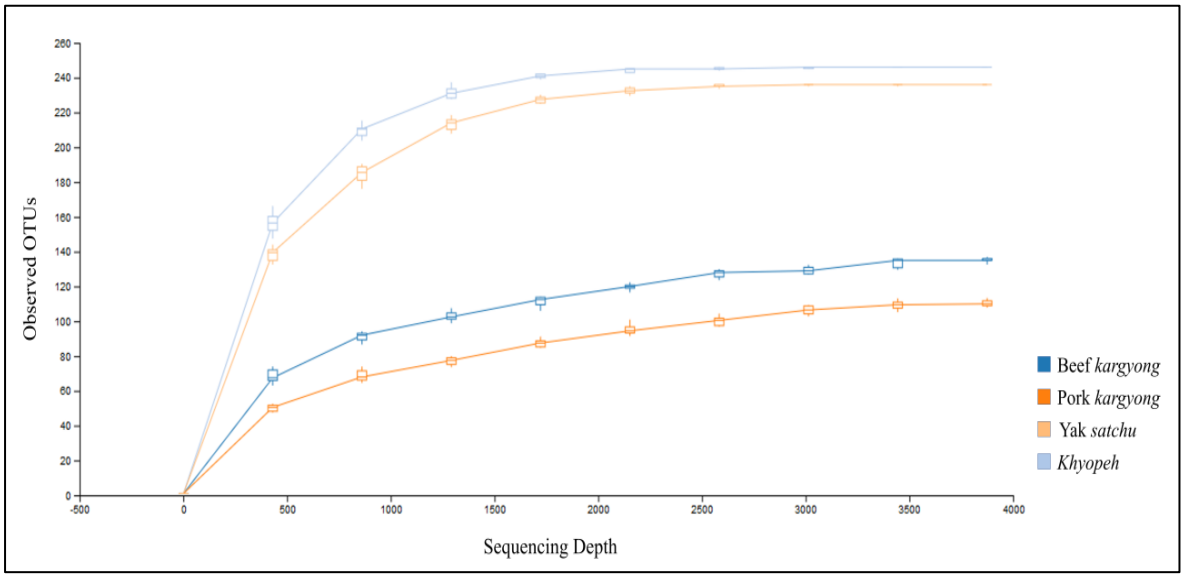


Figure 27: Rarefaction curves showing observed operational taxonomic units (OTUs) of bacterial diversity of beef *kargyong, pork kargyong,* yak *satchu* and *khyopeh*.

Table 31: Diversity indices of bacterial community analysed by High-throughput sequencing in traditionally processed meat products of Sikkim

Samples	Alpha Diversity					
	Observed OTUs	**Chao1**	**Fisher alpha**	**Shannon**	**Simpson**	**Coverage**
Beef *Kargyong*	130	130	24.36	4.78	0.92	1.0
Pork *Kargyong*	121	121	20.92	4.21	0.88	1.0
Satchu	91	19	10.13	4.15	0.94	1.0
Khyopeh	71	21.5	4.37	2.62	0.73	1.0

Profile of Bacterial Diversity in Traditional Meat Products Based on Culture-Dependent and Culture-Independent Methods

We detected 9 genera of bacteria in meat products of Sikkim by phenotypic characterization (Table 32). Similarly, we detected 11 genera with 17 species in same products by 16S rRNA gene sequencing method. Finally, by application of HTS (culture-independent method), we detected 16 genera with 19 species with >1% abundance (Table 32) and 21 genera with 37 species detected at <1% abundance.

Table 32: Profile of bacterial species in traditional meat products of Sikkim revealed by Culture-dependent and-independent Methods

Product	Culture-dependent		Culture-independent
	Phenotypic	Genotypic (16S rRNA gene sequence)	High-throughput Sequencing
	Bacterial genera	Bacterial species	
Beef *Kargyong*	*Escherichia, Klebsiella, Citrobacter, Staphylococcus, Bacillus, Enterobacter*	*Staphylococcus piscifermentans, Bacillus cereus*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Citrobacter europaeus Citrobacter freundii*	*Psychrobacter pulmonis, Psychrobacter spp., Lactococcus spp., Agrobacterium spp., Pseudomonas veronii, Leuconostoc spp., Kurthia zopfii, Chryseobacterium spp., Psychrobacter sanguinis, Staphylococcus sciuri*
Pork *Kargyong*	*Citrobacter, Enterobacter, Staphylococcus, Pseudomonas*	*Staphylococcus aureus*, *Staphylococcus saprophyticus Burkholderia cepacia Citrobacter werkamanii*, *Pseudocitrobacter anthropic*	*Lactobacillus sakei*, *Psychrobacter pulmonis*, *Lactococcus garvieae*, *Lactobacillus* spp., *Psychrobacter* spp., *Lactococcus* spp., *Weissella cibaria*, *Leuconostoc* spp., *Macrococcus caseolyticus*, *Enterococcus* sp., *Weissella ceti*
Satchu	*Klebsiella, Salmonella, Enterobacter*	*Citrobacter freundii*, *Klebsiella grimontii*, *Salmonella enterica*, *Klebsiella aerogens*	*Ignatzschineria* spp., *Chryseobacterium* spp., *Staphylococcus succinus*, *Bdellovibrio bacteriovorus*, *Clostridium* spp., *Novosphingobium* spp., *Staphylococcus equorum*, *Psychrobacter pulmonis*, *Psychrobacter* spp., *Acinetobacter* spp., *Anaerospora* spp., *Brevundimonas* spp., *Enterobacter* spp., *Ketogulonicigenium vulgare*, *Luteolibacter* spp., *Pseudomonas stutzeri*
Khyopeh	*Escherichia, Staphylococcus, Enterococcus*	*Enterococcus faecalis*, *Escherichia fergusonii* and *Macrococcus caseolyticus*	*Enterococcus* spp., *Lactobacillus sakei*, *Carnobacterium divergens*, *Carnobacterium* spp., *Lactobacillus* spp., *Staphylococcus equorum*, *Brochothrix* spp.

Bacterial Gene Functionality

A total of 140 KEGG pathways was obtained from PICRUSt2 analysis which showed the major predictive functionality of metabolism (78.1%) such as carbohydrate metabolism (23.1%), amino acids metabolism (17.5%), metabolism of cofactors and vitamins (17.3%), metabolism of terpenoids and polyketides (12.7%), lipid metabolism (12.5%), metabolism of other amino acids (12.1%), and metabolism of xenobiotics biodegradation (4.4%); genetic information processing (14.5%), environmental information processing (3.5%), cellular processes (3.2%), human diseases (0.5%) and organismal systems (0.2%) (Figure 28). The detailed functional pathways with gene copy numbers (reads) using 16S rRNA sequences profile inferred by PICRUSt2 against KEGG database in meat products of Sikkim are shown in Table 33. The functional pathways with a relative abundance of more than 1% were represented by a Heatmap using GraphPad Prism 8.02 (Figure 29).

Table 33: Abundance of functional pathway with gene copy numbers (reads) using 16S rRNA sequences profile inferred by PICRUSt2 against KEGG database in meat products of Sikkim

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Metabolism	Carbohydrate Metabolism	Glycolysis/Gluconeogenesis	1871.5305	3310.6155	27.977	238.205
		Citrate cycle (TCA cycle)	1767.9811	1383.6393	34.1519	93.3815
		Pentose phosphate pathway	2082.2573	3954.8386	29.5355	264.0873
		Pentose and glucuronate interconversions	899.7185	736.1023	16.165	82.8319
		Fructose and mannose metabolism	1066.8515	2773.3218	15.3321	210.1785
		Galactose metabolism	974.4559	1689.21	13.5852	176.1234
		Ascorbate and aldarate metabolism	699.3472	237.6917	11.7028	70.6839
	Lipid metabolism	Fatty acid biosynthesis	3236.8862	4401.6915	48.2269	266.8685
		Fatty acid degradation	1417.0042	958.2346	21.7125	0
		Synthesis and degradation of ketone bodies	2831.46	2763.0375	45.41	132.415

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Metabolism	Lipid metabolism	Steroid biosynthesis	18.4163	11.4375	1.1875	0.75
		Primary bile acid biosynthesis	41.5344	486.6978	2.3333	38.8144
		Secondary bile acid biosynthesis	370.19	4380.28	15	349.33
	Energy metabolism	Oxidative phosphorylation	829.476	631.6413	15.1507	46.6531
		Photosynthesis	731.7013	1334.6909	0	72.2075
		Nitrogen metabolism	1000.825	725.1244	15.4392	55.4726
		Sulfur metabolism	1364.6938	830.45	22.1125	93.7069
		Nitrogen metabolism	1000.825	725.1244	15.4392	55.4726
		Methane metabolism	642.7295	804.5499	9.7141	59.2752
		Carbon fixation in photosynthetic organisms	1860.5217	2506.7744	32.6089	159.1644
		Photosynthesis - antenna proteins	3.4286	48.5714	0	3.4286

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Metabolism	Nucleotide metabolism	Pyrimidine metabolism	1630.8717	2538.5408	24.318	152.318
		Purine metabolism	1384.9629	2064.566	20.1032	120.3766
	Amino acid metabolism	Alanine, aspartate and glutamate metabolism	2288.2178	2564.2289	33.5715	153.7881
		Glycine, serine and threonine metabolism	1907.4709	1446.7741	27.6935	82.8521
		Cysteine and methionine metabolism	1683.403	1628.3645	25.9827	126.16
		Valine, leucine and isoleucine degradation	1646.6893	1013.7607	27.2666	47.5397
		Valine, leucine and isoleucine biosynthesis	3070.0464	3586.95	43.4355	219.7855
		Lysine biosynthesis	2221.5725	2086.285	31.4475	167.0645
	Metabolism of cofactors and vitamins	Ubiquinone and other terpenoid-quinone biosynthesis	1114.315	679.867	19.5405	49.6495

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Metabolism	Amino acid metabolism	Lysine degradation	837.4419	337.0144	15.3993	0
		Arginine and proline metabolism	1223.9435	655.4933	18.006	58.7611
		Histidine metabolism	2046.9063	702.1979	29.1816	18.5789
		Tyrosine metabolism	920.2803	939.7406	12.9558	33.4942
		Phenylalanine metabolism	803.5238	338.8296	12.7996	18.3715
		Tryptophan metabolism	1028.4797	646.14	15.7939	29.2577
		Phenylalanine, tyrosine and tryptophan biosynthesis	1829.5671	1521.1877	26.7181	87.9345
	Metabolism of terpenoids and polyketides	Geraniol degradation	1640.36	356.9938	19.1438	0
		Terpenoid backbone biosynthesis	1966.5261	3194.6978	28.7011	201.5344
		Carotenoid biosynthesis	70.7382	19.7647	4.1176	17.5094
		Zeatin biosynthesis	722.2325	1254.42	11.0825	66.5825

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Metabolism	Biosynthesis of other secondary metabolites	Penicillin and cephalosporin biosynthesis	339.1929	91.5529	1.9043	0
		Flavonoid biosynthesis	55.212	9.467	0.6	0.6
		Tropane, piperidine and pyridine alkaloid biosynthesis	728.1158	210.9533	0	0
	Xenobiotics biodegradation and metabolism	Chlorocyclohexane and chlorobenzene degradation	458.797	466.179	4.516	25.733
		Benzoate degradation	928.0208	658.3884	11.2926	32.3855
		Fluorobenzoate degradation	554.325	0	0	0
		Caprolactam degradation	856.4171	649.4214	12.4043	0
		Drug metabolism - other enzymes	1623.8155	2359.3273	24.4827	0
	Metabolism of other amino acids	beta-Alanine metabolism	1061.6625	283.335	0	0
		Taurine and hypotaurine metabolism	1132.2575	2108.0962	15.3938	0
		Phosphonate and phosphinate metabolism	432.1293	176.1814	4.7379	28.0471

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Metabolism	Metabolism of other amino acids	Selenocompound metabolism	1576.6293	1668.0333	26.1867	149.0867
		Cyanoamino acid metabolism	1022.5879	0	0	0
		D-Glutamine and D-glutamate metabolism	2978.2633	5071.3467	46.9967	264.9967
		D-Arginine and D-ornithine metabolism	673.66	132.45	10.33	63
		D-Alanine metabolism	2713.74	5010.4567	38.6633	273.6633
		Glutathione metabolism	1855.4484	1880.9789	26.3563	88.3147
	Glycan biosynthesis and metabolism	N-Glycan biosynthesis	27.985	0.6364	1.2273	0
		Other glycan degradation	356.585	313.4575	8.25	78.915
		Glycosaminoglycan degradation	68.7143	91.6957	2.8571	7.2857
	Carbohydrate metabolism	Starch and sucrose metabolism	1017.1055	1571.7832	16.253	117.0235

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Metabolism	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism	1249.7167	2749.6935	18.508	179.3926
		Inositol phosphate metabolism	554.9726	486.8409	5.5857	41.9417
		Glyoxylate and dicarboxylate metabolism	1247.8539	659.0009	20.6485	27.4845
		Lipopolysaccharide biosynthesis	1415.6413	418.2853	24.6867	5.7333
		Peptidoglycan biosynthesis	2513.8379	4693.4116	39.1637	268.7332
		Pyruvate metabolism	2222.7211	3170.0595	33.4832	190.3489
	Lipid metabolism	Glycerolipid metabolism	783.3631	1086.4428	12.6862	74.0928
		Linoleic acid metabolism	512	185.4467	5.11	0
		Sphingolipid metabolism	85.923	43.6	2.833	0
		Biosynthesis of unsaturated fatty acids	1198.6833	936.412	15.1433	45.244

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Metabolism	Xenobiotics biodegradation and metabolism	Aminobenzoate degradation	552.5217	496.1307	7.505	24.833
		Nitrotoluene degradation	247.2255	80.2655	5.2564	0.5455
		Dioxin degradation	451.9071	1272.3029	0	0
		Xylene degradation	0	0	2.65	0
		Toluene degradation	796.2371	566.5664	0	0
		Polycyclic aromatic hydrocarbon degradation	0	0	2.47	0
		Propanoate metabolism	1678.7324	1854.2148	25.4215	88.7364
		Styrene degradation	690.7356	0	0	0
	Biosynthesis of other secondary metabolites	Streptomycin biosynthesis	1741.9644	3513.9956	27.1078	200.8122

‘

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Metabolism	Carbohydrate metabolism	Butanoate metabolism	1711.49	1580.9611	24.8546	83.8086
		C5-Branched dibasic acid metabolism	2701.28	2329.7017	36.58	137.7767
	Metabolism of cofactors and vitamins	One carbon pool by folate	2366.535	3366.1543	38.5086	192.6879
		Thiamine metabolism	1899.6127	3477.5236	34.8473	178.7855
		Riboflavin metabolism	1197.1008	734.7458	19.9983	29.0275
		Vitamin B6 metabolism	1669.1287	1122.795	23.4562	76.4162
		Nicotinate and nicotinamide metabolism	1764.3339	1805.68	24.655	100.9061
		Pantothenate and CoA biosynthesis	2287.62	2680.5993	36.586	175.4867
		Biotin metabolism	1881.544	1496.446	33.066	62.466
		Thiamine metabolism	1899.6127	3477.5236	34.8473	178.7855
		Lipoic acid metabolism	1950.14	2873.01	39.915	150.165
		Folate biosynthesis	2101.565	1557.1517	32.1233	68.1658

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Metabolism	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism	818.3004	394.6981	16.8185	21.6917
	Metabolism of terpenoids and polyketides	Biosynthesis of ansamycins	4222.42	1973.33	65.33	356.66
		Biosynthesis of siderophore group nonribosomal peptides	117.0206	71.965	2.125	0
		Biosynthesis of vancomycin group antibiotics	1728.2	4340.88	36.33	258.33
Environmental Information Processing	Signal transduction	Two-component system	798.6621	602.392	12.9813	46.5729
	Membrane transport	ABC transporters	1604.9841	1177.908	16.3486	99.7307
		Bacterial secretion system	1452.9968	1472.5614	24.1743	71.3324
Genetic Information Processing	Translation	Aminoacyl-tRNA biosynthesis	2309.0156	3943.4116	35.1222	214.1119
		Ribosome biogenesis in eukaryotes	106.6659	135.1415	1.5446	6.6422
		Ribosome	2133.2624	3750.8271	33.5531	205.1317
		RNA transport	74.2439	149.1726	1.0227	8.1312

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Genetic Information Processing	Replication and repair	Base excision repair	1446.5062	2522.5957	23.5619	146.379
		Nucleotide excision repair	985.8492	1638.0233	16.3671	106.3596
		Mismatch repair	2238.5917	3715.4648	36.5557	219.5043
		Homologous recombination	2073.8133	3263.4003	32.4033	176.9533
		Non-homologous end-joining	246.422	4.3	3.5	0
		DNA replication	1725.9404	2754.9	28.5558	160.3569
	Transcription	RNA polymerase	937.261	1208.2697	19.0433	79.6323
	Folding, sorting and degradation	Protein export	2112.944	3213.0955	34.914	173.8645
		Sulfur relay system	1857.6055	2131.5245	32.27	104.7564
		RNA degradation	888.9097	1298.8118	15.9461	75.0166
		Protein processing in endoplasmic reticulum	25.5859	61.4422	0.7971	3.4396

Level 1	Level 2	Pathway Name	Gene copies number (reads)			
			Beef *kargyong*	**Pork *kargyong***	**Yak *satchu***	***Khyopeh***
Cellular Processes	Cell growth and death	Cell cycle – Caulobacter	2085.9019	3195.9562	35.6012	179.7269
	Cell motility’	Bacterial chemotaxis	1749.7808	144.0631	39.5992	0
	Cell motility	Flagellar assembly	732.7705	29.5995	23.2505	0
	Transport and catabolism	Peroxisome	524.0137	480.5597	8.6611	19.8189
Organismal Systems	Environmental adaptation	Plant-pathogen interaction	300.8771	330.712	5.1806	22.3426
	Endocrine system	Insulin signalling pathway	105.8926	44.1277	1.5128	5.6495
Human Diseases	Drug resistance: antimicrobial	beta-lactam resistance	296.2375	150.0075	2.25	1.25
	Infectious disease: bacterial	Epithelial cell signalling in *Helicobacter pylori* infection	130.6029	51.9011	2.0832	8.1546
	Infectious disease: bacterial	*Staphylococcus aureus* infection	237.887	715.7127	2.1515	48.0094

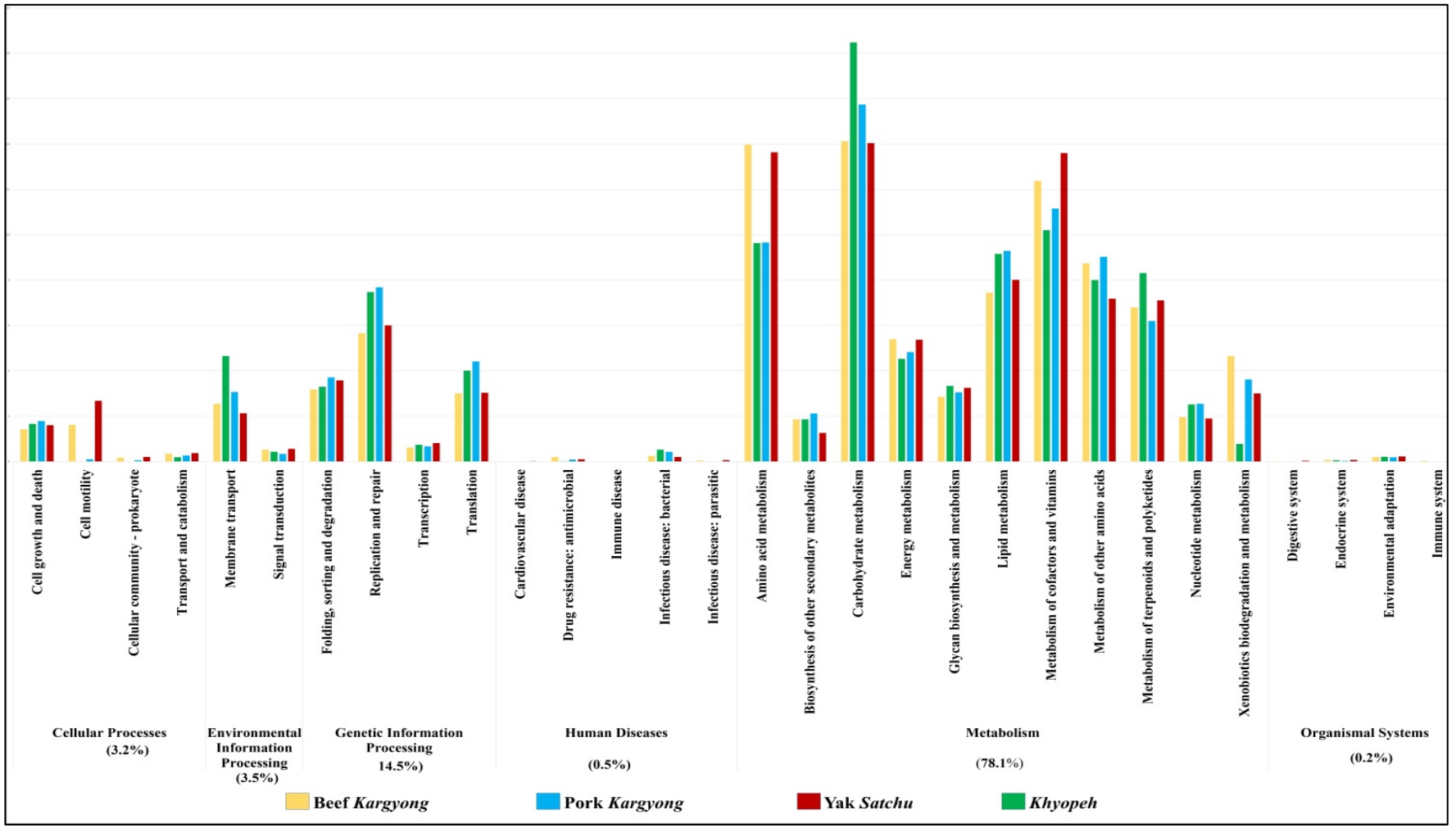


Figure 28: Detailed predictive functionality as inferred by KEGG database using PICRUSt2. Among the six common classes of functionality category, the predictive funtionality is dominated by metabolism category, reflecting the high metabolic rate of the processed meat samples.

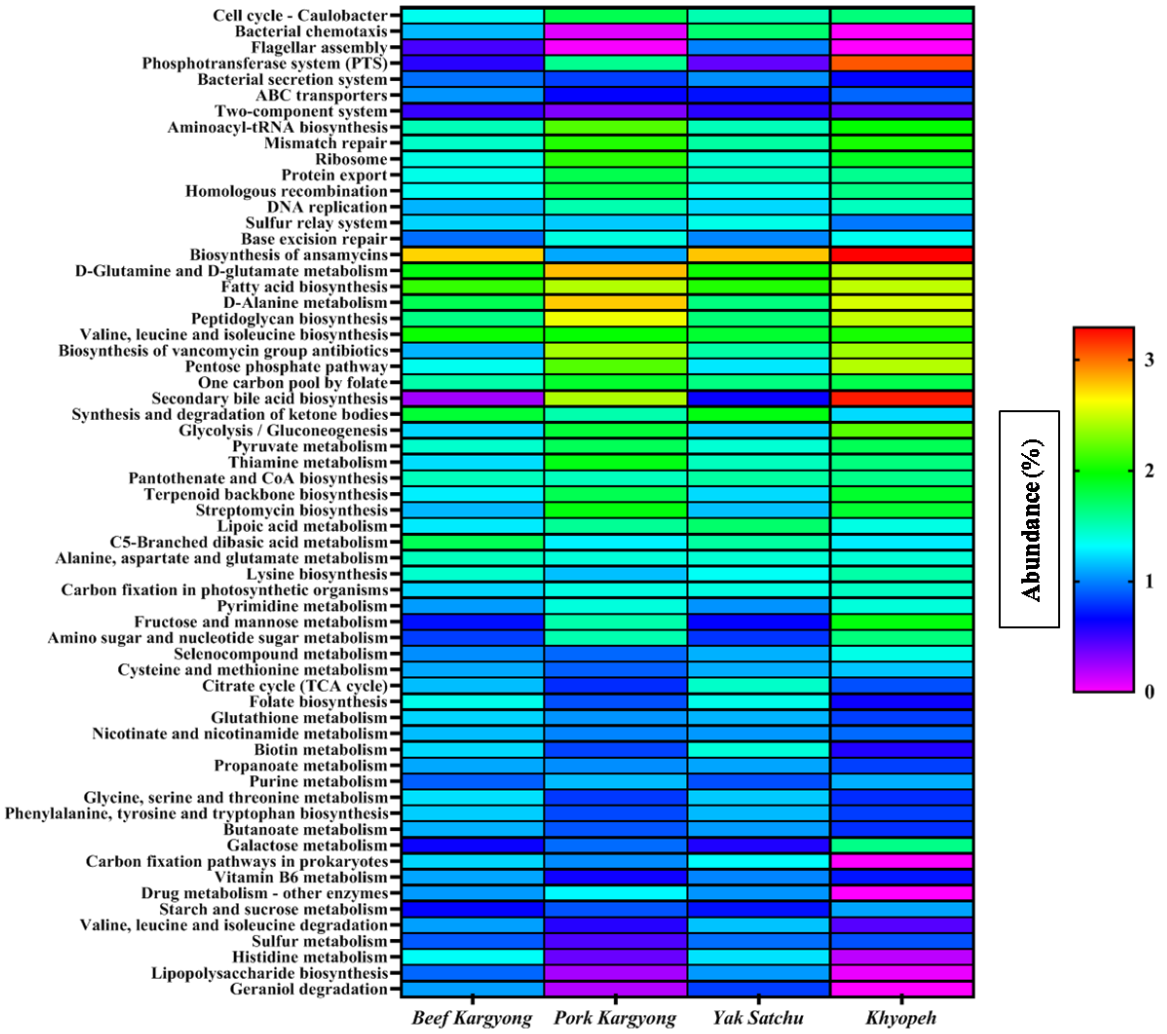


Figure 29: Heatmap (GraphPad Prism 8.02) showing the predictive functional pathway found in traditionally processed meat products through PICRUSt2 against KEGG database.

BACTERIAL DIVERSITY IN TRADITIONAL FISH PRODUCTS

Overall Bacterial Communities

Metagenomic DNA from nine samples of sun-dried fish products (3 samples each from *suka ko maacha*, *sidra* and *sukuti*, respectively) was extracted and was analysed for metataxonomic by high-throughput sequencing method using bioinformatics (QIIME2) software. Metataxonomic of bacterial communities in all nine samples, with >1% abundance, showed the phylum Firmicutes (49.8%) was the most dominant followed by the phyla Proteobacteria (47.6%), Bacteroidetes (1.41%) and other minor phyla, detected at <1% abundance, comprised of 1.19% (Figure 30a). At the Family level, Moraxellaceae (21.26%) was dominant followed by Bacillaceae (20.91%), Enterobacteriaceae (18.21%), Staphylococcaceae (9.75%), Clostridiaceae (6.25%), Planococcaceae (4.35%), Peptostreptococcaceae (3.69%), Enterococcaceae (2.55%), Pseudomonadaceae (2.48%), Vibrionaceae (1.55%), Flavobacteriaceae (1.26%), Bartonellaceae (1.09%), and Alcaligenaceae (1.06%), including other families with <1% abundance (5.59%) (Figure 30b).

Psychrobacter (24.19%) was the most dominant genus followed by *Bacillus* (21.19%), *Staphylococcus* (10.32%), *Serratia* (9.7%), *Clostridium* (5.27%), *Enterobacter* (3.2%), *Pseudomonas* (3.03%), *Clostridium* (2.44%), *Rummeliibacillus* (2.06%), *Enterococcus* (1.98%), *Photobacterium* (1.86%), *Myroides* (1.57%), *Peptostreptococcus* (1.56%), *Plesiomonas* (1.15%), *Achromobacter* (1.11%), and other minor genera <1% abundance of 9.36% (Figure 30c). *Psychrobacter celer* (12.37%) was the most abundant species followed by *Serratia marcescens* (9.7%), *Psychrobacter pulmonis* (6.37%), *Clostridium perfringens* (4.55%), *Clostridium bifermentans* (1.76%), *Myroides odoratimimus* (1.57%), and *Plesiomonas shigelloides* (1.15%). Besides these identified species, some unknown species were also detected such as *Bacillus* spp.

(21.18%), *Staphylococcus* spp. (9.98%), *Psychrobacter* spp. (5.11%), *Enterobacter* spp. (3.19%), *Pseudomonas* spp. (3.02%), *Rummeliibacillus* spp. (2.06%), *Enterococcus* spp. (1.98%), *Peptostreptococcus* spp. (1.56%), *Clostridium* spp. (1.34%), and *Achromobacter* spp. (1.11%). Other minor species occurring at <1% abundance comprised of 11.99% (Figure 30d).

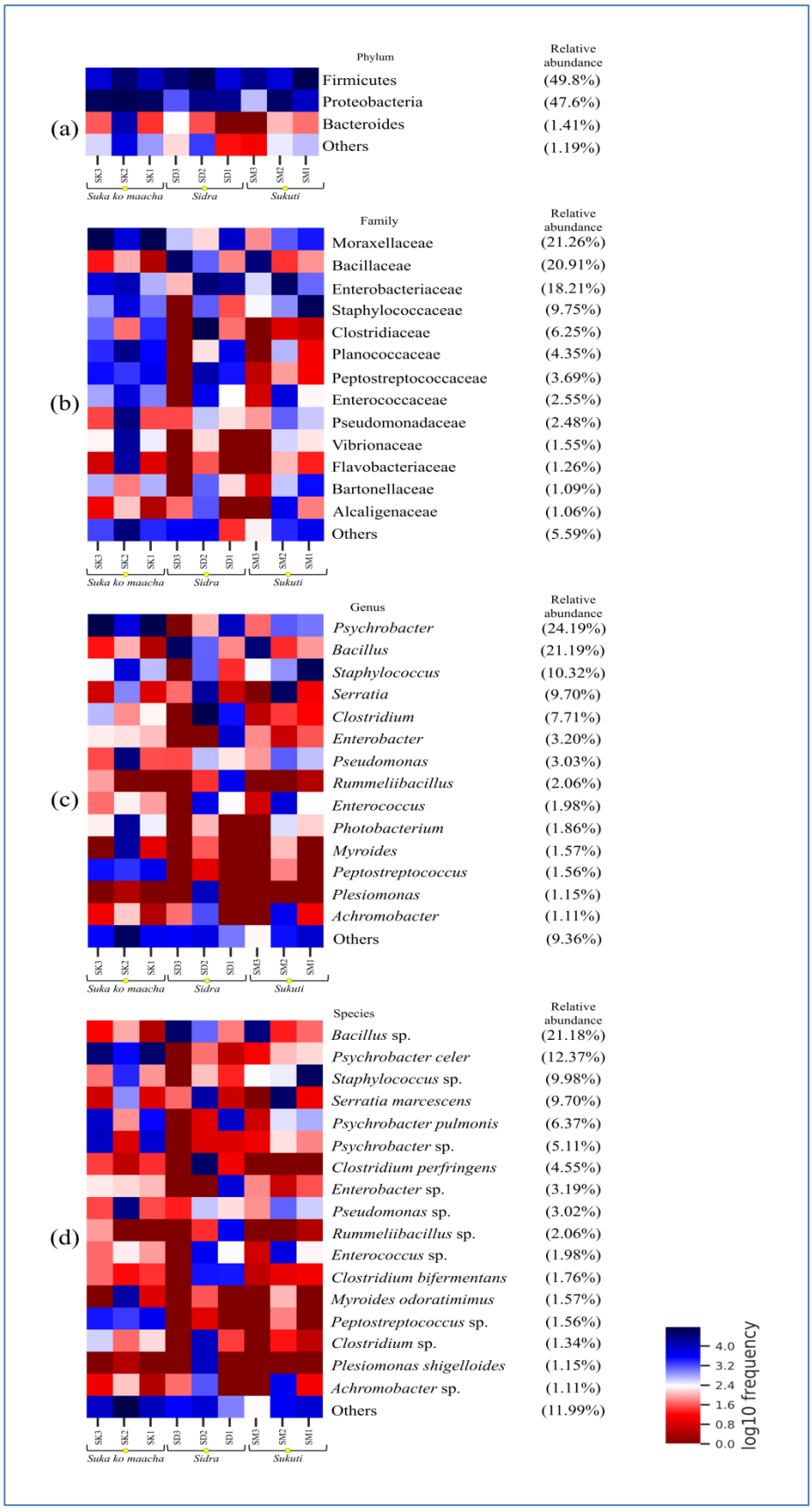


Figure 30: Metataxonomic distribution of bacterial diversity in fish products of Sikkim at (a) Phylum, (b) Family and (c) Genus and (d) species level by High-throughput Sequencing method.

Bacterial communities in *suka ko maacha*

In samples of *suka ko maccha*, Firmicutes (65.9%) was the most abundant phylum followed by phylum Proteobacteria (33.56%) (Figure 31a). Others phyla detected at <1% abundance were comprised of 0.54% which included Actinobacteria, Bacteroidetes, Fusobacteria, and Cyanobacteria. At the family level, we observed Bacillaceae (32.28%) as the dominant family followed by Staphylococcaceae (26.52%), Enterobacteriaceae (23.89%), Enterococcaceae (3.71%), Alcaligenaceae (2.68%), Moraxellaceae (2.56%), Bartonellaceae (2.22%), Halomonadaceae (1.68%) and Pseudomonadaceae (1.08%) (Figure 31a). Other minor families, detected at <1% abundance (3.39%), were Micrococcaceae, Sphingomonadaceae, Xanthomonadaceae, Vibrionaceae, Planococcaceae, Oxalobacteraceae, Leuconostocaceae, Streptococcaceae, Pseudoalteromonadaceae, Shewanellaceae, Lactobacillaceae, Flavobacteriaceae, Methylobacteriaceae, Peptostreptococcaceae, Comamonadaceae, Aeromonadaceae, Methylophilaceae, Brevibacteriaceae, Tissierellaceae, Brucellaceae, Fusobacteriaceae, Weeksellaceae, Exiguobacteraceae, Rhodobacteraceae, Xanthobacteraceae, Corynebacteriaceae, Carnobacteriaceae, Rhizobiaceae, Clostridiaceae, Microbacteriaceae, Erysipelotrichaceae, Caulobacteraceae, Acetobacteraceae, Veillonellaceae, and Propionibacteriaceae.

Bacillus (32.62%) was the most abundant genus in *suka ko maacha* samples followed by *Staphylococcus* (28.33%), *Serratia* (23.07%), *Enterococcus* (3.7%), *Achromobacter* (2.7%), *Kushneria* (1.81%), *Psychrobacter* (1.45%), *Acinetobacter* (1.23%), and *Pseudomonas* (1.11%) (Figure 31a). About 3.98% of more than 64 minor genera occurring at <1% abundance were detected in the samples which included *Kocuria, Morganella, Sphingomonas, Stenotrophomonas, Photobacterium, Leuconostoc, Lactococcus, Janthinobacterium, Shewanella, Vibrio, Enterobacter, Lactobacillus,*

Proteus, Vagococcus, Ralstonia, Myroides, Macrococcus, Vibrio, Jeotgalicoccus, Oceanimonas, Methylotenera, Peptostreptococcus, Brevibacterium, Ochrobactrum, Cetobacterium, Comamonas, Providencia, Clostridium, Chryseobacterium, Exiguobacterium, Delftia, Pseudoalteromonas, Paracoccus, Methylobacterium, Enhydrobacter, Novosphingobium, Hafnia, Flavobacterium, Sporosarcina, Salinicoccus, Xanthobacter, Peptoniphilus, Wohlfahrtiimonas, Corynebacterium, Acidovorax, Carnobacterium, Planomicrobium, Weissella, Agrobacterium, Klebsiella, Clostridium, Cloacibacterium, Microbacterium, Erysipelothrix, Arthrobacter, Asticcacaulis, Micrococcus, Acetobacter, Dialister, Lentibacillus, Rummeliibacillus, Propionibacterium, Pelomonas and *Erwinia*. At the species level with >1% abundance, we detected *Serratia marcescens* (23.05%), and unknown species of *Bacillus* (32.6%), *Staphylococcus* (28.27%), *Enterococcus* (3.7%), *Achromobacter* (2.7%), *Kushneria* (1.81%) and *Pseudomonas* (1.1%) (Figure 31a). Huge diversity of minor species (6.77%) with less than 1% abundance was recovered in samples which included *Psychrobacter pulmonis, Acinetobacter johnsonii*, *Psychrobacter celer*, *Photobacterium damselae*, *Janthinobacterium lividum*, *Acinetobacter guillouiae*, *Photobacterium angustum*, *Kocuria koreensis*, *Myroides odoratimimus*, *Staphylococcus sciuri*, *Bacillus thermoamylovorans*, *Sphingomonas wittichii*, *Jeotgalicoccus psychrophilus*, *Methylotenera mobilis*, *Morganella morganii*, *Leuconostoc mesenteroides*, *Psychrobacter meningitidis*, *Acinetobacter lwoffii*, *Hafnia alvei*, *Corynebacterium stationis*, *Shewanella algae*, *Clostridium bifermentans*, *Psychrobacter sanguinis*, *Psychrobacter arenosus*, *Vibrio harveyi*, *Stenotrophomonas geniculata*, *Paracoccus marcusii*, *Weissella viridescens*, *Kocuria palustris*, *Pseudomonas stutzeri*, *Pseudomonas veronii*, *Carnobacterium viridans*, *Lactococcus garvieae*, *Lactobacillus zeae*, *Vibrio cholerae*, *Staphylococcus pettenkoferi,*

Propionibacterium acnes, *Kushneria avicenniae*, *Brevibacterium aureum,* and several unknown species of genera *Acinetobacter* ssp., *Psychrobacter* spp., *Kocuria* spp., *Stenotrophomonas* spp., *Sphingomonas* spp., *Leuconostoc* spp., *Lactococcus* spp., *Vibrio* spp., *Shewanella* spp., *Enterobacter* spp., *Lactobacillus* spp., *Proteus* spp., *Vagococcus* spp., *Ralstonia* spp., *Macrococcus* spp., *Oceanimonas* spp., *Peptostreptococcus* spp., *Brevibacterium* spp., *Ochrobactrum* spp., *Cetobacterium* spp., *Comamonas* spp., *Providencia* spp., *Chryseobacterium* spp., *Exiguobacterium* spp., *Delftia* spp., *Pseudoalteromonas* spp., *Serratia* spp., *Methylobacterium* spp., *Enhydrobacter* spp., *Novosphingobium* spp., *Morganella* spp., *Flavobacterium* spp., *Clostridium* spp., *Sporosarcina* spp., *Salinicoccus* spp., *Xanthobacter* spp., *Peptoniphilus* spp., *Paracoccus* spp., *Wohlfahrtiimonas* spp., *Acidovorax* spp., *Planomicrobium* spp., *Agrobacterium* spp., *Klebsiella* spp., *Cloacibacterium* spp., *Microbacterium* spp., *Erysipelothrix* spp., *Arthrobacter* spp., *Carnobacterium* spp., *Asticcacaulis* spp., *Micrococcus* spp., *Acetobacter* spp., *Dialister* spp., *Lentibacillus* spp., *Rummeliibacillus* spp., *Pelomonas* spp., *Weissella* spp. and *Erwinia* spp.

Bacterial communities in *sidra*

Firmicutes was the most dominant phylum with 62.67% relative abundance in *sidra* samples followed by phylum Proteobacteria (36.26%), and other minor phyla (1.07%), detected at <1% abundance, which included Bacteroidetes, Actinobacteria, Fusobacteria, Cyanobacteria, Planctomycetes, Verrucomicrobia and Gemmatimonadetes (Figure 31b). At the family level, Bacillaceae (30.42%) was the most dominant family followed by Enterobacteriaceae (24.95%), Clostridiaceae (17.06%), Moraxellaceae (8.88%), Peptostreptococcaceae (6.65%), Planococcaceae (4.64%) and Enterococcaceae (1.64) including minor families (5.78%) with <1%

relative abundance (Figure 31b), which included Leuconostocaceae, Micrococcaceae, Streptococcaceae, Bartonellaceae, Alcaligenaceae, Staphylococcaceae, Sphingomonadaceae, Pseudomonadaceae, Lactobacillaceae, Methylobacteriaceae, Xanthomonadaceae, Comamonadaceae, Paenibacillaceae, Bacteroidaceae, Xanthobacteraceae, Weeksellaceae, Prevotellaceae, Acetobacteraceae, Oxalobacteraceae, Vibrionaceae, Fusobacteriaceae, Corynebacteriaceae, Veillonellaceae, Rhizobiaceae, Ruminococcaceae, Propionibacteriaceae, Aeromonadaceae, Lachnospiraceae, Dermabacteraceae, Flavobacteriaceae, Pseudoalteromonadaceae, Microbacteriaceae, Verrucomicrobiaceae, Brevibacteriaceae, Isosphaeraceae, Exiguobacteraceae, Shewanellaceae, Brucellaceae, Gemmataceae, Rickettsiaceae, Halomonadaceae, Tissierellaceae, Erysipelotrichaceae, Carnobacteriaceae, Methylophilaceae, Rhodobacteraceae, Oceanospirillaceae, Caulobacteraceae and Aerococcaceae.

In *sidra* samples, the dominant genus was *Bacillus* (30.91%) followed by *Clostridium* (15.76%), *Psychrobacter* (11.96%), *Enterobacter* (9.28%), *Clostridium* (6.98%), *Rummeliibacillus* (6.13%), *Serratia* (5.80%), *Plesiomonas* (3.45%), *Enterococcus* (2.12%) and others with <1% abundance was 6.71% (Figure 31b) which included *Kocuria*, *Leuconostoc*, *Acinetobacter*, *Klebsiella*, *Achromobacter*, *Lactococcus*, *Staphylococcus*, *Sphingomonas*, *Pseudomonas*, *Caloramator*, *Lactobacillus*, *Stenotrophomonas*, *Brevibacillus*, *Delftia*, *Bacteroides*, *Macrococcus*, *Xanthobacter*, *Cloacibacterium*, *Prevotella*, *Acetobacter*, *Ralstonia*, *Wohlfahrtiimonas*, *Cetobacterium*, *Vagococcus*, *Corynebacterium*, *Providencia*, *Photobacterium*, *Dialister*, *Arthrobacter*, *Morganella*, *Erwinia*, *Propionibacterium*, *Proteus*, *Myroides*, *Methylobacterium*, *Micrococcus*, *Vibrio*, *Aeromonas*, *Brachybacterium*, *Oceanimonas*, *Ruminococcus*, *Microbacterium*, *Pelomonas*, *Exiguobacterium*, *Brevibacterium*,

Enhydrobacter, *Shewanella*, *Akkermansia*, *Ochrobactrum*, *Sporosarcina*, Weissella, *Faecalibacterium*, *Lentibacillus*, *Rickettsia*, *Comamonas*, *Peptostreptococcus* and *Kushneria*.

Species detected at >1% abundance were *Clostridium perfringens* (13.63%), *Psychrobacter pulmonis* (11.92%), *Serratia marcescens* (5.80%), *Clostridium bifermentans* (5.23%), and *Plesiomonas shigelloides* (3.45%), and several unknown species of genera which included *Bacillus* spp. (30.90%), *Enterobacter* spp. (9.27%), *Rummeliibacillus* spp. (6.13%), *Clostridium* spp. (3.69%) and *Enterococcus* spp. (2.12%) (Figure 31b). About 7.87% of several species in samples were detected <1% abundance which included *Acinetobacter lwoffii*, *Sphingomonas wittichii*, *Brevibacillus laterosporus, Clostridium subterminale*, *Bacteroides uniformis*, *Staphylococcus pettenkoferi*, *Leuconostoc mesenteroides*, *Providencia stuartii*, *Arthrobacter nicotinovorans*, *Morganella morganii*, *Photobacterium damselae*, *Psychrobacter celer, Propionibacterium acnes*, *Photobacterium angustum*, *Enterobacter pulveris*, *Myroides odoratimimus*, *Acinetobacter guillouiae*, *Bacillus flexus*, *Shewanella algae*, *Akkermansia muciniphila*, *Psychrobacter meningitidis*, *Staphylococcus sciuri*, *Brachybacterium conglomeratum*, *Pseudomonas stutzeri*, *Vibrio cholerae*, *Brevibacterium aureum*, *Faecalibacterium prausnitzii*, *Lactococcus garvieae*, *Staphylococcus succinus*, *Acinetobacter johnsonii*, *Cetobacterium somerae, Psychrobacter marincola*, *Acinetobacter venetianus,* and several unknown species *Comamonas* spp., *Peptostreptococcus* spp., *Kushneria* spp. *Morganella* spp., and *Photobacterium* spp.

Bacterial communities in *sukuti*

Phylum Proteobacteria (72.97%) was dominant in samples of *sukuti* followed by Firmicutes (20.84%), Bacteroidetes (3.93%) and other phyla (2.25%) (Figure 31c). Minor phyla recovered at <1% of abundance were Actinobacteria, Fusobacteria, Cyanobacteria, Planctomycetes, Verrucomicrobia and Gemmatimonadetes. Family Moraxellaceae (52.35%) was most abundant followed by families Planococcaceae (8.11%), Pseudomonadaceae (6.05%), Enterobacteriaceae (5.79%), Peptostreptococcaceae (4.37%), Vibrionaceae (4.28%), Flavobacteriaceae (3.71%), Staphylococcaceae (2.32%), Enterococcaceae (2.30%), Aeromonadaceae (2.08%), Fusobacteriaceae (1.70%), Clostridiaceae (1.68%), Tissierellaceae (1.19%) and other minor families with <1% abundance (4.06%) (Figure 31c) which included families Pseudoalteromonadaceae, Xanthomonadaceae, Bartonellaceae, Micrococcaceae, Shewanellaceae, Bacteroidaceae, Erysipelotrichaceae, Carnobacteriaceae, Leuconostocaceae, Halomonadaceae, Streptococcaceae, Corynebacteriaceae, Oxalobacteraceae, Sphingomonadaceae, Alcaligenaceae, Exiguobacteraceae, Bacillaceae, Lactobacillaceae, Methylobacteriaceae, Brevibacteriaceae, Oceanospirillaceae, Methylophilaceae, Prevotellaceae, Comamonadaceae, Dermabacteraceae, Aerococcaceae, Weeksellaceae, Caulobacteraceae, Rhodobacteraceae, Propionibacteriaceae, Paenibacillaceae, Xanthobacteraceae, Acetobacteraceae, Rhizobiaceae, Veillonellaceae, Brucellaceae, Ruminococcaceae, Microbacteriaceae, Lachnospiraceae, Verrucomicrobiaceae, Isosphaeraceae, Gemmataceae and Rickettsiaceae.

Psychrobacter (59.16%) was the most dominant genus followed by *Pseudomonas* (7.57%), *Photobacterium* (5.27%), *Myroides* (4.64%), *Peptostreptococcus* (4.63%), *Vagococcus* (2.65%), *Oceanimonas* (2.61%), *Staphylococcus* (2.16%), *Cetobacterium*

(2.02%), *Proteus* (2.02%), *Vibrio* (1.10%) and other genera at <1% abundance (6.17%) (Fig 31c). Genera occurred at <1% abundance comprised of *Macrococcus*, *Morganella*, *Kocuria*, *Shewanella*, *Wohlfahrtiimonas*, *Bacteroides*, *Clostridium*, *Acinetobacter*, *Serratia*, *Ignatzschineria*, *Enterobacter*, *Erysipelothrix*, *Klebsiella*, *Enterococcus*, *Peptoniphilus*, *Leuconostoc*, *Kushneria*, *Lactococcus*, *Carnobacterium*, *Arthrobacter*, *Corynebacterium*, *Sporosarcina*, *Vibrio*, *Propionigenium*, *Trichococcus*, *Clostridium*, *Providencia*, *Achromobacter*, *Rummeliibacillus*, *Ralstonia*, *Sphingomonas*, *Exiguobacterium*, *Bacillus*, *Lactobacillus*, *Planomicrobium*, *Brevibacterium*, *Marinobacterium*, *Salinicoccus*, *Jeotgalicoccus*, *Prevotella*, *Methylotenera*, *Brachybacterium*, *Micrococcus*, *Stenotrophomonas*, *Facklamia*, *Delftia*, *Acidovorax*, *Cloacibacterium*, *Asticcacaulis*, *Paracoccus*, *Flavobacterium*, *Janthinobacterium*, *Propionibacterium* and *Plesiomonas*. At the species level, *Psychrobacter celer* (36.91%) was the most dominant followed by *Psychrobacter pulmonis* (6.64%), *Myroides odoratimimus* (4.64%), *Photobacterium angustum* (2.73%), *Photobacterium damselae* (2.51%) (Figure 31c). Several unknown species of genera were also detected at >1% abundance which included *Psychrobacter* spp. (14.69%), *Pseudomonas* spp. (7.57%), *Peptostreptococcus* spp. (4.63%), *Vagococcus* spp. (2.62%), *Oceanimonas* spp. (2.61%), *Proteus* spp. (2.02%), *Cetobacterium* spp. (1.89%), *Staphylococcus* (1.28%) and *Vibrio* spp. (1.13%), and other species detected at <1% abundance (8.13%) (Figure 31c), which included *Psychrobacter meningitidis*, *Staphylococcus sciuri*, *Shewanella algae*, *Serratia marcescens*, *Staphylococcus equorum*, *Bacteroides coprosuis, Cetobacterium somerae*, *Corynebacterium stationis*, *Psychrobacter sanguinis*, *Carnobacterium viridans*, *Morganella morganii*, *Clostridium bifermentans*, *Vibrio harveyi*, *Vagococcus salmoninarum*, *Clostridium perfringens*, *Staphylococcus succinus*, *Kushneria avicenniae*, *Psychrobacter marincola*, *Jeotgalicoccus*

psychrophilus, *Prevotella stercorea***,** *Methylotenera mobilis*, *Brachybacterium conglomeratum*, *Lactobacillus helveticus*, *Vibrio shilonii*, *Acinetobacter venetianus*, *Sphingomonas wittichii*, *Arthrobacter psychrolactophilus*, *Leuconostoc mesenteroides*, *Acinetobacter guillouiae*, *Kocuria palustris*, *Acinetobacter johnsonii*, *Janthinobacterium lividum*, *Acinetobacter lwoffii*, *Propionibacterium acnes*, *Plesiomonas shigelloides* and *Lactococcus garvieae,* and several unknown species.

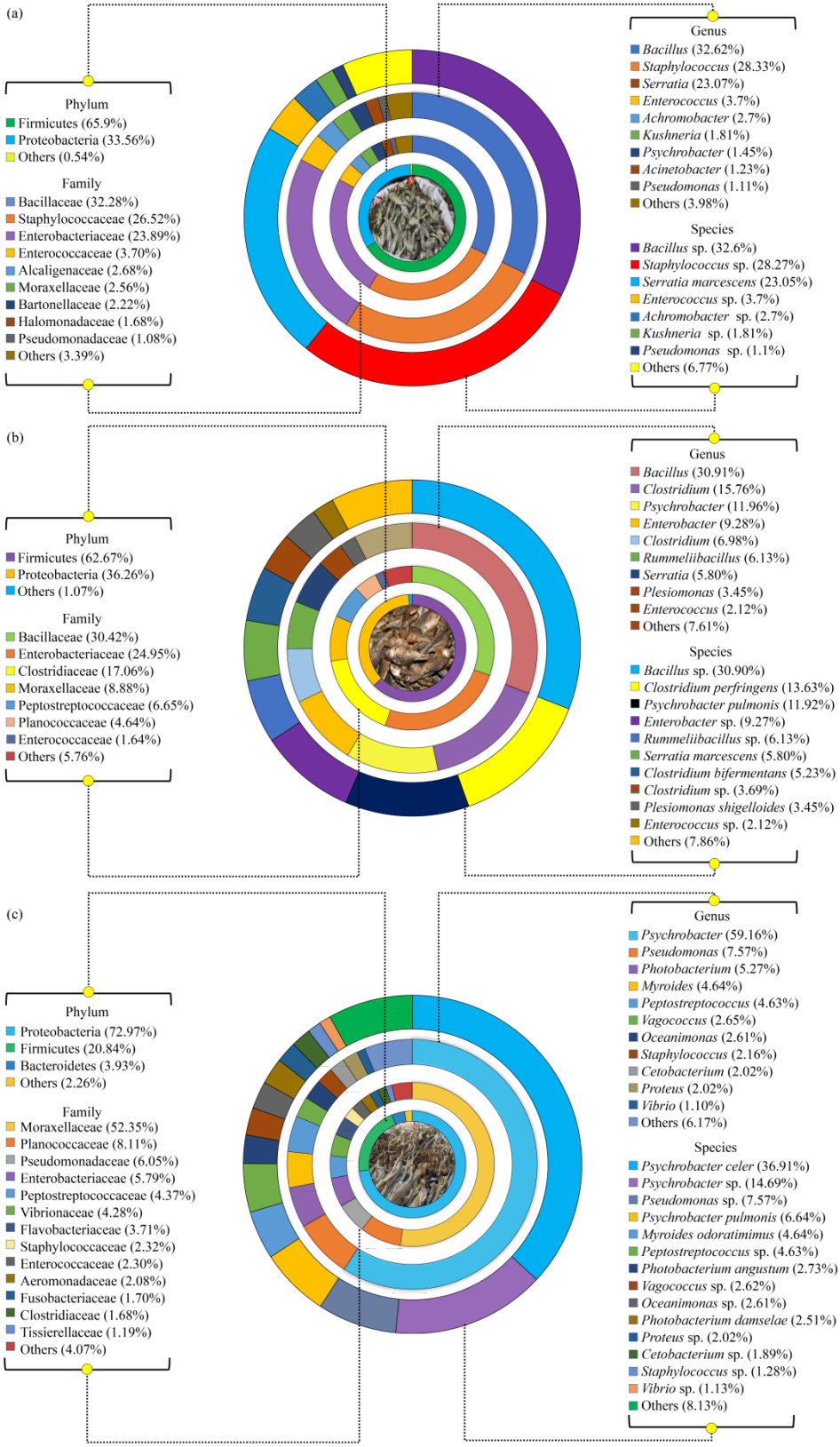


Figure 31: Dough-nut chart showing the bacterial composition in the traditionally processed fish products of Sikkim (a) *Suka ko maacha* (b) *Sidra* and (c) *Sukuti*. Each doughnut chart represents the relative abundance of the Phylum, Family and Genus level of the individual samples.

Unique species

We observed several genera as unique or rare to each product based on analysis of shared OTUs content among the samples. Analysis was performed based on the genera content of those prominent in all samples with relative abundance above 1%. *Acinetobacter*, *Achromobacter* and *Kushneria* were found to be unique genera in *suka ko maacha* samples. *Rummeliibacillus*, *Clostridium, Enterobacter* and *Plesiomonas* were observed as unique genera in *sidra*. *Cetobacterium*, *Proteus*, *Oceanimonas*, *Peptostreptococcus*, *Vagococcus*, *Photobacterium*, *Myroides* and *Vibrio* were unique genera in *sukuti*. *Enterococcus*, *Serratia* and *Bacillus* were shared genera between *suka ko maacha* and *sidra*. *Staphylococcus* and *Pseudomonas* were shared between *suka ko maacha* and *sukuti*. The core genus shared amongst all nine samples of sun-dried fish products was *Psychrobacter* (Figure 32).

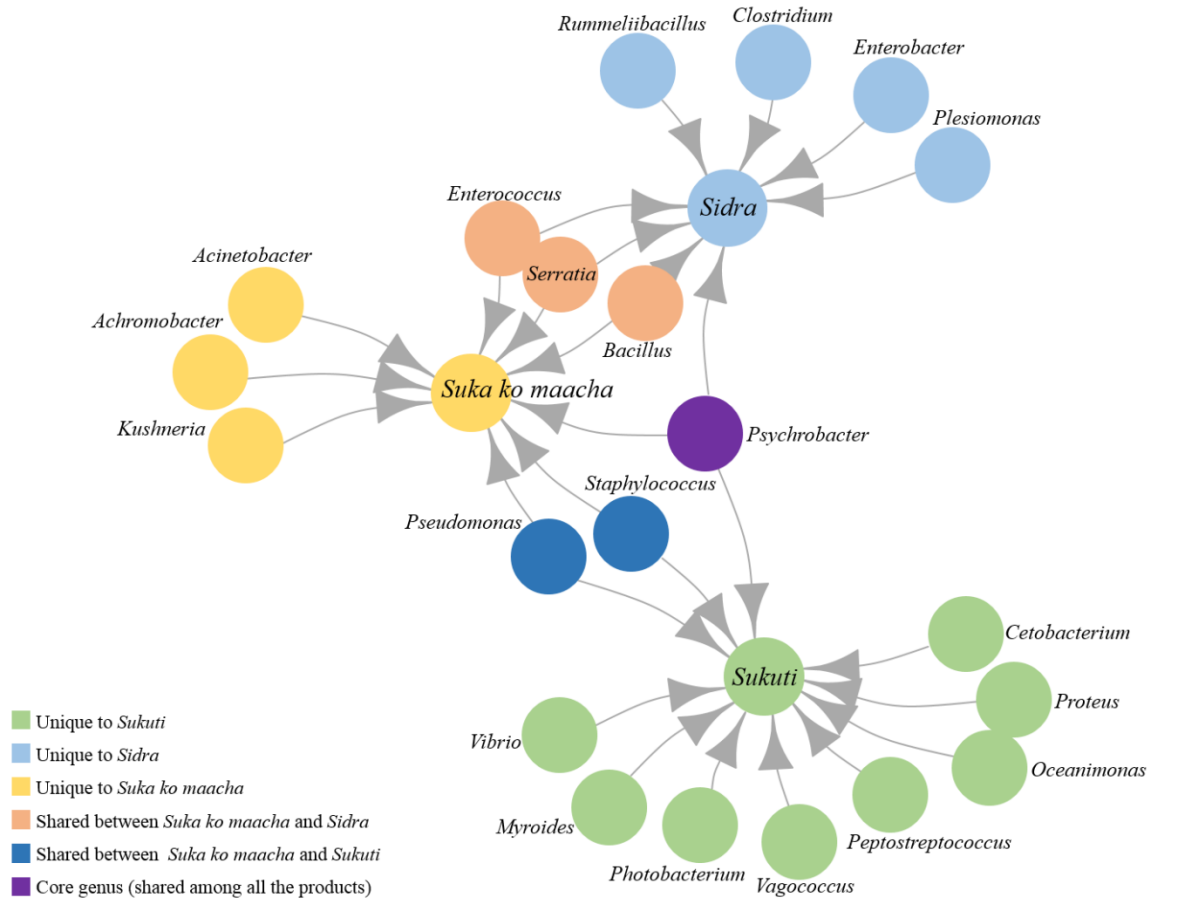


Figure 32: Graphical representation of the shared and unique genera among *Suka ko maacha*, *Sidra* and *Sukuti* visualized using iGraph R-package. Each circle represents the genera that was obtained from the 16S-targeted amplicon sequencing study where the difference in the shared and unique genera amongst the tree products are shown. *Psychrobacter* was the only shared genera among these samples.

Bacterial Diversity

To understand the bacterial community structure of traditionally processed fish products, alpha and beta diversities were calculated in QIIME2-2018.10 environment. Alpha diversity indices showed significance ($p>0.05$) between *sidra* and *sukuti* as per ace, chao1, fisher_alpha metrices indices (Table 34). However, we observed significance ($p>0.05$) in terms of Shannon indices between *suka ko maacha* and *sukuti*. It is noteworthy that the sequencing of the samples showed maximum coverage as per goods_coverage (0.99) (Table 33), an indication of maximum diversity being captured from the samples. Beta diversity showed clustering of bacterial components between *suka ko maacha* and *sidra,* whereas *sukuti* showed scattering pattern among the other samples, indicating a diverse population from *suka ko maacha* and *sidr*a samples as represented by PCA plot (Figure 33).

Table 34: Alpha diversity profiles of bacterial community analysed by High-throughput sequencing in traditionally processed fish products of Sikkim

Alpha diversity Metrices	*Suka ko maacha*			*Sidra*			*Sukuti*			p-value		
	SM1	SM2	SM3	SD1	SD2	SD3	SK1	SK2	SK3	SM Vs SD	SD Vs SK	SM vs SK
Ace	282.062	397.732	106.047	136.591	384.985	122.791	339.043	496.834	343.525	0.444	0.034	0.138
chao1	278.028	393.918	101.870	137.143	378.043	122.143	337.906	491.857	340.460	0.450	0.032	0.136
fisher_alpha	34.235	53.319	12.690	17.039	46.751	15.117	44.273	61.470	45.328	0.337	0.037	0.164
Shannon	3.010	3.305	1.813	4.417	4.764	2.043	5.208	6.020	5.408	0.124	0.151	0.020
Simpson	0.748	0.702	0.471	0.916	0.914	0.501	0.947	0.960	0.953	0.131	0.329	0.068
goods_coverage	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999			
observed_otus	271	378	99	132	370	122	330	482	338			

*SM = *Suka ko maacha*, SD = *Sidra*, SK = *Sukuti*

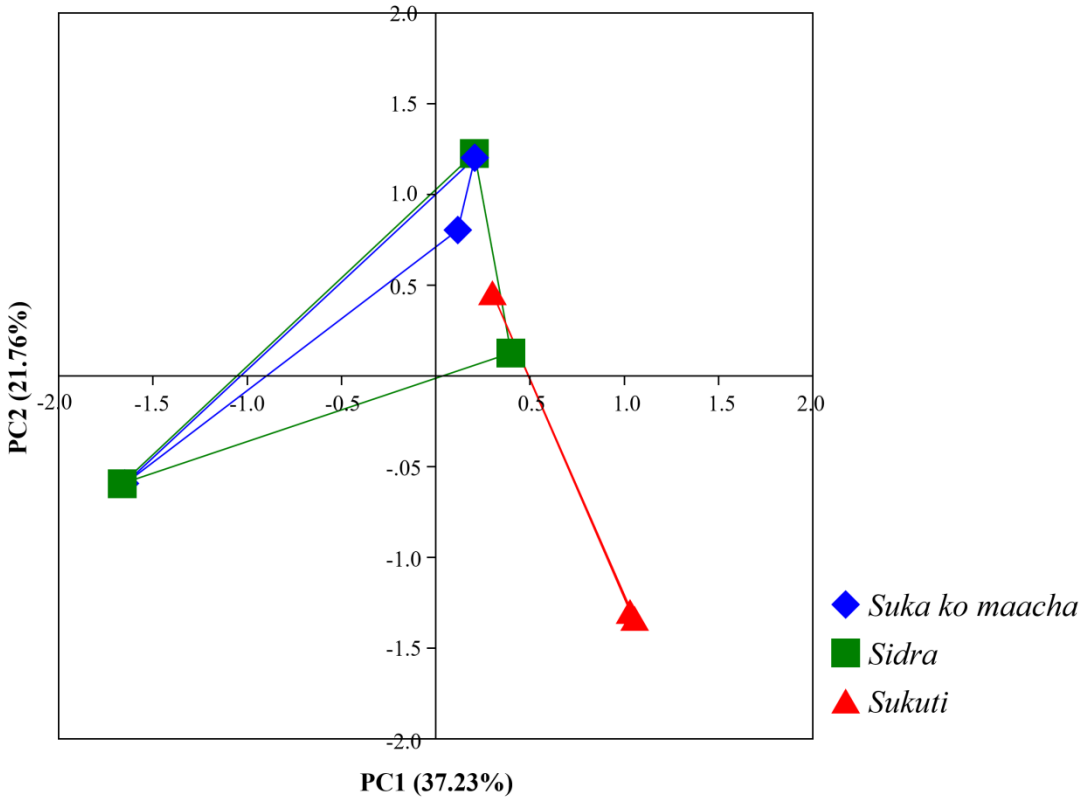


Figure 33: Beta diversity comparison as per Bray-Curtis dissimilarities matrix represented by Princrial Component Analysis (PCA).

Profile of Bacterial Diversity in Traditional Fish Products Based on Culture-Dependent and Culture-Independent Methods

By phenotypic characterization, 9 genera without any species were isolated from traditionally processed fish products of Sikkim. By using Sanger Sequencing method of 16S rRNA gene sequence, we detected 9 genera with 18 species in same products. Finally, by application of HTS (culture-independent method), we detected 15 genera and 17 species occurring at >1% abundance (Table 35) and 74 genera 120 species detected at <1% abundance in fish products of Sikkim.

Table 35: Profile of bacterial species in traditional fish products of Sikkim revealed by Culture-dependent and-independent Methods

Product	Culture-dependent		Culture-independent
	Phenotypic	Genotypic (16S rRNA gene sequence)	High-throughput Sequencing
	Bacteria genera		Bacterial species
Suka ko maacha	Staphylococccus, *Enterobacter, Escherichia*	*Staphylococcus sciuri*, *Enterobacter, hormaechei*, *Escherichia coli*	*Serratia marcescens*, *Bacillus* spp., *Staphylococcus* spp., *Enterococcus* spp., *Achromobacter* spp., *Kushneria* spp., *Pseudomonas* spp.
Sidra	*Bacillus, Staphylococcus, Klebsiella, Enterobacter,Vibrio, Salmonella*	*Bacillus cereus*, *Staphylococcus nepalensis*, *Staphylococcus aureus*, *Staphylococcus edaphicus*, *Staphylococcus sciuri*, *Klebsiella pneumoniae*, *Enterobacter clocae*, *Escherichia fergusonii*, *Providencia rettgeri*, *Salmonella enterica*, *Providencia vermicola*, *Providencia stuartii*, *Escherichia coli*	*Clostridium perfringens*, *Psychrobacter pulmonis*, *Serratia marcescens*, *Clostridium bifermentans*, *Plesiomonas shigelloides*, *Bacillus* spp., *Enterobacter* spp., *Rummeliibacillus* spp., *Clostridium* spp., *Enterococcus* spp.
Sukuti	*Bacillus, Staphylococcus, Enterobacter, Salmonella, Pseudomonas, Klebsiella, Vibrio, Citrobacter*	*Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus vitulinus*, *Staphylococcus sciuri*, *Enterobacter hormaechei*, *Salmonella enterica*, *Enterobacter cancerogenus*, *Providencia vermicola*, *Pseudomonas plecoglossicida*, *Acinetobacter radioresistens*, *Klebsiella pneumoniae*	*Psychrobacter celer*, *Psychrobacter pulmonis*, *Myroides odoratimimus, Photobacterium angustum*, *Photobacterium damselae* *Pseudomonas* spp., *Peptostreptococcus* spp., *Vagococcus* spp., *Oceanimonas* spp., *Proteus* spp., *Cetobacterium* spp., *Staphylococcus Vibrio* spp.

Gene Functionality

The functional potentials of the annotated genes inferred by PICRUSt2 were mapped against KEGG database for systematic analysis of gene functions (Table 36). The predictive functionality of samples of *suka ko maacha, sidra* and *sukuti* were primarily classified into six categories - metabolism (79.87%), genetic information processing (11.89%), cellular processes (4.35%), environmental information processing (3.21%), human diseases (0.34%), and organismal systems (0.31%). The percentile distribution of functional pathways in each fish product at Level 1 category is shown in Figure 34. The sub-pathways with relative high abundance were found to be biosynthesis of

ansamycins (2.69%), fatty acid biosynthesis (1.97%), valine, leucine and isoleucine biosynthesis (1.86%), synthesis and degradation of ketone bodies (1.85%), D-glutamine and D-glutamate metabolism (1.7%), C5-branched dibasic acid metabolism (1.7%), D-alanine metabolism (1.66%), lipoic acid metabolism (1.6%), pantothenate and CoA biosynthesis (1.56%), peptidoglycan biosynthesis (1.56%), alanine, aspartate and glutamate metabolism (1.42%), pyruvate metabolism (1.42%), folate biosynthesis (1.4%), biotin metabolism (1.36%), one carbon pool by folate (1.35%), biosynthesis of vancomycin group antibiotics (1.35%), thiamine metabolism (1.33%), pentose phosphate pathway (1.32%), lysine biosynthesis (1.28%), histidine metabolism (1.23%), glycolysis/gluconeogenesis (1.21%), carbon fixation in photosynthetic organisms (1.21%), citrate cycle (TCA cycle) (1.2%), terpenoid backbone biosynthesis (1.19%), glycine, serine and threonine metabolism (1.19%), cysteine and methionine metabolism (1.18%), vitamin b6 metabolism (1.17%), carbon fixation pathways in prokaryotes (1.16%) selenocompound metabolism (1.1%), streptomycin biosynthesis (1.07%), nicotinate and nicotinamide metabolism (1.04%), valine, leucine and isoleucine degradation (1.04%), propanoate metabolism (1.03%), butanoate metabolism (1.02%), phenylalanine, tyrosine and tryptophan biosynthesis (1.02%), glutathione metabolism (1%).

Table 36: Abundance of functional pathway with gene copy numbers (reads) using 16S rRNA sequences profile inferred by PICRUSt2 against KEGG database in fish products of Sikkim

Level 1	Level 2	Pathway name	Samples								
			SM1	SM2	SM3	SD1	SD2	SD3	SK1	SK2	SK3
			Gene copy numbers (reads)								
Metabolisms	Polyketides and Terpenoids metabolism	Vancomycin-group antibiotics biosynthesis	10591.95	24485.8	28990.71	28246.41	61577.44	45648.66	59460.64	144972.8	62921.07
		Biosynthesis of siderophore group nonribosomal peptides	3093.793	4231.691	11023.24	3303.141	3324.129	16472.82	1129.637	6014.173	1651.961
		Biosynthesis of ansamycins	63961.21	68107.06	30684.97	66048.62	202176.2	48529.29	96868.52	199625.1	97519.88
		Zeatin biosynthesis	13939.81	6727.18	7465.908	6260.098	18483.17	11658.35	18686.7	36729.77	18235.95
		Carotenoid biosynthesis	12967.59	689.4194	1793.427	223.4776	1125.472	2797.667	122.5753	2421.634	138.6476
		Geraniol degradation	19091.3	21409.2	7827.595	14065.74	16705.16	12368.45	47842.03	107096	49360.54
	Lipid metabolism	Biosynthesis of unsaturated fatty acids	19218.24	15936.13	16000.65	13042.29	23177.56	24314.18	36541.05	75224.77	36664.07
		Sphingolipid metabolism	1858.396	2128.231	4376.267	1236.348	5124.066	6738.712	826.0845	1226.655	952.3875
		Linoleic acid metabolism	3179.217	2242.823	4906.25	1595.715	12004.34	7778.277	4769.213	11633.75	3587.965
		Glycerophospholipid metabolism	22060.46	12167.9	13440.56	11635.35	29815.78	20644.1	25079.36	63595.92	25191.12
		Steroid hormone biosynthesis	110.9161	1102.024	9.8889	0	437.4033	62.0956	798.3361	503.5833	704.7178
		Secondary bile acid biosynthesis	45089.55	16099.61	29399.25	6954.1	53845.67	44163	5567.36	98086.49	3758.6
		Primary bile acid biosynthesis	5026.339	1921.382	3268.806	772.7889	6017.852	4937.389	619.2622	10900.31	419.0667
		Steroid biosynthesis	2712.429	97.1188	17.1306	332.6738	23.6806	50.0731	613.2175	1596.818	511.9506
		Synthesis and degradation of ketone bodies	67806.71	30020.31	37367.68	33885.65	31838.17	56902.77	96889.69	168930.4	98945.78
		Fatty acid degradation	28204.41	18420.18	21002.24	14972.88	24241.12	32129.1	40858.85	88200.57	41290.21
		Fatty acid biosynthesis	65526.29	37202.9	50251.26	31719.45	75443.76	76657.16	72178.63	184109.5	70360.49
		Drug metabolism - other enzymes	32292.14	18105.68	16333.46	16544.38	59660.4	25398.93	34010.27	92059.83	32309.79
		Glycerolipid metabolism	26663.4	9143.679	15728.38	10857.43	25010.22	23766.01	19917.63	42083.34	20291.34
	Biosynthesis of other secondary metabolites	Caprolactam degradation	6348.946	14434.75	236.4771	6195.736	10211.37	1134.806	17729.29	41325.41	19078.53
		Atrazine degradation	22778.07	3844.621	0	0	6715.866	19277.45	0	0	0
		Styrene degradation	4860.784	10898.83	326.6578	7102.36	7647.081	848.0556	15320.75	41058.52	15732.61

Metabolism		Ethylbenzene degradation	18140.69	6952.383	5111.07	0	9734.133	0	0	0	13398.76
		Nitrotoluene degradation	2413.096	1682.861	5337.171	4617.387	12860.73	8251.796	7792.294	18304.66	7976.086
		Aminobenzoate degradation	7605.89	7442.801	7937.823	5400.465	7947.256	12276	7246.919	25235.54	7991.165
		Chloroalkane and chloroalkene degradation	27414.97	11649.58	22567.5	9878.935	23970.52	34461.32	21582.14	56237.45	20544.66
		Polycyclic aromatic hydrocarbon degradation	1355.834	2356.361	38.3971	1089.7	1865.275	155.5182	261.71	3653.256	523.0594
		Fluorobenzoate degradation	4079.536	7353.851	216.0688	5090.303	5761.103	777.4812	774.7888	26162.81	2540.698
		Benzoate degradation	11753.97	11025.44	4131.353	9422.802	12498.92	6656.726	17092.04	41992.17	18808.56
		Chlorocyclohexane and chlorobenzene degradation	7572.484	6226.176	3043.83	3434.889	4770.154	4843.4	1566.129	14107.43	2097.368
		Toluene degradation	14470.87	13259.23	6551.844	9722.839	14241.06	10245.87	19695.15	56636.55	21311.68
		Xylene degradation	362.425	1819.889	85.046	4208.118	7616.213	215.916	0	6578.391	2038.365
		Dioxin degradation	8334.467	5538.031	4358.81	7444.371	13311.09	6847.416	2799.011	19938.56	3709.27
		Betalain biosynthesis	143.55	143.5	0	0	45.5	0	5.125	516.915	50.085
		Tropane, piperidine and pyridine alkaloid biosynthesis	11561.59	10011.78	7461.218	10508.12	22320.38	11647.79	25127.9	41324.3	25507.61
		Isoflavonoid biosynthesis	2.2143	0.2857	0	2.8571	97.8571	4.2857	0.1429	7.0714	1
		Flavonoid biosynthesis	179.4	26.1	6.067	5.2	35.043	101.3	40.2	799.4	28.467
		Penicillin and cephalosporin biosynthesis	257.6914	3364.041	4201.911	827.9314	1328.157	6520.189	550.9114	12742.17	434.7814
		Streptomycin biosynthesis	24670.49	22456.79	35690.92	15869.49	56895.81	55124.89	25079.61	89464.74	26614.68
	Energy metabolism	Sulfur metabolism	37698.85	18585.81	27730.01	13810.96	36008.49	42266.12	31730.35	88621.75	32484.38
		Nitrogen metabolism	17040.42	14550.92	18375.9	11449.11	31387.68	27911.67	21788.73	66986.87	21709.31
		Porphyrin and chlorophyll metabolism	20661.73	7705.243	10191.6	12213.11	33536.93	15587.01	22796.44	71162.5	21820.8
		Retinol metabolism	0	3905.088	0	0	3253.748	0	7563.204	13107.63	0
		Folate biosynthesis	49956.49	18549.7	32131.96	21995.12	53971.86	49049.17	63378.1	125098.4	63384.74
		Lipoic acid metabolism	78375.01	25111.9	44437.42	20857.65	36432.06	66830.49	63396.69	122668.5	65473.73
		Ubiquinone and other terpenoid-	23641.52	16310.69	14952.09	13325.2	21871.78	22568.17	28285.94	77191.12	29538.81

Metabolism		quinone biosynthesis									
		Biotin metabolism	26670.89	20772.05	29622.8	23634.96	56124.45	45060.16	68402.75	130752.6	69103.7
		Pantothenate and CoA biosynthesis	61295.33	29943.28	35720.39	23323.66	68183.41	54734.05	60053.93	126711.7	59785.73
		Nicotinate and nicotinamide metabolism	24247.5	18240.15	16652.16	18557.21	40884.58	25867.99	59649.48	93085.18	59860.24
		Vitamin B6 metabolism	30452.32	23028.23	29458.17	18156.23	37674.56	45136.17	51999.8	102905.9	52810.78
		Riboflavin metabolism	21252.13	14382.98	10069.39	14035.75	37838.02	15683.79	34002.65	84153	33618.09
		Thiamine metabolism	51158.86	20102.69	35024.89	20832.21	61481.26	53414.91	45977.33	110754.9	44443.64
		One carbon pool by folate	46494.71	23248.98	25617.48	20699.42	57192.7	39725.1	62202.09	133219	61383.47
		Carbon fixation pathways in prokaryotes	40496.87	20623.45	21140.4	19463.95	39911.39	32568.95	55946.77	111397.9	55941.44
		Carbon fixation in photosynthetic organisms	40327.51	23206.39	23108.47	21881.58	60828.32	35857.31	47729.86	104958.4	47336.17
		Photosynthesis	14092.58	6895.566	0	0	18594.9	11669.01	0	0	18348.96
		Oxidative phosphorylation	14866.51	9945.894	9287.258	8215.178	18130.53	14308.84	24409.46	51909.44	24546.48
		Methane metabolism	14867.35		9278.425	7096.724	20642.82	14304.76	16590.78	40509.38	16600.32
	Glycan biosynthesis and metabolism	C5-Branched dibasic acid metabolism	64850.74	39097.65	34896.65	26896.84	56176.78	53476.56	69603.05	144038.7	71946.21
		Butanoate metabolism	36331.96	21416.5	23812.97	17345.52	36488.44	36506.4	37992.72	91539.26	38933.94
		Metabolism of pyruvic acid	55332.01	25403.82	35331.21	22056.31	56114.98	53887.42	52295.77	122397	51974.88
		Metabolism of amino and nucleotide sugars	28182.37	18441.81	25100.32	13600.22	53517.91	38337.32	24452.61	63935.49	24026.34
		Metabolism of propanoate	37993.14	17807.43	26046.23	15905.33	35605.69	39782.04	40849.75	91776.05	40584.83
		Metabolism sucrose and starch	18887.55	14152.23	27235.74	10748.09	40696.8	41448.22	15154.3	38683.8	15644.48
		Ascorbate and aldarate metabolism	18738.3	13385.75	19622.58	11788.28	18390.93	29461.99	13507.55	34170.44	15394.86
		Galactose metabolism	21126.28	12666.25	16390.06	10327.68	43927.67	25036.71	10075.71	39175.31	10358.99
		Fructose and mannose metabolism	24804.01	16613.17	23198.26	13695.41	44611.68	35470.5	13962.48	45960.07	14539.96
		Pentose and glucuronate interconversions	33776.44	14818.08	33879.51	12815.69	27420.84	50822.36	15640.88	38601.9	17232.11
		Pentose phosphate pathway	51171.09	26557.6	34968.94	21305.76	69967.93	53390.43	35011.76	105667	34252.1
		Citrate cycle (TCA cycle)	43917.38	22345.26	27569.51	19977.51	32824.19	42140.94	51710.17	111406.8	52423.63

Metabolism		Glycolysis / Gluconeogenesis	48831.74	23415.74	31905.3	19466.86	57338.21	48780.69	34904.56	100287.7	34929.98
		Glyoxylate and dicarboxylate metabolism	26864.78	18461.49	17250.78	15365.88	26515.7	26423.42	31848.23	85841.42	33145.65
		Inositol phosphate metabolism	7692.411	7707.992	16550.3	6063.45	12568.52	25066.32	9330.997	20185.82	10312.82
		Peptidoglycan biosynthesis	54765.31	26153.2	29796.15	26990.43	71634.53	46035.89	69656.55	136184.8	68950.28
		Lipopolysaccharide biosynthesis	10318.85	17681.01	365.7827	14692.55	18859.68	1152.124	49535.83	98648.23	52039.04
		Glycosaminoglycan degradation	2358.636	3507.876	0	48.8386	4750.756	90.3957	878.8271	13254.63	638.8186
		N-Glycan biosynthesis	50.6891	112.5023	1327.894	0.9395	158.2223	2016.677	29.9927	1216.004	20.0764
	Amino acid metabolism	Phenylalanine, tyrosine and tryptophan biosynthesis	34859.33	17968.35	20199.66	17141.55	42073.4	31119	45578.56	95159.63	45128.72
		Tryptophan metabolism	21603.38	12338.08	15325.64	10446.42	16033.75	23345.41	27504.47	58819.54	27640.22
		Phenylalanine metabolism	10598.06	12008.13	8102.136	10623.07	16088.58	12524.6	21666.27	40360.65	23065
		Tyrosine metabolism	14162.67	12817.41	6810.326	9275.143	17719.53	10712.27	21440.95	46247.79	22506.13
		Histidine metabolism	46480.66	17807.67	32683.77	18656.27	46898.85	49729.73	47991.79	103922.9	47352.12
		Arginine and proline metabolism	26682.68	15682.04	23020.53	14660.86	34510.79	34954.15	26922.38	75019.26	26724.73
		Lysine degradation	19805.42	8991.17	13173.94	8213.758	12401.58	19991.67	22860.29	46528.08	23220.81
		Lysine biosynthesis	45825.84	22792.09	26815.86	20827.37	55667.82	41471.85	53396.46	115079	52209.63
		Biosynthesis of valine, leucine and isoleucine	67897.31	39714.39	40647.71	27839.3	79109.73	62822.57	72611.69	153395.2	73243.98
		Degradation of valine, leucine and isoleucine	34568.4	17936.24	26541.59	16326.99	24051.22	40383.22	45509.6	100217.3	45556.74
		Cysteine and methionine metabolism	35246.05	20967.72	33128.01	19332.03	54565.5	50287.76	39223.86	97906.17	38625.46
		Glycine, serine and threonine metabolism	42201.47	22064.92	26271.19	19681.44	39373.34	40058.69	50305.26	109908.7	50642.43
		Alanine, aspartate and glutamate metabolism	43369.19	24268.22	37194.01	21321.64	62957.9	56876.9	54951.42	126213.2	53614.55
		Pyrimidine metabolism	31325.54	16547.63	18304.5	15618.15	47601.8	28408.39	38728.33	88575.16	37822
		Purine metabolism	28006.94	14551.49	15806.81	13613.55	37477.66	24502.07	34562.72	75428.66	34212.88

Genetic Information Processing	Folding, sorting and degradation	Protein processing in endoplasmic reticulum	0.043896	0.02393	0.037507	0.020669	0.034338	0.03714	0.025442	0.026193	0.026193
	Replication and repair	Non-homologous end-joining	0.041157	0.084783	0.255508	0.125472	0.027738	0.252477	0.010236	0.087841	0.007647
		Homologous recombination	1.221222	1.053392	0.829561	1.212732	1.298764	0.853551	1.500706	1.246518	1.466552
		Mismatch repair	1.357793	1.2105	1.076652	1.36581	1.602374	1.100805	1.581129	1.405552	1.529963
		Nucleotide excision repair	0.531515	0.572937	0.540408	0.59063	0.763841	0.551565	0.619756	0.593169	0.588021
		Ribosome biogenesis in eukaryotes	0.050673	0.057931	0.03227	0.060987	0.053804	0.033275	0.087285	0.071207	0.087108
		Aminoacyl-tRNA biosynthesis	1.315055	1.205923	1.138593	1.362063	1.418667	1.165781	1.610673	1.329414	1.563082
Environmental Information Processing	Membrane transport	Bacterial secretion system	20576.24	20456.74	11493.51	16749.31	32531.89	17956.49	47059.61	93419.08	48144.1
		Phosphotransferase system (PTS)	26484.46	20791.69	21155.9	14709.93	56732.05	31966.14	7263.22	40537.83	8582.527
Human Diseasess	Immune disease	Systemic lupus erythematosus	11.8696	6.8261	0	0	2.7826	1.3043	0.1739	0	0
	Infectious disease: bacterial	Staphylococcus aureus infection	11585.29	2711.818	6267.932	2021.518	5389.296	9462.028	1225.094	5648.782	947.8194
	Infectious disease: parasitic	Amoebiasis	1206.073	191.6482	2249.203	0	373.5208	3356.703	0	1276.652	100.2623
		Epithelial cell signaling in Helicobacter pylori infection	3745.101	1419.814	3183.377	2499.284	5844.608	4796.041	5136.396	12134.61	5182.148
		Vibrio cholerae infection	42.335	16.9304	0.0417	0	10.9858	5.0488	178.9546	451.5017	115.2675
		Bacterial invasion of epithelial cells	66.5606	17.6885	2.3333	9.7636	35.8273	6.6667	11.9394	663.0455	22.1273
	Drug resistance: antimicrobial	Beta-Lactam resistance	11089.96	3840.46	116.1375	1044.618	2051.77	506.375	145.1975	11495.26	662.3775

SM1, *Suka ko maacha* 1; SM2, *Suka ko maacha* 2; SM3, *Suka ko maacha* 3; SD1, *Sidra* 1; SD2, *Sidra* 2; SD3; *Sidra* 3; SK1, *Sukuti* 1; SK2, *Sukuti* 2; SK3, *Sukuti* 3

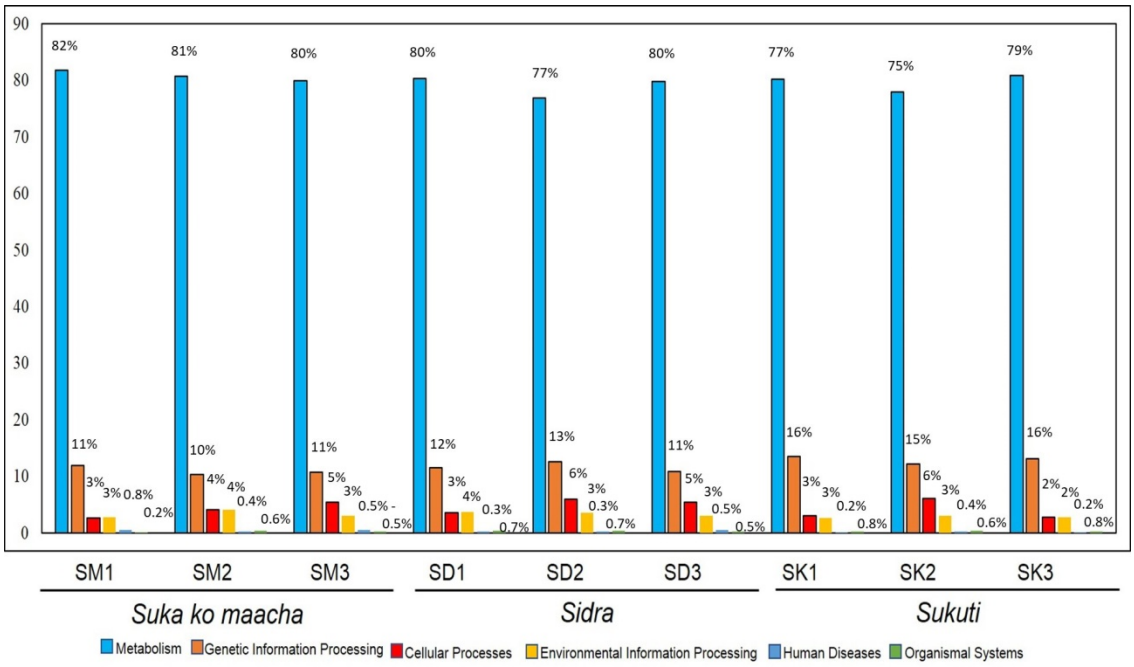


Figure 34: Percentile distriution of predictive functional pathways in different fish products at level 1 category inferred by KEGG database using PICRUSt2.

The predictive functionality across the products were relatively similar. Statistical significance amongst the predicted pathways were analyzed by STAMP ($p<0.05$) (Table 37). Exploratory visualization of the gene predictive functionality was computed using BURRITO, which depicted the paired- taxonomic and functional information, showing the genera-predictive functionality relationship (Figure 35). Metagenome contribution of genera with >1% abundance was also computed to acquire the OTU-functionality. Geraniol degradation was contributed only by *Psychrobacter*, *Bacillus*, *Staphylococcus*, *Serratia*, *Clostridium*, *Enterobacter*, *Pseudomonas*, *Rummeliibacillus*, *Photobacterium*, *Myroides*, *Plesiomonas* and *Achromobacter*. Phosphotransferase system (PTS) was contributed by *Bacillus*, *Staphylococcus*, *Serratia*, *Clostridium*, *Enterobacter*, *Pseudomonas*, *Rummeliibacillus*, *Enterococcus*, *Photobacterium*, *Peptostreptococcus*, *Plesiomonas* and *Achromobacter*. Among the human diseases-related pathways, *Staphylococcus aureus* infection was found to be associated with the

genera *Bacillus*, *Staphylococcus*, *Clostridium*, *Pseudomonas*, *Rummeliibacillus*, *Enterococcus*, *Peptostreptococcus* and *Achromobacter*. However, only one genus *Photobacterium* was associated with *Vibrio cholerae* infection pathway. STAMP analysis resulted in the following significant pathways: amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism, galactose metabolism, pentose phosphate pathway, glycolysis/gluconeogenesis, carbon fixation pathways in prokaryotes, oxidative phosphorylation, biotin metabolism, nicotinate and nicotinamide metabolism, one carbon pool by folate, seleno-compound metabolism, geraniol degradation), protein export, ribosome biogenesis in eukaryotes and phosphotransferase system (Figure 36).

Table 37: Differed levels of predictive functionality of bacteria in fish products of Sikkim inferred by PICRUSt2 against KEGG databases

Level-1	Level-2	Level-3	p-values	p-values (corrected)
Environmental Information Processing	Membrane transport	Phosphotransferase system (PTS)	0.004100095	0.004100095
Genetic Information Processing	Folding, sorting and degradation	Protein export	0.017225473	0.017225473
Genetic Information Processing	Translation	Ribosome biogenesis in eukaryotes	0.024514723	0.024514723
Human Diseases	Infectious disease: bacterial	Vibrio cholerae infection	0.000949123	0.000949123
Human Diseases	Infectious disease: bacterial	Staphylococcus aureus infection	0.04382201	0.04382201
Metabolism	Carbohydrate metabolism	Fructose and mannose metabolism	0.001831465	0.001831465
Metabolism	Energy metabolism	Oxidative phosphorylation	0.006333703	0.006333703
Metabolism	Carbohydrate metabolism	Pentose phosphate pathway	0.009704916	0.009704916
Metabolism	Carbohydrate metabolism	Galactose metabolism	0.01291235	0.01291235
Metabolism	Metabolism of cofactors and vitamins	Biotin metabolism	0.017135023	0.017135023
Metabolism	Metabolism of carbohydrate	Glycolysis/ Gluconeogenesis	0.019863004	0.019863004
Metabolism	Metabolism of cofactors and vitamins	One carbon pool by folate	0.019958879	0.019958879
Metabolism	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism	0.033236692	0.033236692
Metabolism	Energy metabolism	Carbon fixation pathways in prokaryotes	0.038897757	0.038897757
Metabolism	Metabolism of cofactors and vitamins	Nicotinate and nicotinamide metabolism	0.038997973	0.038997973
Metabolism	Metabolism of terpenoids and polyketides	Geraniol degradation	0.042965511	0.042965511
Metabolism	Other amino acids metabolism	Seleno-compound metabolism	0.049397571	0.049397571

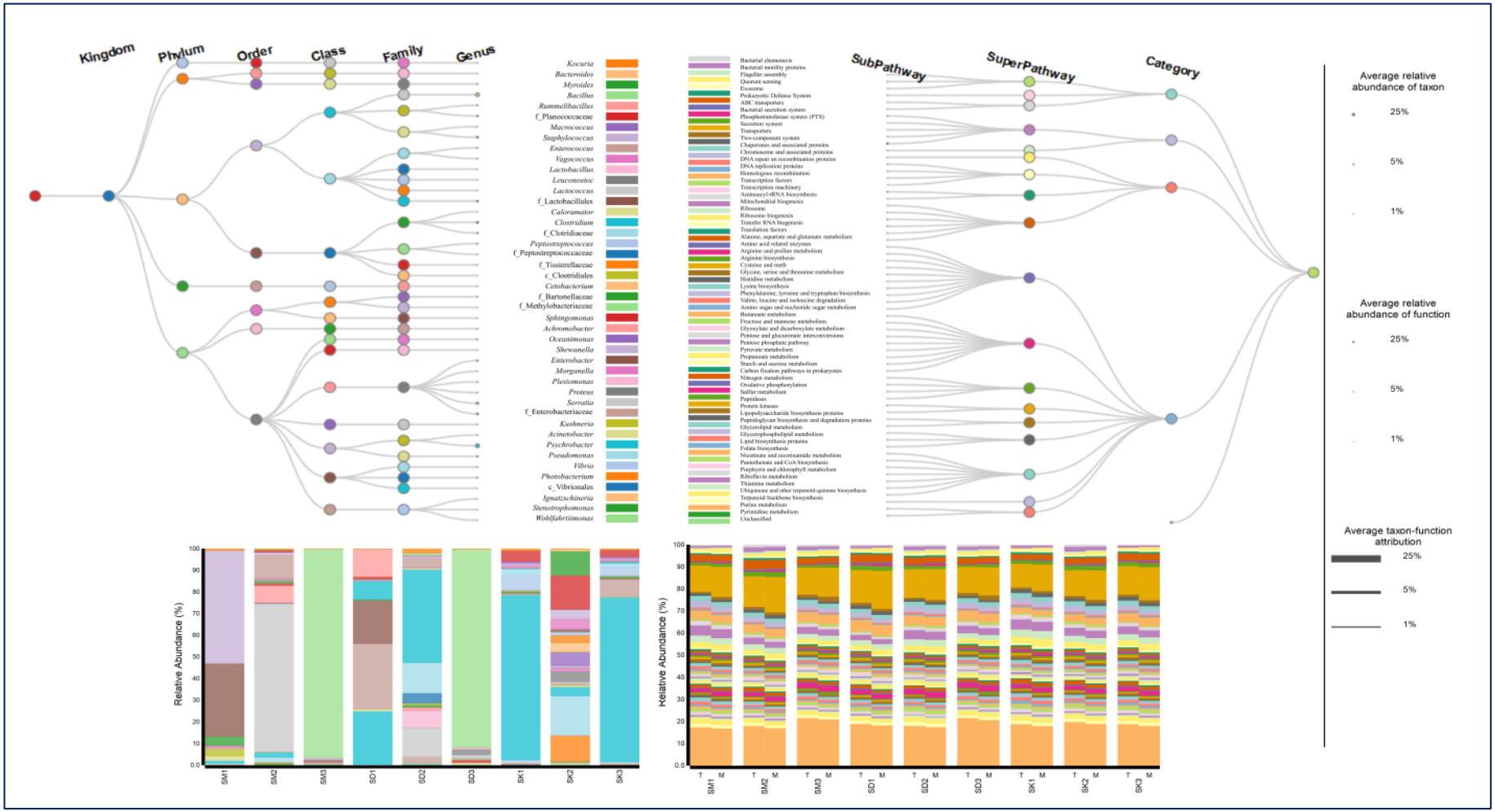


Figure 35: Relative abundance of bacterial composition with the predictive functionality as per PICRUSt2 visualized by BURRITO.

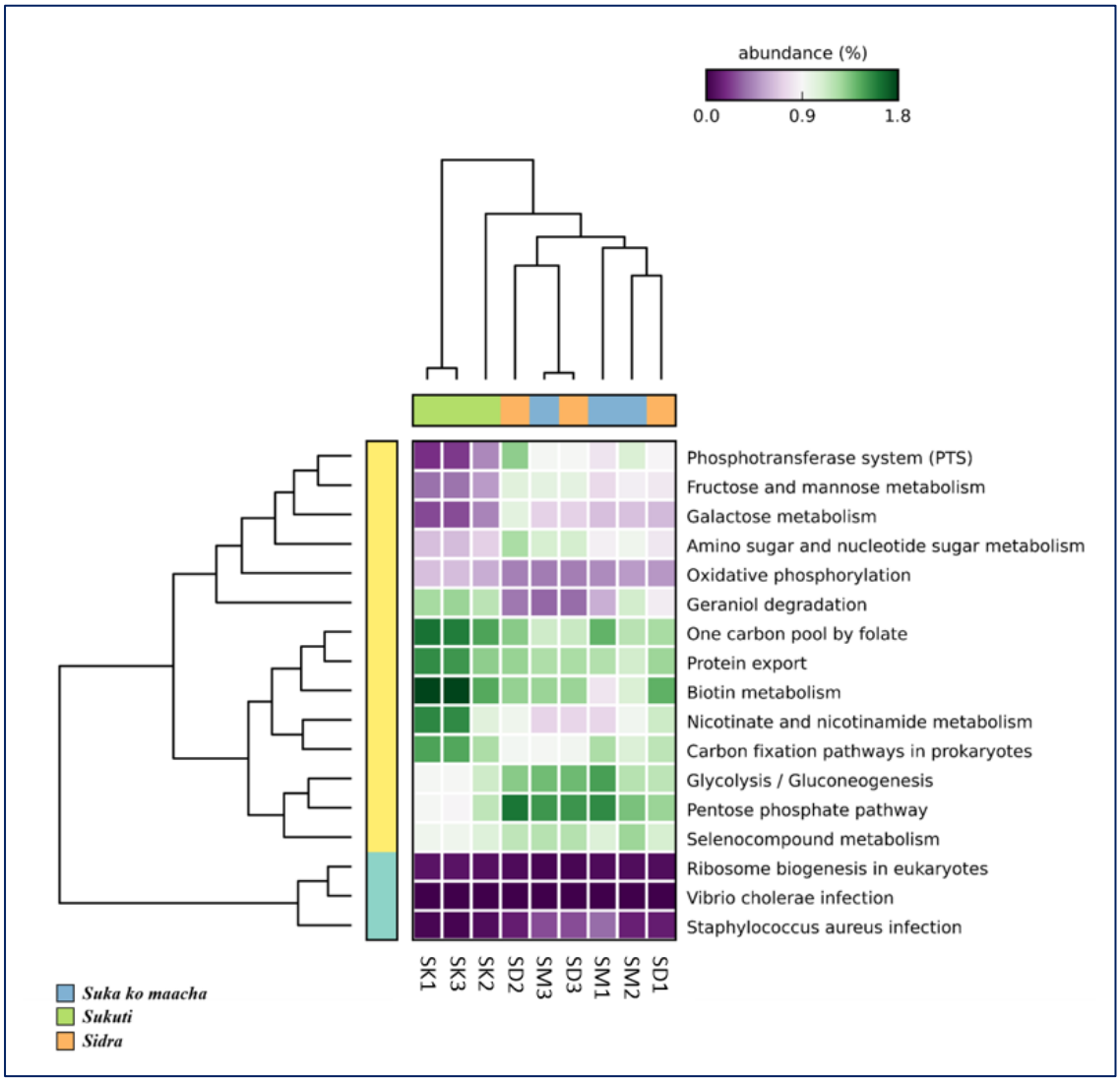


Figure 36. Heatmap (using STAMP software) showing the statistically significant ($p<0.05$) predictive functionality observed in the traditionally prepared fish products through PICRUSt2 against KEGG database.

Some traditionally processed meat and fish products of Sikkim have been studied earlier based on limited phenotypic and biochemical tests and reported some species of lactic acid bacteria (LAB), and few non-LAB genera (Rai et al. 2010a; Thapa et al. 2006). However, the occurrence of foodborne pathogens in these traditionally processed meat and fish products of Sikkim have not been assessed for safety measures. Conventional method of identification of bacteria are not reliable, hence application of 16S rRNA gene sequencing method using PCR products (Srinivasan et al. 2015) by Chain-termination DNA (Sanger et al. 1977) to check the sequence (Heather and Chain 2016) is more reliable. Sequence-based taxonomy and proper identification of microorganism associated with fermented meats and fish are major components of the microbial systematics in fermented foods (Ceuppens et al. 2014; Tamang 2014; Poirier et al. 2018). High-throughput sequencing method has been applied to reveal the colossal diversity of bacterial community in various meat and fish products (Połka et al. 2015; Wang et al. 2018; Song et al. 2018; Du et al. 2019; Jiang et al. 2019), and also inferred predictive gene functionality of dominance bacteria using computational biology (Wilkinson et al. 2018). Hence, this Thesis was intended to assess the safety of traditionally processed meat and fish products by characterising the potential bacterial food-borne pathogens, their toxins and the antimicrobial resistance pattern. Additionally, high-throughput sequencing method was applied to profile the bacterial community in different types of meat and fish products and also to predict various functional features of dominance bacteria in the products.

Traditional Processing Methods for Meat and Meat Products

Sikkim is a mountainous state in India where livestock and animal husbandry are major components in hill farming systems including domesticated animals such as poultry, cow, ox, pigs, goats, sheep, yaks, etc., used for meat, milk and milk products (Tamang 2005). Different types of traditionally processed meat products are prepared and consumed in Sikkim by ethnic people for centuries which include sausage-like products prepared from beef and pork meat, locally called *kargyong*, smoked and sun-dried beef/yak meat product called *satchu* and fermented yak meat product known as *khyopeh*. *Kargyong* is a sausage like product which is traditionally prepared by mixing lean meats of pork/beef/yak with required amount of salt, garlic and ginger and is stuffed into the intestine of animals locally called *gyuma* as natural casings. Both ends of casing are then tied, boiled hooked in a bamboo stick and smoked for 10-15 days (Rai et al. 2009). Natural casings of sausage are made from the sub-mucosa of the small intestine consist of collagen, giving shape, size and integrity to the sausage (Djordjevic et al. 2015). Unlike European sausage, nitrates and nitrites are not used during *kargyong* preparation. Nitrates and nitrites have been traditionally used as curing agents in the production of cured meat products for the development of the distinct flavour, retaining red colour, and the protection against lipid oxidation in cured meat products (Govari and Pexara 2018). *Satchu* or *suka ko masu* is prepared by slicing red meat (yak/beef/buffalo) into long strands and mixed with turmeric, salt and oil which is then dried or smoked for 7-10 days (Rai et al. 2009). Smoking of meat breaks down collagen (a protein found in muscle tissue), making the meat much more tender (Chang et al. 2011) and also melts fat making the product tender and delicious (Theobald et al. 2012). *Khyopeh* is a unique spontaneously fermented yak meat product of Sikkim which is prepared inside the rumen of yak for 4-6 months above earthen oven. Such

type of rumen-fermented meat product for human consumption has not been reported elsewhere. People living nearby mountain rivers, streams and lakes traditionally preserve perishable fish for consumption by smoking and drying in Sikkim. Three different traditionally processed fish products prepared by sun-drying and smoking viz. *suka ko maacha, sidra* and *sukuti* are common in Sikkim. Sun drying of perishable fish is one of the oldest methods of perseveration of fish in Asia (Thapa 2016) and also effects the biochemical properties of fish for flavour and taste (Patterson et al. 2018).

Product Characteristics

In present study we collected four types of traditionally processed meat products viz. beef *kargyong*, pork *kargyong*, *satchu* and *khyopeh* and three different types of traditionally prepared sun-dried fish products locally known as *suka ko maacha, sidra* and *sukuti* which are popular meat and fish products in Sikkim. The pH value in meat products was 5.3-5.9, showing slightly acidic in nature, which is similar to the pH in traditional dry cured lacon (5.0-6.0) (Lorenzo et al. 2015), beef and pork jerky (5.5-6.07) (Yang et al. 2009) and *biltong* (5.0-6.2) (Petit et al. 2014). The average moisture content was 18% in beef *kargyong*, which is almost same as on other sausages (Kerr et al. 2005). The average pH of fish products was slightly higher as compared to meat products with a value ranging from 6.6 to 7.0. Dried fish products with pH of 6.0-6.9 are considered to be of good quality (FAO 2012). Moisture contents in these fish products were low due to dehydration which may be good for increasing shelf life of the perishable fish for preservation (Mei et al. 2019).

Aerobic bacterial population was highest in beef *kargyong,* 7.4 log10 cfu/g whereas he staphylococcal count was found highest in beef *kargyong* with 6.4 log10 cfu/g. Similar count of *Staphylococcus* was recorded in traditional fermented sausage of France

ranging from 6-8 log cfu/g (Morot et al. 2006). *Bacillus* count was found highest in pork *kargyong* with 5.2 log10 cfu/g whereas the coliform count was highest in *satchu* with 6.5 log10 cfu/g. Similarly, in fish sample, the highest count of aerobic bacteria was observed in *sidra* with 7.32 log10 cfu/g) and staphylococcal count in *sukuti* with 6.4 og10 cfu/g whereas *Bacillus* count was found highest in *sukuti* 6.60 log10 cfu/g. Total coliform count was found highest in *sidra* with 7.41 log10 cfu/g and lowest in *sukuti* with 5.07 log10 cfu/g. *Enterobacteriaceae* counts of ≥4 log10 cfu/g are indicative of unacceptable contamination levels in food (van Schothorst et al. 2009).

Bacterial Taxonomy

We isolated 388 bacterial isolates from traditional meat products (128 isolates) and fish products (283 isolates) of Sikkim, respectively, and phenotypic characterization of was performed, were tentatively identified genera as *Enterobacter*, *Klebsiella*, *Escherichia, Salmonella*, *Enterococcus*, *Bacillus, Staphylococcus*, *Citrobacter* and *Pseudomonas* from beef *kargyong*, pork *kargyong, satchu* and *khyopeh.* Similarly, bacteria isolated from samples of *suka ko maacha*, *sidra* and *sukuti* were tentatively identified as *Enterobacter*, *Klebsiella*, *Escherichia, Salmonella*, *Vibrio, Bacillus, Staphylococcus*, *Citrobacter* and *Pseudomonas*. We used the selective media for rapid detection of pathogenic bacteria in traditionally processed meats and fish, which are common method for detection of foodborne bacteria using selective media (Mandal et al. 2011; Priyanka et al. 2016). However, conventional method of identification of bacteria is not authenticate and accurate (Cocolin at el. 2013), it may lead to wrong identity of bacteria (Gill 2017). A total of 19 representative isolates from meat samples and 38 bacterial strains from fish samples were randomly selected on the basis of phenotypic characteristics. All 57 representative bacterial strains (19 isolates from meat products

and 38 from fish products) were identified by 16S rRNA gene sequencing. In culture-dependent method, application of 16S rRNA gene sequencing method using Sanger Sequencing method (Sanger et al. 1977) is more reliable for molecular identification of bacteria (Clarridge III 2004; Srinivasan et al. 2015).

Bacterial Diversity in Meat Products

Based on molecular phylogeny the following bacterial species were identified from traditionally processed meat products: *Bacillus cereus, Staphylococcus aureus, Staphylococcus piscifermentans Citrobacter freundii*, *Enterococcus faecalis*, *Salmonella enterica*, *Citrobacter werkamanii*, *Klebsiella pneumonia*, *Macrococcus caseolyticus*, *Klebsiella aerogenes*, *Staphylococcus saprophyticus*, *Pseudocitrobacter anthropi*, *Citrobacter europaeus*, *Shigella sonnei, Escherichia fergusonii, Klebsiella grimontii* and *Burkholderia cepacia*. *Staphylococcus aureus* is usually considered as food poisoning bacterium (Hennekinne et al. 2012), since the illness caused by it is mild, hence it is frequently underreported (Paparella et al. 2018). Outbreak of staphylococcal food poisoning is often associated with the consumption of high proteinaceous food like meat, fish, poultry, eggs, etc. (Bennett et al. 2013). Some coagulase-negative staphylococci (CNS) *Staphylococcus saprophyticus* and *Staphylococcus piscifermentans* were also detected in samples, which are considered as a flavouring microorganisms capable of reducing nitrite, enhance colour stability and prevent rancidity by inhibiting oxidation of unsaturated free fatty acids (Papamanoli et al. 2012; Talon and Leroy 2006). Coagulase-negative staphylococci are also reported as dominant microbiota of dried/ salted/fermented meat products (Pinto et al. 2002; Leroy et al. 2015; Lorenzo et al. 2015) that may contribute to bio-protection against food-borne pathogens by producing bacteriocins (Mainer et al. 2017). *Bacillus cereus* is an

endospore forming bacteria responsible for food-borne illness in humans and is frequently involved in food-borne outbreaks (Hall et al. 2001).

We have also identified various bacteria belonging to family *Enterobacteriaceae* which is commonly present in the intestine human (Martinson et al. 2019) and animal (Schierack et al. 2007) that can survive in a diverse of environments (Martinson et al. 2019). However, they may also cause a variety of community acquired (foodborne) and nosocomial infections (Al-Mutairi 2011; Bereket et al. 2012). *Salmonella* was detected in only one sample (*satchu*), which may be associated with inadequate cooking, cross contamination from unhygienic environment and food handlers as well as poor handling practices (Liu et al. 2018). *Burkholderia cepacia* is opportunistic pathogen and is reported to cause pneumonia in immunocompromised individual with cystic fibrosis and chronic granulomatous disease (Mahenthiralingam et al. 2005). *Klebsiella* and *Enterococcus* have often been reported as contaminants in meat products occurring from various sources such as water, containers, personnel and faeces of animal or human origin which may contribute to disease and spoilage (Clegg and Sebghati, 2002; Messaoudi et al. 2009; Gundogan et al., 2013). However, despite its concern about pathogenicity, enterococci isolated from sausages has the ability to produce enterocins harbouring antimicrobial activity against pathogens *Listeria monocytogenes* (Herranz et al. 2001; Garcia et al. 2004). Foods are most commonly contaminated with *Shigella* by an infected food handler who practices poor hygiene (Garedew et al. 2016).

Bacterial Diversity in Fish Products

In traditionally processed fish products of Sikkim, identified bacteria on the basis of 16S rRNA gene sequence, were *Bacillus cereus, Salmonella enterica*, *Staphylococcus sciuri*, *Enterobacter hormaechei*, *Staphylococcus aureus*, *Enterobacter cloacae*,

Klebsiella pneumonia, *Escherichia coli*, *Providencia vermicola*, *Escherichia fergusonii*, *Providencia stuartii*, *Providencia rettgeri*, *Staphylococcus edaphicus*, *Staphylococcus nepalensis*, *Enterobacter cancerogenus*, *Acinetobacter radioresistens, Pseudomonas plecoglossicida* and *Staphylococcus vitulinus.* During traditional processing of fish products, many pathogenic, non-pathogenic and opportunistic microorganism may appear from the aquatic environment (Ashie et al. 1996; Lee et al. 2014; Zhang et al. 2016), which may metabolize organic compounds to produce distinct flavour, texture, and taste in fermented/smoked/dried fish products (Wang et al. 2017; Bao et al. 2018). Varying proportion of Gram-positive and Gram-negative bacteria has been isolated from seafood (Gram and Huss 1996). Frequent isolation of coagulase-positive and coagulase-negative staphylococci from fish products has been reported by many researchers worldwide (Simon and Sanjeev 2007; Moon et al. 2017; Moura et al. 2017; Hussein et al. 2019). *Salmonella enterica* has been reported in several fish products worldwide (Valyasevi and Rolle 2002; Bernbom et al, 2009; dos Santos et al. 2019). *Bacillus cereus* is responsible for increasing number of food-borne illness in industrialized countries as it may easily survive heating and pasteurization by forming the spores (Kotiranta et al. 2000). Several food-borne outbreak of gastrointestinal diseases caused by *Bacillus cereus* have been reported in many European countries (Osimani et al. 2018), USA (Bennet et al. 2014) and Australia (Thirkel et al. 2019). We also detected many species of *Providencia* in fish samples some of which are opportunistic pathogens in humans causing traveler's diarrhoea and urinary tract infections (Ryan and Ray 2004; Yoh et al. 2005). *Providencia* species is often isolated from diseased farm fish like *rohu* (*Labeo rohita*) and *namsing* (fermented fish of Assam) of India (Ramkumar et al. 2014; Chowdhury et al. 2019) and Nile *tilapia* (*Oreochromis niloticus*) of Brazil (Souza et al. 2019*). Acinetobacter*

radioresistens has been associated with nosocomial infections but very few cases in the literatures are available describing the isolation of *Acinetobacter radioresistens* from clinical specimen (Wang et al. 2019). However, this bacterium often harbours multidrug resistant genes that have been reported from humans (Poirel et al. 2008) aquatic environment (Miranda and Zemelman 2002) and well as from Antartica (Opazo et al. 2019*)*. *Enterobacter* is also ubiquitous in nature and has been reported as opportunistic pathogen because of its ability to resist multiple antibiotics (Davin-Regli et al. 2019). Fish inhabiting contaminated water bodies were reported to be infected with enteric bacterial species (Thillai et al. 2008). These enteric bacteria have been known to be the most prolific producers of biogenic amines which serve as an indicator of food intoxication (Ladero et al. 2010; Visciano et al. 2012). *Pseudomonas plecoglossicida* is considered pathogenic to fish as it causes haemorrhagic ascites and was first isolated from *ayu* or sweetfish (*Plecoglossus altivelis*) (Nishimori et al. 2000). *Pseudomonas pleccoglosscida* is industrially important bacteria because of its ability to degrade pesticides residue and also found to possess promising antitumor activity (Boricha and Fulekar 2009; Ni et al. 2009). *Escherichia* species are generally non-pathogenic microflora of the intestine of humans and animals and produces many vitamins as well as suppresses the pathogenic organism (Stromberg et al. 2018). However, some strains of *Escherichia* possess a virulent genes which may affects gastrointestinal, central nervous or urinary system thereby causing a life threatening disease and a large outbreak as well (Farrokh et al. 2013). There have been many reports on the isolation of *Escherichia* species as contaminants in fish products with some harbouring the virulence factors (Aberoumand 2010; Gupta et al. 2013; Sekhar et al. 2017).

Prevalence of Bacterial Toxins

In our study, we analyzed the sample for the presence of three different bacterial toxins viz. *Bacillus* diarrhoeal enterotoxin, *Salmonella* enterotoxin and Staphylococcal enterotoxin by Enzyme-Linked Immunosorbent Assay (ELISA) test. Additionally, molecular detection of few enterotoxin genes were also performed which included gene *sea* of *Staphylococcus* sp., genes *nhea* and *nheb* of *Bacillus cereus* and genes *inv* and *stn* of *Salmonella* sp. by PCR technique using strain specific primers. The production of toxins investigated in this study varied according to the types of sample, type of toxin and bacterial isolates.

Out of 26 samples of meat products, *Bacillus* diarrhoeal enterotoxin tested positive for 4 samples (beef *kargyong* and *satchu*) (15.3%), *Salmonella* enterotoxin tested positive for 4 samples (*satchu* and pork *kargyong*) (15.3%) and Staphylococcal enterotoxin tested positive for 4 samples (beef *kargyong* and pork *kargyong*) (15.3%) as well. *B. cereus* is commonly found in soil and is easily spread to many types of food such as dairy, egg and meat products (Kramer et al. 1989). *Bacillus* enterotoxins has been reported from fermented legume products like *soumbala* and *bikalga* of Africa (Ouoba et al. 2008), *doenjang,* fermented soybean food of Korea (Lee et al. 2017), dairy milk (Saleh-Lakha et al. 2017), some street food of Indian Himalayas (Kharel et al. 2016) and meat product (Soleimani et al. 2018). *B. cereus* causes two types of illness i.e. emetic and diarrhoeal diseases, the latter is often related to meat and fish products with required infective dose of 10^5 to 10^8 (McDowell et al. 2019). Strains of *B. cereus* from beef *kargyong* when tested for the occurrence of virulent genes (*nhea* and *nheb*), showed negative result. *Salmonella* is the most common bacterial pathogens in laboratory confirmed food-borne illness cases causing gastroenteritis, typhoid fever and bacteremia (Eng et al. 2015). The major identified virulence genes like *inv, stn, fimA*

and *spv* responsible for salmonellosis are linked to a combination of plasmid and chromosomal factors (Chaudhary et al. 2015). Although few meat samples were found positive for *Salmonella* enterotoxin by ELISA test but the identified bacterial isolates were found negative for *inv* and *stn* virulent genes indicating its absence in the isolated strain. Staphylococcal food poisoning is major cause of foodborne infections in the world with a very high rate of infections in country like India due to warm and humid climate (Bhatia and Zahoor 2007). *Staphylococcus* produces different types of enterotoxins which include classical enterotoxins (*sea-see*) and the newer enterotoxins (*seg-sely*) group (Fisher et al. 2018) and strains producing *sea* are implicated in the majority of cases of staphylococcal food poisoning (Kerouanton et al. 2007; Wallin-Carlquist 2010). Our ELISA test showed 15.3% meat products to be contaminated with staphylococcal enterotoxins. Further, molecular detection of staphylococcal enterotoxins showed the presence of *sea* virulent gene in coagulase negative *Staphylococcus,* which is well supported by the similar studies where the enterotoxin and biofilm genes in coagulase negative *Staphylococcus* was detected in ground beef indicating it as potential pathogen (Pavan et al. 2019). Few studies have been conducted on the survival of *S. aureus* at different temperatures in meat products such as vacuum-packaged and dried beef jerky and biltong (Ingham et al. 2006; Burnham et al. 2008; Naidoo and Lindsay 2010). However, the risk of toxin production by these traditional processed meat products of Sikkim has not been assessed previously. Interestingly, *khyopeh* was the only meat product with absence of enterotoxins, indicating it as a safe food for consumption (Zeaki et al. 2019).

In Fish products, 16 samples out of 30 were found positive for *Bacillus cereus* diarrhoeal enterotoxin, 20 samples were positive for *Salmonella* and 8 were positive for Staphylococcal enterotoxins by ELISA test. We detected 26.6% of tested samples of

fish products were contaminated with staphylococcal enterotoxins. Similar kind of study was performed in Turkey to investigate the prevalence of staphylococcal enterotoxins in retail fish products by ELISA test which showed 9% of samples were contaminated (Onmaz et al. 2015). The virulent gene *sea* of staphylococcus was detected in 62% of bacterial strains isolated from studied fish products. Outbreak of food poisoning caused by staphylococcal enterotoxins in France was confirmed by the recovery of $>10^5$ cfu/g of *S. aureus* from food remnants with presence of *sea* (Kerouanton et al. 2007), whereas in India food poisoning caused by staphylococcal enterotoxins was *seb* gene followed by *sea* and others (Zeaki et al. 2019). In our studies, *Salmonella* enterotoxin was prevalent in 66.6% of tested fish products by ELISA tests. In contrast, the PCR analysis showed negative for enterotoxin genes (*inv* and *stn*) in strains isolated from these samples indicating its absence. The specific target for detection of *Salmonella* is the molecular identification of *inv*A and *stn* as they are conserved among the salmonellae irrespective of serotype (Tekale et al. 2015). There are several reports on the prevalence of *Salmonella* in fish products and their enterotoxin genes indicating the need for hygienic practices in handling and processing operations (Lotfy et al. 2011; Traore et al. 2015; Li et al. 2019). Presence of *Salmonella* in fish indicates a vehicle for a growing number of enteric disease outbreaks due to deteriorated quality of water sources which may have originated from discharged of partially treated sewage, leakage of animal waste, etc. (Olgunoglu et al. 2012; Traore et al. 2015). In our study, ELISA tests revealed 53.3% of fish products to be contaminated with *Bacillus* diarrhoeal enterotoxin. Similar results were observed in retail seafood of United States where 50% of tested sample produces diarrhoeal enterotoxins (Rahmati and Labbe 2008). The presence of *nhea* and *nheb* genes in all strains of bacteria signifies the prevalence of *Bacillus cereus* enterotoxins in fish products of Sikkim.

There are many types of virulent genes in *Bacillus cereus* such as *ces*, *hbl*A, *hbl*D, *cyt*K, *bceT*, *sph*, *ent*S but *nhe*a and *nhe*b contributes to 89% of virulent genes in food products (Gdoura-Ben Amor et al. 2019). *Bacillus cereus* enterotoxins causes diarrhoea after ingestion of spores or vegetative cells and the virulence is associated with the production of reactive proteins in the tissues (Bottone 2010). In India, majority of foodborne outbreaks go unreported, unrecognized and un-investigated which makes it difficult to detect and control the outbreaks (Tewari and Abdullah 2015).

Prevalence of Antimicrobial Resistant Bacteria

In our study, we have tested 56 bacterial species for their sensitivity pattern against the various classes of antibiotics according to the CLSI guidelines (CLSI 2015). The antimicrobial susceptibility testing confirms whether the chosen empirical antimicrobial agent is sensitive or resistance towards individual bacterial isolates (Reller et al. 2009). Previous studies have reported that widespread use of antibiotics in animals for the treatment of diseases and other purposes may develop antibiotic resistance in bacteria resulting into cross transmission of resistance genes from animals to humans through food, direct contact and contaminated water (Tang et al. 2017). A total of 19 bacterial isolates from meat products of Sikkim were examined for antimicrobial sensitivity tests. All bacterial strains showed 100% sensitivity towards gentamicin, cotrimoxazole, norfloxacin and trimethoprim. Similar reports was observed in bacterial isolates from street vended foods which showed 90% of sensitivity towards gentamicin and triomethoprim (Amare et al. 2019). Most of the strains showed resistance pattern towards one or more antibiotics however two bacterial strains viz. PSKE30 (*Burkholderia cepacia*) and PSST53 (*Staphylococcus aureus*) isolated from pork *kargyong* were found sensitive towards all the tested antibiotics. About 58% of the

isolates were found resistant to amoxicillin-clavulanate followed by tetracycline (27%), cefoxitin (16%) cotrimoxazole (10%). Increasing prevalence of amoxicillin-clavulanate resistance has been reported due to continued spread of β-lactamase-mediated resistance (White et al. 2004). Amoxicillin-clavulanate resistant bacteria were also reported from retail sausage in Malaysia which exhibited 100% resistance along with penicillin followed by ampicillin (83.3%) and cefotazime (71.4%) (Tew et al. 2016). Nitrofurantoin was found resistant to only one isolates from meat sample i.e. BSE27 (*Klebsiella pneumoniae*) isolated from beef *kargyong*. Nitrofurantoin is a broad-spectrum antibiotic which is being used for the treatment of uncomplicated urinary tract infections (UTI) and *Klebsiella pneumoniae* is one of the major causes of UTIs (Osei 2018). Cotrimoxazole was found resistant to *Enterococcus faecalis* (KHE57) only isolated from *khyopeh*. Most of the Gram-positive cocci were oxacillin resistant, however, the resistant pattern was not observed in any cephalosporin class of antibiotics except for *B. cereus*. The maximum resistance pattern towards antibiotics was shown by *Bacillus cereus* towards ampicillin, cotrimoxazole, tobramycin, penicillin, oxacillin, cefuroxime, ceftazidime, rifampicin and cefepime but found sensitive to gentamicin, streptomycin, ciprofloxacin chloromphenical and tetracycline. Rather et al. (2012) reported that *Bacillus cereus* from meat products showed 100% of sensitivity towards gentamicin, 98% towards ciprofloxacin, chloramphenicol (89%) streptomycin (85%) and tetracycline (70%) (Rather et al. 2012).

The antibiotic sensitivity tests were performed in 38 bacterial strains isolated from fish products which showed more than 90% of sensitivity pattern towards gentamicin, norfloxacin, streptomycin, ciprofloxacin, tobramycin and trimethoprim. Only one isolated strain *Escherichia coli* ASE42 isolated from *suka ko maacha* was found susceptible towards all the tested antibiotics. Overall, the highest resistant pattern was

observed towards amoxycillin-clavulanate (53%) followed by nitrofurantoin (45%), ampicillin (37%), aztreonam (30%) and cefoxitin (26%). Among the Gram-negative bacteria, *Pseudomonas plecoglossicida* SULT15 isolated from *sukuti* contributed to 70% of resistant patterns towards various tested antibiotics followed by *Salmonella enterica* (35%), *Klebsiella pneumoniae* (25%), *Enterobacter cloacae* (25%), *Acinetobacter radioresistens* (20%), *Providencia rettgeri* (15%) and least in *Escherichia fergusonii* contributing to only 5% of resistance towards antimicrobials. The Gram-positive bacteria isolated from fish samples showed the highest sensitivity patterns towards Clindamycin (100%), followed by erythromycin (79%). Oxacillin was found resistant to all the tested Gram-positive bacteria followed by penicillin (57%). *Staphylococcus* isolated from street foods also showed highest sensitivity pattern towards clindamycin (88.2%) and highest resistance towards penicillin (73.5%) (Amare et al. 2019). Many coagulase negative staphylococci showed resistance pattern towards oxacillin, penicillin and vancomycin. Similar studies was reported from Germany where coagulase-negative staphylococcus associated with food were tested for antibiotic resistance pattern and found highest level of resistance towards penicillin, oxacillin, tetracycline and ampicillin and some level of resistance towards cefoxitin (Resch et al. 2008). The resistant pattern was observed in *Bacillus cereus* strain isolated from *sidra and sukuti* contributing to 19-42% of resistance towards antimicrobials. The least antimicrobial resistance was observed in *Staphylococcus edaphicus* SIJST46 isolated from *sidra* which showed resistant toward oxacillin only.

Bacterial Community Structure in Meat Products

Unlike Sanger sequencing which is based on low-throughput technique, time consuming and costly reactions, high-throughput sequencing technology enables

sequencing of hundreds of millions of DNA molecules at a short time and at relatively low cost (Soon et al. 2013). Application of high-throughput sequencing method facilitates a better understanding on the knowledge of microbial community structure in foods by providing a detail profile of food microbiota responsible for food fermentation or food spoilage (Ercolini 2013; De Filippis et al. 2018). Moreover, with the advancement in bioinformatics software such as PICRUSt (Douglas et al. 2019), the predictive functional profiling of microbial communities was made possible using 16S rRNA marker gene sequences and database of reference genomes (Langille et al. 2013). In this study, high-throughput sequencing technique was applied to profile bacterial communities present in four different traditional meat products of India viz. beef *kargyong,* pork *kargyong* yak *satchu* and *khyopeh. Staphylococcus* was the core genus present in all samples of meat products. Coagulase negative group of *Staphylococcus* genus was isolated from fermented and dried meat products like *kitoza* of Madagascar (Ratsimba et al. 2017), dry fermented sausage of Spain (Quijada et al. 2018) and Chinese dry/smoked-cured sausage (Wang et al. 2018a,b). *Psychrobacter pulmonis* and other *Psychrobacter* species were dominant in beef *kargyong*. *Psychrobacter* is psychrotolerant and halotolerant bacterium (Wu et al. 2013) and is also associated with meat products when stored at cold temperature (Zhang et al. 2012). The unique genera observed in beef *kargyong* were 66% to the total bacterial diversity. Among these rare genus found in beef *kargyong*, *Vitreoscilla*, a Gram-negative bacterium, has been reported as the first source of bacterial haemoglobin (VHb) with antibiotics production (Veseli et al. 2018; Mejía et al. 2018).

Lactobacillus sakei was the most abundant bacterium in pork *kargyong.* Though *Lb. sakei* is generally isolated from vegetable sources (Lee et al. 2018), however, it was also reported from meat products such as sausage prepared from llama meat of

Northwest Argentina (Fontana et al. 2016) and *mum,* fermented sausage of Thailand (Wanangkarn et al. 2014). *Lb. sakei* strain has several probiotic attributes such as acidification, tolerance to bile salt, reduction of biogenic amine levels and adherence to intestinal cells (Laranjo et al. 2019). *Psychrobacter pulmonis* (18.33%) was also present in pork *kargyong*. *Rothia*, *Micrococcus*, *Stenotrophomonas*, *Corynebacterium* and *Citrobacter* were found only in pork *kargyong* among the four different meat samples analysed by HTS at lesser extent (<1%). Probably these genera are residential bacteria in the source of the end products (Møretrø and Langsrud 2017).

Novosphingobium, Bdellovibrio, Brevundimonas, Clostridium, Ketogulonicigenium, Ignatschineria, Enterobacter and *Luteolibacter* were detected by OTUs as rare genera in yak *satchu*. Among these unique species, *Bdellovibrio bacteriovorus* is a predatory bacterium that has the ability to invade and parasitize the Gram-negative pathogenic bacteria (Negus et al. 2017) and also has antibiotic and probiotic characteristics (Shatzkes et al. 2017). Detection of predatory *Bdellovibrio bacteriovorus* in traditionally smoked meat *satchu* by OTUs sequences is remarkable findings in this paper. Our finding has also motivated us to isolate *Bdellovibrio bacteriovorus* from traditional meat products of India by culture method using selective medium (Ottaviani et al. 2019) and study further in future.

Interesting Firmicutes was detected by OTUs in *khyopeh* as the predominant phylum with 100% abundance. During the preparation of *khyopeh*, chopped innards of yak are stuffed into the rumen of dead yak, and fermented spontaneously for 4-6 months. The predominance of phylum Firmicutes may be due to supplement of gut microbiome from rumen, where Firmicutes is the predominant bacterial phylum in the yak rumen (Liu et al. 2019). The dominant bacterium in *khyopeh* was *Enterococcus* spp. followed by *Lactobacillus sakei* and *Carnobacterium divergens.* Species of *Enterococcus* have

been reported from rumen of yak (Li et al. 2018), which stimulates the growth of rumen microbes (Mamuad et al. 2019), and also produces antimicrobial agents in rumen (Wang et al. 2018a,b). *Lactobacillus sakei* has been reported in fermented sausage as well as frozen raw meat products (Zagorec and Champomier 2017). *Carnobacterium divergens* can tolerate to freezing and high pressure conditions and grow anaerobically (Leisner et al. 2007) and has been reported from frozen vacuum packaged meat product (Mokrani et al. 2018). *Carnobacterium divergens* is considered as a biopreservative due to its ability to inhibit pathogenic bacteria in foods (Zhang et al. 2019). Based on our metataxonomic result, some bacterial genera detected in traditionally processed meat products of Sikkim belonged to beneficial groups of bacteria in meat fermentation (Fontana et al. 2016; Laranjo et al. 2019) such as *Lactobacillus*, *Enterococcus*, *Lactococcus, Leuconostoc*, *Bdellovibrio*, *Novosphingobium*, and *Leuconostoc*. However, some spoilage bacteria such as *Pseudomonas* (Stellato et al. 2017)*, Brochothrix* (Illikoud et al. 2019) and *Clostridium* (Adam et al. 2010) were also detected in some samples of meat products. Our studies did not detect any major food borne bacterial pathogens such as *Salmonella, Listeria, Campylobacter* etc. in any meat product of Sikkim by high-throughput sequencing method. *Campylobacter*, *Salmonella* and *Listeria monocytogenes* are frequently present in livestock causing disease zoonoses which are transmitted from animal to human, posing a great threat to the health and life of people (Chlebicz and Śliżewska 2018).

Bacterial Community Structure in Fish Products

Application of high-throughput sequencing method using QIIME2 plateform, which provides an interactive platform for amplicon-based surveillance (Bolyen et al. 2019), in samples of *suka ko maacha, sidra* and *sukuti* revealed a diverse bacterial community structure. Metataxonomic approach of these fish products showed the dominance of

bacteria belonging to class Bacilli and Gamma-Proteobacteria with higher abundances of *Bacillus* (21.1%) followed by *Psychrobacter* (12.3%). Other genera prevalent in the samples with >1% relative abundance were *Peptostreptococcus*, *Enterobacter*, *Rummeliibacillus*, *Plesiomonas*, *Clostridium*, *Enterococcus*, *Staphylococcus*, *Pseudomonas*, *Myroides*, *Serratia* and *Achromobacter*. Similarly, these organisms were also recorded in other fish products of the world (Ohshima et al. 2019; Keisam et al. 2019). Gram-negative bacterium *Psychrobacter* was found to be the common genus detected in all samples of *suka ko maacha, sidra* and *sukuti*, however, it was predominant in *sukuti* samples. *Psychrobacter* is known to have osmotolerant and cryotolerant characteristics which are abundantly found in fish, meat and cold environment considering it as an evolutionary successful group of bacteria (Azevedo et al. 2013). *Psychrobacter* sp. has proteolytic properties which enhances the flavor of fermented fish in addition to non-biogenic amines producing activities and has been used as a starter culture in fish sauce of Korea (Zheng et al. 2017). *Bacillus* sp. was also found as dominant bacteria in *rakfisk*, which is a fermented fish product of Norway (Zheng et al. 2017). Food safety of traditional fish products remains an important concern since these are traditionally prepared with poor sanitary practices. Metagenomics allow the detection, identification and characterization of wide range of pathogens which is considered as powerful tool in food safety (Grützke et al. 2019). Microbiota obtained from samples of *suka ko maacha, sidra* and *sukuti* showed several species of pathogenic bacteria which included *Clostridium perfringens*, *Plesiomonas shigelloides, Photobacterium damselae, Enterobacter* spp. *Vibrio* spp., *Klebsiella* spp. and *Proteus* spp. Occurrence of *Enterobacter* indicates human source of fish contamination indicating the cross-contamination of microflora between terrestrial and marine/river ecosystem (Naik et al. 2018). Interesting, we observed a significant

presence of Clostridiaceae family which is minutely detected in *suka ko maacha* sample. *Clostridium perfringens* is a spore-forming bacterium which has the ability to grow at elevated temperatures and produces enterotoxins that may cause food-borne illness ranging from mild diarrhea to severe human necrotic enteritis (Dodd et al. 2017). Prevalence of toxins producing *Clostridium* sp. in traditionally fermented/smoked/salted/dried fish has often been reported (Sabry et al. 2016; Wani et al. 2018) which clearly indicates the need for proper quality standardization of a product to make it available commercially. *Plesiomonas shigelloides* is often recovered from acute gastroenteritis cases which are associated with ingestion of contaminated water or fish (Behera et al. 2018). *Photobacterium*, *Oceanimonas*, *Myroides*, *Peptostreptococcus*, *Vagococcus*, *Cetobacterium*, *Proteus* and *Vibrio* are found to be distinctive genera in *sukuti* among the three fish products of Sikkim. *Photobacterium* is a food spoilage bacterium reported in fresh meat and fish (Fuertes-Perez et al. 2019). Many psychrotolerant species of *Photobacterium* are reported to cause greater capability of histamine fish poisoning incident (Bjornsdottir-Butler et al. 2016).

Despite the fact that our metagenomic studies revealed abundances of many food spoilage and disease causing bacteria, interestingly we also detected some bacteria which may have several functional properties. Detection of *Rummeliibacillus* in samples of *sidra* is novel findings in this study. Administration of *Rummeliibacillus* considerably reduces the potential pathogenic bacteria, improves the growth, immunity and gut microflora of a fish signifying it as a potent probiotics (Tan et al. 2019). Additionally, there has also been a report on the effective application of *Rummeliibacillus* in biodiesel formation from effluent of palm oil mill (Junpadit et al. 2017) and biomineralization potential (Mudgil et al. 2018). Hence, our findings encouraged us to isolate *Rummeliibacillus* from this fish product and screen for its

industrial applications. Many *Oceanimonas* species has the degradation potential of polycyclic aromatic hydrocarbons, phenol, xenobiotics and also in denitrifying process by removing the nitrogen from polluted wastewater (Tan et al. 2017; Lee et al. 2018). Some strains of *Myroides* spp. have been reported to have a bioremediation potential by detoxifying the aflatoxin contamination and degradation ability of marine collagen fibre (Mwakinyali et al. 2019). Increasing risk associated with *Vibrio* which has become the leading cause of seafood related illness globally (Baker-Austin et al. 2017; Taylor et al. 2018).

In *suka ko maacha*, we found rare genera such as *Acinetobacter*, *Kushneria* and *Achromobacter*, which have the industrial applications (Yun et al. 2018; Bunnoy et al. 2019). Some strains of *Acinetobacter* are protective against *Aeromonas hydrophila* which is a pathogenic bacterium in fish (Bunnoy et al. 2019). *Kushneria* spp., isolated from Korean fermented sea food, is reported as phosphate solubilizing bacteria (Yun et al. 2017, 2018). *Achromobacter* spp. has ability to degrade sulfamethoxazole and phthalate against the environmental pollutants (Nguyen et al. 2019; Liang et al. 2020).

Diversity

Microbial diversity is a measure for analysis of microbiome in samples where alpha/intra and beta/inter diversity metrices are computed to test the differences in the microbial composition within and between the samples (Goodrich et al. 2014). Based on the alpha diversity recorded, we observed abundance of observed OTUs in beef *kargyong* and pork *kargyong* which reflected more diversity as per Chao1 and Fisher alpha metrices in comparison to yak *satchu* and *khyopeh*. Goods coverage reflected the maximum coverage of sequencing depth in samples, which indicated the maximum diversity captured (Sims et al. 2014). Qualitative species-based measures, Chao1 and

ACE (Chao et al. 1984) showed a significant difference between *sidra* and *sukuti* due to a higher microbial abundance obtained in *sukuti* in comparison to *sidra*. Quantitative species-based measures as per Simpson's indices showed significance between *suka ko maacha* and *sukuti* indicating *sukuti* has a higher diversity of *sukuti* in comparison to *suka ko maacha*. Beta diversity measures species turnover which overemphasizes the role of unique species in the samples as the difference in species composition between communities reflecting the presence/absence of some rare/unique species in the group (Baselga 2010). PCA-plot computed as per Bray-Curtis dissimilarities showed a clear cluster of *sukuti* samples, however, *suka ko maacha* and *sidra* forms a distinct scattering pattern. This reflects the differences in the bacterial composition (Erb-Downward et al. 2012) of *sukuti* from *suka ko maacha* and *sidra*, respectively. However, ANOSIM analysis of the Bray-Curtis dissimilarity matrices showed no significance ($p=0.3437$) indicating that there is no significant difference among the total bacterial composition (Anderson 2017) in all fish products of Sikkim.

Predictive Functionality

In our study, we tried to predict the possible functionality in OTUs of bacteria present in beef *kargyong*, pork *kargyong, satchu* and *khoypeh* by PICRUSt2 software (Douglas et al. 2019). Our study revealed six functional gene groups at Level 1 category including metabolism, genetic information processing, environmental information processing, cellular processes, human diseases and organismal systems which are similar to the previous reports on predictive functionality of beef steaks with marker genes encoding for amino acid and lipid metabolism (Yang et al. 2018). At Level 2 category, our findings indicated the higher abundance of carbohydrate metabolism followed by metabolisms of amino acids, cofactors and vitamins, terpenoids,

polyketides, lipids, and xenobiotics biodegradation. High carbohydrate metabolism in meat is correlated with *Firmicute*s, whereas high metabolism of amino acid and lipid is due to abundance of *Proteobacteria* in meat samples (Stellato et al. 2016). We observed the abundance of amino acid metabolism in the samples, which may be due to prolong fermentation or drying/smoking during traditional processing of the meat samples resulting into development of the distinct aroma in the products (Perea-Sanz et al. 2019). Amino acid metabolism is one the main factors contributing to development of the organoleptic property of meat products (Flores, 2018). We assumed that genes related to carbohydrate metabolism were overrepresented in traditional meat products of Sikkim, indicating the microbial community associated with it might be more essential to carbohydrate degradation (Leroy et al. 2017). We also predicted genes responsible for metabolism of vitamins such biotin, vitamin B6, folate and thiamine. Production of vitamin B-complex has been reported in meat products (Gille and Schmid 2015). Our findings revealed the predictive genes encoding for biogenic amines such as histidine and tyramine at relatively low abundance. Presence of biogenic amines in fermented food has been reported (Gardini et al. 2016), moreover, quantification of biogenic amines content in food is very important to indicate its safety by analytical methods (Kim et al. 2012) which may be studied in future to ensure the safety of meat products of Sikkim. Beta phenylethylamie and tyramine are abundant in food from animal origin since these matrices are rich in the precursor amino acids, tyrosine and phenylalanine to synthesize catecholamines that may cause adverse effects like allergies, hypertensions and headache on consumers (Pessione and Cirrincione 2016; Gardini et al. 2016). PICRUSt2 analysis of OTUs of bacteria in our meat samples predicted the pathway associated with human diseases but relatively at lesser abundance (<1%) such as genes encoding for *Staphylococcus aureus* infection, beta-

lactam resistance and epithelial cell signalling in *Helicobacter pylori* infection whereas the metataxonomic did not identify these organisms at the diversity level. Hence, it could be other factors probably proteins which may be triggering the signalling pathway causing the diseases (Sebastian et al. 2014).

Similarly, we analysed predictive gene in bacteria from 16S rRNA amplicon data of fish products viz. *sukako maacha, sidra* and *sukuti* of Sikkim by PICRUSt2 software (Douglas et al. 2019), which is a novel approach to understand the predictive gene fucntionality (Ortiz-Estrada et al. 2019). Application of PICRUSt2 algorithm showed a significant observation of metabolism (79.88%) category from the traditionally processed fish microbiome data. The abundance of *Psychrobacter* and *Pseudomonas* in these traditional fish products may contribute to the amino acid metabolism (Zhao and Eun 2020). Carbohydrate and amino metabolism are also associated with taste and aroma of the fermented products (Lee et al. 2014). Fermented foods are said to contain considerable amounts of free galactose (Liu 2000). The higher presence of carbohydrate metabolism, energy metabolism in these samples indicates high metabolic activity of the microbial composition present. The presence of nicotinate and nicotinamide metabolisms also signifies the importance of bacterial group (Liu 2015) present in these samples. Fish samples harbour a high level of carbohydrate and amino acid metabolisms (Zhang et al. 2016). The presence of *Bacillus*, *Staphylococcus*, *Clostridium*, *Pseudomonas*, *Rummeliibacillus*, *Enterococcus*, *Peptostreptococcus* and *Achromobacter* showed functional links to *Staphylococcus aureus* infection, which is an important aspect of food safety in fermented foods as *Staphylococcus aureus* is food poisoning bacterium (Keisam et al. 2019). *Bacillus* sp. isolated from fish products is reported to synthesize many enzymatic properties like glucanase, protease, cellulase, fibrinolytic and keratinolytic activities as well as probiotic attributes (Ray et al. 2007;

Soltani et al. 2019). The present study revealed huge unexplored microorganisms in environment which may synchronize the ecological diversity in fish ecosystems. We attempted to show the relationship of microbiota present with predictive metabolic pathways in traditionally processed fish products using BURRITO software, which visualized the taxa–function relationships in microbiome data (McNally et al. 2018). Gene functionality of diverse bacterial community with some unique or rare species present in traditionally prepared meat and fish products of Sikkim in India may help to predict the several metabolism and secondary metabolites even signalling disease prediction to consumers.

Although we found potent food-borne bacterial pathogens like *Bacillus cereus*, *Salmonella enterica* and *Staphylococcus aureus* in some traditional meat and fish products of Sikkim, however, many isolated bacterial strains showed absence of tested virulent genes which depicted low virulence factors of identified bacteria. High-throughput sequencing of meat products revealed relative higher abundances of lactic acid bacteria whereas in fish products HTS revealed the presence of *Clostridium perfringens* in *sidra* and *Vibrio* sp. in *sukuti* which are considered potent bacterial pathogens, however, no such potent pathogens were detected in *suka ko maacha*. Since we found few enterotoxin genes in these meat (except in *khyopeh*) and fish products (except in *suka ko maacha*), quantification of enterotoxin genes expression and estimation of biogenic amines may be studied in future. Traditionally processed meat and fish products are cooked or fried before consumptions, these products are considered safe for consumption, as heating generally kills the pathogens (Fryer and Robbins 2005) and frying the products in oil also kills the pathogens (Channaiah et al. 2018).

CONCLUSION

Preservation of perishable meat and fish into flavour-enhanced products by traditional methods such as drying, smoking, and fermentation are are culturally and sensory accepted products for consumption by ethnic people of Sikkim. Molecular identification revealed some potential Gram-positive bacterial pathogens and few Gram-negative bacteria mostly belonging to various Enterobacteriaceae. The ELISA tests also showed presence of toxins produced by these pathogens, however, the virulent genes (*inv* and *stn*) of *Salmonella* were not be detected by PCR method. The enterotoxin gene for *Bacillus cereus* was found in fish products but absent in meat products, whereas, virulent gene of *Staphylococcus* (sea) was present in few isolates of both meat and fish sample. Pathogenic strains and virulence factors were not found in *khyopeh,* an unique fermented yak meat. Most of the isolates were sensitive to all classes of antibiotics. High-throughput sequencing approaches revealed complex bacterial community structure in meat and fish products mostly dominated by phyla Firmicutes and Proteobacteria. Interestingly, *Bdellovibrio* sp. was detected only in yak *satchu* which is also called predatory bacterium as it can prey on other pathogenic organisms. We also found some industrially important bacteria such as *Rumemliibacillus, Oceanimonas, Myroides, Kushneria* in traditional fish products which are reported to have a probiotic attributes and protective against environmental pollutants. The predictive functionalities of meat and fish products showed highest reads of carbohydrate metabolism pathway followed by amino acid and other metabolism pathways. Additionally, we also found many organisms linked to degradation capabilities of polycyclic hydrocarbons and xenobiotics. HTS provides the first complete database on bacterial communities from traditionally processed meat and fish products of Sikkim by culture-independent techniques (NGS) which can be used as

reference data base for the further research. Although, we found some food-borne pathogens by molecular identifications and some virulent genes as well, further research is needed to evaluate and quantify gene expression by detecting mRNA by RT-PCR method. Proper food handling, avoiding cross-contamination, implementing personal hygiene, and educating the public about safe handling of foods and proper sanitation can help in lowering the contamination rate. Further research into traditionally prepared meat and fish products of North East India may be important for industrialization as functional foods.

Meat and fish are dietary culture of many non-vegetarians people in Sikkim. Different types of traditionally processed meat products are prepared and consumed in Sikkim by ethnic people for centuries which include sausage-like products prepared from beef and pork meat, locally called *kargyong*, smoked and sun-dried beef or yak meat product locally called *satchu* and fermented yak meat product known as *khyopeh*, which is rare and unique region-specific fermented meat of North Sikkim. Similarly, three various types of traditionally preserved fish products prepared by sun-drying and smoking viz. *suka ko maacha, sidra* and *sukuti* are common in Sikkim. Hence, this Thesis aimed to evaluate the microbiological safety of traditionally processed meat products viz. beef/pork *kargyong, satchu and khyopeh* and fish products viz. *suka ko maacha*, *sidra* and *sukuti* of Sikkim. The study assessed the occurrence of bacterial indicators, pathogens, their toxigenicity and antimicrobial resistance patterns. Additionally, complete bacterial communities were investigated by high-throughput sequencing tool. The average population of aerobic bacteria in all four meat and three fish products of Sikkim were 3.1 x 10^6 cfu/g and 2.8 x 10^6 cfu/g. Average load of *Staphylococcus*, *Bacillus* and total coliform in meat products were 2.6 x 10^5 cfu/g, 3.4 x 10^5 cfu/g and 30.4 x 10^6 cfu/g, respectively. The average count of *Staphylococcus, Bacillus* and total coliform in fish products were recorded as 5.4 x 10^5 cfu/g, 4.9 x 10^5 cfu/g and 6.2 x 10^6 cfu/g, respectively. A total of 388 bacteria were isolated from 57 samples of meat and fish products using selective culture media followed by phenotypic characterisation and presumptive identification. The isolates were tentatively identified as *Enterobacter, Staphylococcus, Bacillus, Klebsiella, Enterococcus, Salmonella, Escherichia, Pseudomonas, Vibrio* and *Citrobacter*. 57 Representative isolates of 57 isolates were randomly selected on the basis of phenotypic and biochemical tests. Genomic DNA of these 57 isolates were extracted for 16S rRNA gene by Sanger Sequencing Method.

Identified bacteria from traditionally processed meat products (19 isolates) were *Staphylococcus piscifermentans* (12%), *Citrobacter freundii* (10%), *Enterococcus faecalis* (5%), *Salmonella enterica* (5%), *Staphylococcus aureus*, *Citrobacter werkamanii* (5%), *Klebsiella pneumonia* (5%), *Macrococcus caseolyticus* (5%), *Klebsiella aerogenes* (5%), *Staphylococcus saprophyticus* (5%), *Pseudocitrobacter anthropi* (5%), *Citrobacter europaeus* (5%), *Shigella sonnei* (5%), *Escherichia fergusonii* (5%), *Klebsiella grimontii* (5%), *Burkholderia cepacia* (5%) and *Bacillus cereus* (5%). Shannon-H index was recorded highest in beef *kargyong* with 1.748 and lowest in *khyopeh* with 1.009. Similarly, 38 bacterial strains isolated from traditionally processed fish products were identified as *Bacillus cereus* (13%), *Salmonella enterica* (11%), *Staphylococcus sciuri* (9%), *Enterobacter hormaechei* (8%), *Staphylococcus aureus* (8%), *Enterobacter cloacae* (8%), *Klebsiella pneumonia* (8%), *Escherichia coli* (6%), *Providencia vermicola* (6%), *Escherichia fergusonii* (5%), *Providencia stuartii* (3%), *Providencia rettgeri* (3%) *Staphylococcus edaphicus* (3%), *Staphylococcus nepalensis* (3%), *Enterobacter cancerogenus* (3%), *Acinetobacter radioresistens* (3%), *Pseudomonas plecoglossicida* (3%) and *Staphylococcus vitulinus* (3%). Shannon-H index value was recorded highest in *sidra* (2.476) and lowest in *suka ko maacha* (1.099).

An enzyme-linked immunosorbent assay (ELISA) tests were performed in 26 meat and 30 fish products for *Bacillus* Diarrhoeal Enterotoxin, *Salmonella* Enterotoxin and *Staphylococcus* Enterotoxin. Out of which 4 samples of meat were found positive for *Bacillus* Diarrhoeal Enterotoxin, *Salmonella* Enterotoxin and Staphylococcal Enterotoxin. *khyopeh* of North Sikkim tested negative for all the three bacterial enterotoxins. Among the fish products 16 samples were found positive for *Bacillus* Diarrhoeal Enterotoxin, 20 samples were found positive for *Salmonella* and 8 samples

were found positive for staphylococcal enterotoxin. Furthermore, prevalence of some enterotoxin genes were determined from molecularly identified bacterial strains, and 24 strains of bacteria were tested for the presence of some potent virulent genes of *Bacillus cereus* (*nhea, nheb*), *Salmonella* (*stn, inv*) and *Staphylococcus* (sea) by PCR analysis using specific primers. *Bacillus cereus* from meat products showed absence of both *nhea* and *nheb* genes whereas 5 isolates from fish products showed presence of *nhea* and *nheb* genes. The isolated strains from these food products did not show the presence of *Salmonella* enterotoxin genes. Staphyloccal enterotoxin gene (*sea*) were found positive in 3 out of 5 bacterial strains of meat sample and out of 8 isolates from fish products, 5 were found presence of sea gene. Interestingly, among all the meat and fish samples, *khyopeh* was the only product that did not show presence of any enterotoxins.

The antibiotic susceptibility test performed on 57 bacterial isolates by Kirby-Bauer disk diffusion method revealed many bacterial strains were susceptible towards several tested antibiotics. However, some bacteria exhibited characteristics resistance to certain tested antibiotics. Overall, bacteria isolated from meat products showed high level of resistance in amoxicillin-clavulanate (58%) followed by ampicillin (27%). Gram-positive bacteria showed sensitive against clindamycin and erythromycin, however, 50% of resistance pattern was observed in oxacillin followed by penicillin (33%). The bacterial isolates from fish products showed high sensitivity patterns (>90%) towards most of tested antibiotics, however, 53% bacterial strains showed resistant patterns towards amoxycillin-clavulanate followed by nitrofurantoin (45%), ampicillin (37%), aztreonam (30%) and cefoxitin (26%). All Gram-positive bacterial strains were found to be resistant to oxacillin and penicillin.

Apart from assessing the safety of these food products, the present study also sheds light on the bacterial ecology and their relationships which may play an important role in improving the quality and functionality of a traditionally processed meat and fish products of Sikkim. Application of high-throughput sequencing technique revealed a diverse bacterial community structure along with their predictive gene functionalities using machine learning tolls by biological computation which could not be possible by conventional microbiological methods. Microbial diversity was achieved through the application of amplicon 16S-targeted method via the MiSeq Illumina platform targeting the V3-V4 region of the 16S rRNA genes and analysis of amplicon library data were performed using Quantitative Insights Into Microbial Ecology (QIIME2) platform. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software was applied to predict the functionality from 16S rRNA amplicon data. Altogether in meat products bacterial diversity based on >1% relative abundance showed Firmicutes (50.11%) as the most dominant phylum followed by Proteobacteria (43.99%), Bacteroidetes (5.72%), and other phyla in including both classified and unclassified with <1% relative abundance (0.31%). At the family level, Moraxellaceae (21.83%) was the dominant family followed by Enterococcaceae (19.77%), Lactobacillaceae (15.32%), Weeksellaceae (13.18%), Staphylococcaceae (8.94%), Xanthomonadaceae (3.38%), Leuconostocaceae (3.05%), Carnobacteriaceae (2.79%), Pseudomonadaceae (1.74%), Rhizobiaceae (1.35%), Enterobacteriaceae (1.27%), Sphingobacteriaceae (1.21%), Rhodobacteraceae (1.13%), Bdellovibrionaceae (1.12%), and Clostridiaceae (1.12%). At the genera level, *Psychrobacter* (21.93%) was the most abundant followed by *Enterococcus* (19.67%), *Lactobacillus* (15.46%), *Lactococcus* (6.77%), *Ignatzschineria* (5.36%), *Staphylococcus* (3.94%), *Chryseobacterium* (3.82%), *Carnobacterium* (2.79%), *Pseudomonas* (2.09%), *Bdellovibrio* (1.79%),

Clostridium (1.79%), *Novosphingobium* (1.79%), *Leuconostoc* (1.72%), *Weissella* (1.37%), *Agrobacterium* (1.32%), *Acinetobacter* (1.28%) and other genera with <1% abundance (7.13%). Alpha diversity metrics showed a maximum bacterial diversity in beef *kargyong* with Fisher alpha value of 24.36 and lowest in *khyopeh* (4.37), Shannon index was found highest in *beef kargyong* (4.78) and lowest in *khyopeh* (2.62) but Chao1 value was found lowest in satchu (19). A total of 140 Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways were obtained from PICRUSt2 analysis which showed the major predictive functionality of metabolism (78.1%) such as carbohydrate metabolism (23.1%), amino acids metabolism (17.5%), metabolism of cofactors and vitamins (17.3%), metabolism of terpenoids and polyketides (12.7%), lipid metabolism (12.5%), metabolism of other amino acids (12.1%), and metabolism of xenobiotics biodegradation (4.4%); genetic information processing (14.5%), environmental information processing (3.5%), cellular processes (3.2%), human diseases (0.5%) and organismal systems (0.2%).

Metataxonomic of bacterial communities in fish samples with >1% abundance, showed the phylum Firmicutes (49.8%) as the most dominant followed by the phyla Proteobacteria (47.6%), Bacteroidetes (1.41%) and other minor phyla, detected at <1% abundance, comprised of 1.19%. At the Family level, Moraxellaceae (21.26%) was dominant followed by Bacillaceae (20.91%), Enterobacteriaceae (18.21%), Staphylococcaceae (9.75%), Clostridiaceae (6.25%), Planococcaceae (4.35%), Peptostreptococcaceae (3.69%), Enterococcaceae (2.55%), Pseudomonadaceae (2.48%), Vibrionaceae (1.55%), Flavobacteriaceae (1.26%), Bartonellaceae (1.09%), and Alcaligenaceae (1.06%), including other families with <1% abundance (5.59%). *Psychrobacter* (24.19%) was the most dominant genus followed by *Bacillus* (21.19%), *Staphylococcus* (10.32%), *Serratia* (9.7%), *Clostridium* (5.27%), *Enterobacter* (3.2%),

Pseudomonas (3.03%), *Clostridium* (2.44%), *Rummeliibacillus* (2.06%), *Enterococcus* (1.98%), *Photobacterium* (1.86%), *Myroides* (1.57%), *Peptostreptococcus* (1.56%), *Plesiomonas* (1.15%), *Achromobacter* (1.11%), and other minor genera <1% abundance of 9.36%. *Psychrobacter celer* (12.37%) was the most abundant species followed by *Serratia marcescens* (9.7%), *Psychrobacter pulmonis* (6.37%), *Clostridium perfringens* (4.55%), *Clostridium bifermentans* (1.76 %), *Myroides odoratimimus* (1.57%), and *Plesiomonas shigelloides* (1.15%). Besides these identified species, some unknown species were also detected such as *Bacillus* spp. (21.18%), *Staphylococcus* spp. (9.98%), *Psychrobacter* spp. (5.11%), *Enterobacter* spp. (3.19%), *Pseudomonas* spp. (3.02%), *Rummeliibacillus* spp. (2.06%), *Enterococcus* spp. (1.98%), *Peptostreptococcus* spp. (1.56%), *Clostridium* spp. (1.34%), and *Achromobacter* spp. (1.11%). Other minor species occurring at <1% abundance comprised of 11.99%. Alpha diversity indices showed significance ($p>0.05$) between *sidra* and *sukuti* as per ace, chao1, fisher_alpha metrices indices. However, in terms of Shannon indices, significance ($p>0.05$) between *suka ko maacha* and *sukuti* was observed. Beta diversity showed a diverse population from *suka ko maacha* and *sidr*a samples. The PICRUSt2 analysis showed an interesting pattern in the predictive functionality against KEGG pathway. It showed a highest metabolism pathway (79.87%) with several sub-pathways responsible for metabolism of terpenoids and polyketides, lipid metabolism, amino acid metabolism, carbohydrate metabolism, metabolism of cofactors and vitamins. Other pathways included genetic information processing (11.89%), cellular processes (4.35%), environmental information processing (3.21%), human diseases (0.34%), and organismal systems (0.31%). Exploratory visualization of the gene predictive functionality was computed using a web-based tool BURRITO, which depicted the paired- taxonomic and functional information, showing

the genera-predictive functionality relationship. Metagenome contribution of genera with >1% abundance was also computed to acquire the OTU (Operational Taxonomic Unit)-functionality. Geraniol degradation was contributed only by *Psychrobacter*, *Bacillus*, *Staphylococcus*, *Serratia*, *Clostridium*, *Enterobacter*, *Pseudomonas*, *Rummeliibacillus*, *Photobacterium*, *Myroides*, *Plesiomonas* and *Achromobacter*. Phosphotransferase system (PTS) was contributed by *Bacillus*, *Staphylococcus*, *Serratia*, *Clostridium*, *Enterobacter*, *Pseudomonas*, *Rummeliibacillus*, *Enterococcus*, *Photobacterium*, *Peptostreptococcus*, *Plesiomonas* and *Achromobacter*. Among the human diseases-related pathways, *Staphylococcus aureus* infection was found to be associated with the genera *Bacillus*, *Staphylococcus*, *Clostridium*, *Pseudomonas*, *Rummeliibacillus*, *Enterococcus*, *Peptostreptococcus* and *Achromobacter*. However, only one genus *Photobacterium* was associated with *Vibrio cholerae* infection pathway. STAMP analysis resulted in the following significant pathways: amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism, galactose metabolism, pentose phosphate pathway, glycolysis/gluconeogenesis, carbon fixation pathways in prokaryotes, oxidative phosphorylation, biotin metabolism, nicotinate and nicotinamide metabolism, one carbon pool by folate, seleno-compound metabolism, geraniol degradation), protein export, ribosome biogenesis in eukaryotes and phosphotransferase system.

Although, this study found prevalence of some spoilage bacteria and pathogens along with some enterotoxins producing organism as these food products are not prepared and handled in hygienic conditions. Interestingly, Next Generation Sequencing by uisng high-throughput sequencing tool revealed a huge bacterial diversity with a dominance of few lacic acid bacteria in some meat products and few spoilage bacteria as well in some meat and fish products. Major food-borne pathogens like *Listeria* and

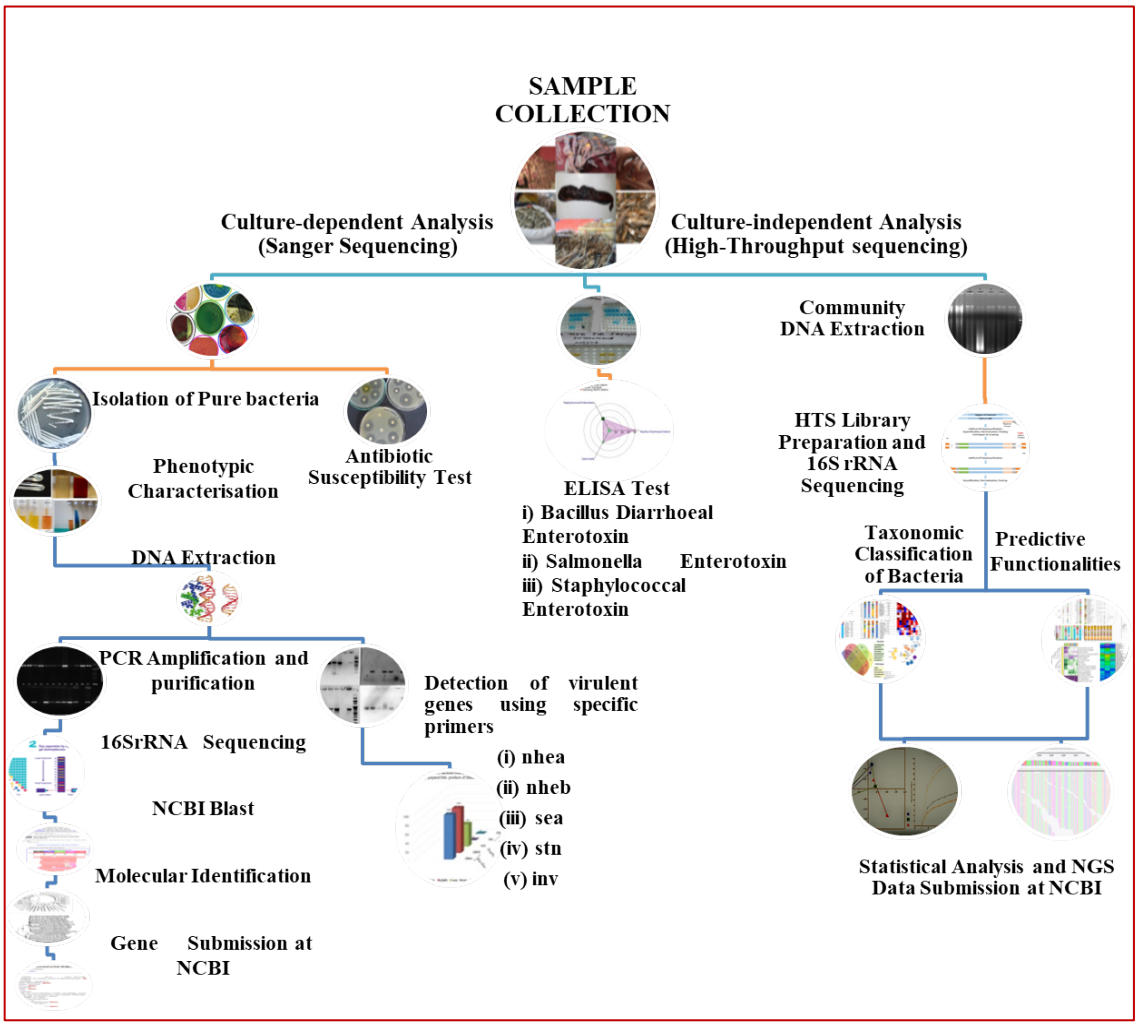
Salmonella were not found in these traditional products Also, various possible functionalities from these products are being reported for the first time. Hence, this study enriches the information on the possible contaminants present in traditionally prepared meat and fish products. Most importantly, this study revealed the complete bacterial community structures with some predictive functionalities that may be used for development of starters to improve the quality of the traditional products.

Highlights of Main Findings

- Traditionally processed meat products viz. beef *kargyong*, pork *kargyong*, *satchu*, *khyopeh* and fish products viz. *sukako maacha, sidra* and *sukuti* of Sikkim were studied.
- Dominant species of bacteria reported in meat and fish products were Gram-positive bacteria: *Staphylococcus aureus*, *Bacillus cereus*, *Macrococcus caseolyticus* and *Enterococcus faecalis*; Gram-negative bacteria: *Salmonella enterica, Citrobacter* spp., *Klebsiella pneumonia, Enterobacter* spp., *Escherichia coli* and *Providencia* spp.
- No enterotoxin gene of *Bacillus cereus* was detected in meat products, but detected in fish products; staphyloccal enterotoxin gene detected in some strains whereas no *Salmonella* enterotoxin genes were detected in any sample.
- Bacterial strains were sensitive towards various classes of antibiotics and few were found resistance to one or more of tested antibiotics.
- Colossal diversity of bacterial community structure dominated by phyla Firmicutes and Proteobacteria were observed by HTS tool. Abundant bacteria were *Psychrobacter*, *Lactobacillus*, *Ignatzschineria* and *Enterococcus* in meat products; *Bacillus* and *Psychrobacter* in fish products.

- Presence of *Bdellovibrio* in yak *satchu* as a predatory bacterium against Gram-negative pathogens is a novel finding.
- *Rumemliibacillus, Oceanimonas, Myroides, Kushneria* in fish products which are reported to have industrial applications.
- KGGE database showed predictive functionality of many metabolic pathways with metabolism of carbohydrate and amino acids as the highest abundances followed by vitamins and antioxidants synthesis pathways.

Schematic Representation of Complete PhD work



Aarti, C., Khusro, A., Arasu, M.V., Agastian, P. and Al-Dhabi, N.A. (2016). Biological potency and characterization of antibacterial substances produced by *Lactobacillus pentosus* isolated from *Hentak*, a fermented fish product of North-East India. *Springer Plus* 5:1743. doi: 10.1186/s40064-016-3452-2.

Aarti, C., Khusro, A., Varghese, R., Arasu, M.V., Agastian, P., Al-Dhabi, N.A., Ilavenil, S. and Choi, K.C. (2017). *In vitro* studies on probiotic and antioxidant properties of *Lactobacillus brevis* strain LAP2 isolated from *Hentak*, a fermented fish product of North-East India. *LWT-Food Science and Technology* 86:438-446.

Abdallah, M.R., Mohamed, M.A., Mohamed, H.M. and Emara, M.M. (2017). Improving the sensory, physicochemical and microbiological quality of pastirma (A traditional dry cured meat product) using chitosan coating. *Food Science and Technology* 86 (1): 247-253.

Abdhul, K., Ganesh, M., Shanmughapriya, S., Kanagavel, M., Anbarasu, K. and Natarajaseenivasan, K. (2014). Antioxidant activity of exopolysaccharide from probiotic strain *Enterococcus faecium* (BDU7) from *Ngari*. *International Journal of Biological Macromolecules* 70: 450-454.

Aberoumand, A. (2010). Estimation of microbiological variations in minced lean fish products. *World Journal of Fish and Marine Sciences* 2(3): 204-207.

Adam, K.H., Flint, S.H. and Brightwell, G. (2010). Psychrophilic and psychrotrophic clostridia: sporulation and germination processes and their role in the spoilage of chilled, vacuum-packaged beef, lamb and venison. *International Journal of Food Science and Technology* 45(8): 1539-1544.

Adams, M. (2010). Fermented Meat Products. In: *Fermented Foods and Beverages of the World*. (Ed: Tamang, J.P. and Kailasapathy, K.), pp. 309-319. CRC press, Taylor & Francis Group, New York.

Adams, M.R. (1998). Fermented Fish. In: *Microbiology Handbook Fish and Seafood* (Vol. **3**), (Eds. Lawley, R.A. and Gibbs, P.), pp. 157– 177. Surrey, United Kingdom.

Aksoy, A., Karasu, S., Akcicek, A. and Kayacan, S. (2019). Effects of different drying methods on drying kinetics, microstructure, color, and the rehydration ratio of minced meat. *Foods* 8(6): 216. doi: 10.3390/foods8060216.

Aksu, M.I. and Kaya, M. (2005). Effect of storage temperatures and time on shelf-life of sliced and modified atmosphere packaged Pastırma, a dried meat product, produced from beef. *Journal of the Science of Food and Agriculture* 85(8): 1305-1312.

Alahi, M.E.E. and Mukhopadhyay, S.C. (2017). Detection methodologies for pathogen and toxins: a review. *Sensors* 17: 1885. doi:10.3390/s17081885

Albano, H., van Reenen, C.A., Todorov, S.D., Cruz, D., Fraga, L., Hogg, T. and Teixeira, P. (2009). Phenotypic and genetic heterogeneity of lactic acid bacteria isolated from "Alheira", a traditional fermented sausage produced in Portugal. *Meat Science* 82(3): 389-398.

Al-Mutairi, M.F. (2011). The incidence of Enterobacteriaceae causing food poisoning in some meat products. *Advance Journal of Food Science and Technology* 3(2): 116-121.

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.

Amare, A., Worku, T., Ashagirie, B., Adugna, M., Getaneh, A. and Dagnew, M. (2019). Bacteriological profile, antimicrobial susceptibility patterns of the isolates among street vended foods and hygienic practice of vendors in Gondar town, Northwest Ethiopia: a cross sectional study. *BioMed Central Microbiology* 19(1): 120.doi: 10.1186/s12866-019-1509-4.

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

Ammor, M.S. and Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Science* 76(1): 138-146.

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

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom 2010; available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc.

Andrews, W.H., Jacobson, A. and Hammack, T. (2011). *Salmonella.* In: *Bacteriological Analytical Manual*. U.S. Food and Drug Administration. Maryland, United States.

Andrews, W.H., Flowers, R.S. Silliker, J. and Bailey J.S. (2001). *Salmonella.* In: Compendium of methods for the microbiological examination of foods (Eds:

Downes, F.P., Ito K), pp. 357-380. American Public Health Association (APHA), Washington, DC.

Anihouvi, V., Kindossi, J. and Hounhouigan, D. (2012). Processing and quality characteristics of some major fermented fish products from Africa: a critical review. *International Research Journal of Biological Sciences* 1: 72-84.

Anihouvi, V.B., Sakyi-Dawson, E., Ayernor, G.S., and Hounhouigan, J.D. (2007). Microbiological changes in naturally fermented cassava fish (*Pseudotolithus* sp.) for lanhouin production. *International Journal of Food Microbiology* 116(2): 287-291.

Anitha, D.P.M., Selvam, S.P., Muhamad, I.I. and Kirubanandan, S. (2019). Fermentation Methods and Fermented Fish Products. In: *Technological Processes for Marine Foods, From Water to Fork: Bioactive Compounds, Industrial Applications, and Genomics* (Ed: Goyal, M.R; Suleria, H.A.R; Kirubanandan, S.) pp 171. CRC Press, Taylor& Francis Group. New York. ISBN: 13-978-1-77188-758-8.

Antara, N.S., Sujaya, I.N., Yokota, A., Asano, K. and Tomita, F. (2004). Effects of indigenous starter cultures on the microbial and physicochemical characteristics of Urutan, a Balinese fermented sausage. *Journal of Bioscience and Bioengineering* 98(2): 92-98.

Aquilanti, L., Garofalo, C., Osimani, A. and Clementi, F. (2016). Ecology of lactic acid bacteria and coagulase negative cocci in fermented dry sausages manufactured in Italy and other Mediterranean countries: an overview. *International Food Research Journal* 23(2): 429-445.

Arcales, J.A.A. and Alolod, G.A.L. (2018). Isolation and Characterization of Lactic Acid Bacteria in Philippine Fermented Milkfish Chanos chanos-Rice Mixture

(*Burong Bangus*). *Current Research in Nutrition and Food Science Journal* 6(2):500-508.

Arfatahery, N., Davoodabadi, A. and Abedimohtasab, T. (2016). Characterization of toxin genes and antimicrobial susceptibility of *Staphylococcus aureus* isolates in fishery products in Iran. *Scientific Reports* 6(1): 1-7.

Argudín, M.Á., Mendoza, M.C., and Rodicio, M.R. (2010). Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins* 2(7): 1751-1773.

Aryanta, W.R. 1996. Utilization of lactic acid bacteria to improve the quality of Balinese traditional fermented sausage: characteristics of Balinese traditional fermented sausage. *Annual Report of International Center for Biotechnology* 19: 610–616.

Ashie, I.N.A., Smith, J.P., Simpson, B.K. and Haard, N.F. (1996). Spoilage and shelf-life extension of fresh fish and shellfish. *Critical Reviews in Food Science and Nutrition* 36(1-2): 87-121.

Aspri, M., Papademas, P. and Tsaltas, D. (2020). Review on non-dairy probiotics and their use in non-dairy based products. *Fermentation 6:* 30. doi:10.3390/fermentation6010030.

Astudillo-Melgar, F., Ochoa-Leyva, A., Utrilla, J. and Huerta-Beristain, G. (2019). Bacterial diversity and population dynamics during the fermentation of palm wine from Guerrero Mexico. *Frontiers in Microbiology* 10: 531. doi: 10.3389/fmicb.2019.00531.

Azevedo, J.S., Correia, A. and Henriques, I. (2013). Molecular analysis of the diversity of genus *Psychrobacter* present within a temperate estuary. *FEMS Microbiology Ecology* 84(3): 451-460.

Bae, D., Kweon, O. and Khan, A.A. (2016). Isolation and characterization of antimicrobial-resistant nontyphoidal *Salmonella enterica* serovars from imported food products. *Journal of Food Protection* 79(8): 1348-1354.

Baker-Austin, C., Trinanes, J., Gonzalez-Escalona, N. and Martinez-Urtaza, J. (2017). Non-cholera *Vibrios*: the microbial barometer of climate change. *Trends in Microbiology* 25(1): 76-84.

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

Baniardalan, S., Mohammadzadeh, A., Pajohi-Alamoti, M., Mahmoodi, P. and. Sadeghinasab, A. *(2017).* Detection of toxic shock toxin (tst) gene in *Staphylococcus aureus* isolated from bovine milk samples**.** *Bulgarian Journal of Veterinary Medicine* 20: 236-243.

Bao, R., Liu, S., Ji, C., Liang, H., Yang, S., Yan, X. and Zhu, B. (2018). Shortening fermentation period and quality improvement of fermented fish, Chouguiyu, by co-inoculation of *Lactococcus lactis* M10 and *Weissella cibaria* M3. *Frontiers in Microbiol*ogy 9: 3003. doi: 10.3389/fmicb.2018.03003.

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

Bartkiene, E., Bartkevics, V., Mozuriene, E., Lele V., Zadeike, D. and Juodeikiene, G. (2019). The safety, technological, nutritional, and sensory challenges associated with lacto-fermentation of meat and meat products by using pure lactic acid bacteria strains and plant-lactic acid bacteria bioproducts. *Frontiers in Microbiology* 10: 1036. doi: 10.3389/fmicb.2019.01036.

Baselga, A. (2010). Partitioning the turnover and nestedness components of beta

diversity. *Global Ecology and Biogeo*graphy 19 (1): 134–143.

Beales, N. (2004). Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. *Comprehensive Reviews in Food Science and Food Safety* 3(1):1-20.

Beattie, S.H. and Williams, A.G. (2002). Growth and diarrhoeagenic enterotoxin formation by strains of *Bacillus cereus* in vitro in controlled fermentations and in situ in food products and a model food system. *Food Microbiology* 19(4): 329-340.

Beddows, C.G. 1985. Fermented fish and fish products. In: Wood, B.J.B. (Ed.). Microbiology of fermented foods vol.2, Elsevier Applied Science Publishers, London. 1-39.

Behera, B.K., Bera, A.K., Paria, P., Das, A., Parida, P.K., Kumari, S. and Das, B.K. (2018). Identification and pathogenicity of *Plesiomonas shigelloides* in silver carp. *Aquaculture* 493: 314-318.

Benabbou, R., Subirade, M., Desbiens, M. and Fliss, I. (2018). The impact of chitosan-divergicin film on growth of *Listeria monocytogenes* in cold-smoked salmon. *Frontiers in Microbiology* 9: 2824. doi: 10.3389/fmicb.2018.02824.

Bennett, J.E., Dolin, R. and Blaser, M.J. (2014). In: *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases* (eight edition) (Vol. 2). Elsevier Health Sciences, Philadelphia.

Bennett, R.W. (2005). Staphylococcal enterotoxin and its rapid identification in foods by Enzyme-linked Immunosorbent assay–based methodology. *Journal of Food Protection* 68(6): 1264-1270.

Bennett, R.W., Yeterian, M., Smith, W., Coles, C.M., Sassaman, M. and McClure, F.D. (1986). *Staphylococcus aureus* identification characteristics and enterotoxigenicity. *Journal of Food Science* 51(5): 1337-1339.

Bennett, S.D., Walsh, K.A. and Gould, L.H. (2013). Foodborne disease outbreaks caused by *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus*-United States, 1998–2008. *Clinical Infectious Diseases* 57(3): 425-433.

Bereket, W., Hemalatha, K., Getenet, B., Wondwossen, T., Solomon, A., Zeynudin, A. and Kannan, S. (2012). Update on bacterial nosocomial infections. *European Review for Medical and Pharmacological Sciences* 16(8): 1039-44.

Bernbom, N., Ng, Y.Y., Paludan, M.C. and Gram, L. (2009). Survival and growth of *Salmonella* and *Vibrio* in *som-fak*, a Thai low-salt garlic containing fermented fish product. *International Journal of Food Microbiology* 134(3): 223-229.

Beuchat, L.R., Komitopoulou, E., Beckers, H., Betts, R.P., Bourdichon, F., Fanning, S. and Ter Kuile, B.H. (2013). Low–water activity foods: increased concern as vehicles of foodborne pathogens. *Journal of Food Protection* 76(1): 150-172.

Bhandare, S.G. Sherikar, A.T., Paturkar, A.M., Waskar, V.S. and Zende, R.J. (2007). A comparison of microbial contamination on sheep/goat carcasses in a modern Indian abattoir and traditional meat shops. *Food Control* 18(7): 854-858.

Bhatia, A. and Zahoor, S. (2007). *Staphylococcus aureus* enterotoxins: a review. *Journal of Clinical and Diagnostic Research* 3(1): 188-197.

Bhunia, A.K. (2018). *Foodborne Microbial Pathogens.* Springer, New York.

Biji, K.B., Ravishankar, C.N., Venkateswarlu, R., Mohan, C.O. and Gopal, T. S. (2016). Biogenic amines in seafood: a review. *Journal of Food Science and Technology*, 53(5): 2210-2218.

Bintsis T. (2017). Foodborne pathogens. *AIMS Microbiology* 3(3): 529–563.

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

Bis-Souza, C. V., Barba, F. J., Lorenzo, J. M., Penna, A. B. and Barretto, A. C. S. (2019). New strategies for the development of innovative fermented meat products: a review regarding the incorporation of probiotics and dietary fibers. *Food Reviews International* 35(5): 467-484.

Bjornsdottir-Butler, K., McCarthy, S.A., Dunlap, P.V. and Benner, R.A.Jr. (2016). *Photobacterium angustum* and *Photobacterium kishitanii*, psychrotrophic high-level histamine-producing bacteria indigenous to tuna. *Applied Environmental Microbiology* 82: 2167–2176.

Blaiotta, G., Ercolini, D., Pennacchia, C., Fusco, V., Casaburi, A., Pepe, O. and Villani, F. (2004). PCR detection of staphylococcal enterotoxin genes in *Staphylococcus* spp. strains isolated from meat and dairy products. Evidence for new variants of seG and seI in *S. aureus* AB-8802. *Journal of Applied Microbiology* 97(4):719-730.

Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O. and Piddock, L.J. (2015). Molecular mechanisms of antibiotic resistance. *Nature Review in Microbiology* 13(1): 42-51.

Boada, L.D., Henríquez-Hernández, L.A. and Luzardo, O.P. (2016). The impact of red and processed meat consumption on cancer and other health outcomes: Epidemiological evidences. *Food and Chemical Toxicology* 92: 236-244.

Bohaychuk, V.M., Gensler, G.E., King, R.K., Manninen, K.I., Sorensen, O., Wu, J.T. and McMullen, L.M. (2006). Occurrence of pathogens in raw and ready-to-eat meat and poultry products collected from the retail marketplace in Edmonton, Alberta, Canada. *Journal of Food Protection* 69(9): 2176-2182.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A. and Bai, Y. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* 37(8): 852-857.

Boricha, H. and Fulekar, M.H. (2009). *Pseudomonas plecoglossicida* as a novel organism for the bioremediation of cypermethrin. *Biology and Medicine* 1(4): 1-10.

Bottone, E.J. (2010). *Bacillus cereus*, a volatile human pathogen. *Clinical Microbiology Reviews* 23(2): 382-398.

Brink, B. (2010). Urease Test Protocol. In: *American Society for Microbiology*. Washington, DC. Available at: https://www.asmscience.org/content/ education/protocol/ protocol.3223.

Bunnoy, A., Na-Nakorn, U. and Srisapoome, P. (2019). Probiotic effects of a novel strain, *Acinetobacter* KU011TH, on the growth performance, immune responses, and resistance against *Aeromonas hydrophila* of bighead catfish (*Clarias macrocephalus* Günther, 1864). *Microorganisms* 7(12): 613. doi:10.3390/microorganisms7120613.

Burnham, G.M., Hanson, D.J., Koshick, C.M. and Ingham, S.C. (2008). Death of *Salmonella serovars*, *Escherichia coli* O157: H7, *Staphylococcus aureus* and *Listeria monocytogenes* during the drying of meat: A case study using *biltong* and *droëwors*. *Journal of Food Safety* 28(2): 198-209.

Busani, L., Cigliano, A., Taioli, E., Caligiuri, V., Chiavacci, L., Di Bella, C. and Ricci, A. (2005). Prevalence of *Salmonella enterica* and *Listeria monocytogenes* contamination in foods of animal origin in Italy. *Journal of Food Protection* 68(8): 1729-1733.

Buxton, R. (2011). Nitrate and Nitrite Reduction Test Protocols. In: *American Society for Microbiology*. Washington, DC. Available at: https://www.asmscience.org/content/education/protocol/protocol.3660.

Cachaldora, A., Fonseca, S., Franco, I. and Carballo, J. (2013). Technological and safety characteristics of Staphylococcaceae isolated from Spanish traditional dry-cured sausages. *Food Microbiology* 33(1): 61-68.

Casquete, R., Benito, M.J., Martín, A., Ruiz-Moyano, S., Aranda, E. and Córdoba, M.G. (2012). Microbiological quality of *salchichón* and *chorizo*, traditional Iberian dry-fermented sausages from two different industries, inoculated with autochthonous starter cultures. *Food Control* 24 (1-2):191-198.

Casquete, R., Castro, S.M. and Teixeira, P. (2017). Evaluation of the combined effect of chitosan and lactic acid bacteria in *alheira* (fermented meat sausage) paste. *Journal of Food Processing and Preservation* 41(2): 0145-8892. doi:10.1111/jfpp.12866.

Cauchie, E., Delhalle, L., Taminiau, B., Tahiri, A., Korsak, N., Burteau, S., Fall, P.A., Farnir, F., Baré, G. and Daube, G. (2020). Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packed minced pork meat samples by 16S rDNA metagenetic analysis. *Frontiers in Microbiology* 10: 3074. doi:10.3389/fmicb.2019.03074.

Ceuppens, S., Li, D., Uyttendaele, M., Renault, P., Ross, P., Ranst, M.V., Cocolin, L. and Donaghy, J. (2014). Molecular methods in food safety microbiology: interpretation and implications of nucleic acid detection. *Comprehensive Reviews in Food Science and Food Safety* 13: 551-577.

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: 139-148.

Chang, H., Wang, Q., Xu, X.L., Li, C., Huang, M., Zhou, G.H. and Dai, Y. (2011). Effect of heat-induced changes of connective tissue and collagen on meat texture properties of beef semitendinosus muscle. *International Journal of Food Properties* 14: 381-396.

Channaiah, L.H., Michael, M., Acuff, J., Lopez, K., Vega, D., Milliken, G., Thippareddi, H. and Phebus, R. (2018). Validation of a simulated commercial frying process to control *Salmonella* in donuts. *Foodborne Pathogens and Disease* 15(12): 763–769.

Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics* 11(4): 265-270.

Chao, A. and Chiu, C. (2016). *Nonparametric Estimation and Comparison of Species Richness.* Chichester: John Wiley & Sons. doi: 10.1002/9780470015902.a0026329.

Chaudhary, J.H., Nayak, J.B., Brahmbhatt, M.N. and Makwana, P.P. (2015). Virulence genes detection of *Salmonella serovars* isolated from pork and slaughterhouse environment in Ahmedabad, Gujarat. *Veterinary World* 8(1): 121-4.doi: 10.14202/vetworld.2015.121-124.

Chen, M.J., Tu, R.J. and Wang, S.Y. (2014). Chapter 37. Asian Products. In: *Handbook of Fermented Meat and Poultry* (Ed: Fidel Toldrá), pp 321-327. John Wiley & Sons, New Jersey. doi:org/10.1002/9781118522653.

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

Chlebicz, A. and Śliżewska, K. (2018). Campylobacteriosis, salmonellosis, yersiniosis,

and listeriosis as zoonotic foodborne diseases: a review. *International Journal of Environmental Research and Public Health* 15(5): 863. doi.org/10.3390/ijerph15050863.

Cho, H.R., Park, U.Y. and Chang, D.S. (2002). Studies on the shelf-life extension of *jeotkal*, salted and fermented seafood. *Korean Journal of Food Science and Technology* 34(4): 652-660.

Chokesajjawatee, N., Pornaem, S., Zo, Y.G., Kamdee, S., Luxananil, P., Wanasen, S. and Valyasevi, R. (2009). Incidence of *Staphylococcus aureus* and associated risk factors in *Nham*, a Thai fermented pork product. *Food Microbiology* 26(5): 547-551.

Chowdhury, N., Goswami, G., Hazarika, S., Pathak, S. S., and Barooah, M. (2019). Microbial dynamics and nutritional status of namsing: a traditional fermented fish product of Mishing community of Assam. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences* 89(3): 1027-1038.

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.

Clegg, S. and Sebghati, T.A.S. (2002). *Klebsiella pneumoniae*, In: *Molecular Medical Microbiology* (vol. 3) (Ed: Sussman, M.), pp. 1655–1680. Academic Press, San Diego, CA.

Clinical and Laboratory Standard Institute (2015). Disk diffusion. Performance Standards for Antimicrobial Susceptibility Testing; 15th Informational Supplement, Wayne, PA, USA, 35, M100S15.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R. and Rantsiou, R. (2013). Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *International Journal of Food Microbiology* 167: 29–43.

Collignan, A., Bohuon, P., Deumier, F., and Poligné, I. (2001). Osmotic treatment of fish and meat products. *Journal of Food Engineering* 49(2-3): 153-162.

Coloretti, F., Tabanelli, G., Chiavari, C., Lanciotti, R., Grazia, L., Gardini, F. and Montanari, C. (2014). Effect of wine addition on microbiological characteristics, volatile molecule profiles and biogenic amine contents in fermented sausages. *Meat Science* 96(3): 1395-1402.

Comi, G., Muzzin, A., Corazzin, M. and Iacumin, L. (2020). Lactic acid bacteria: variability due to different pork breeds, breeding systems and fermented sausage production technology. *Foods 9*: 338. doi.org/10.3390/foods9030338

Cox, J.W., Ballweg, R.A., Taft, D.H., Velayutham, P., Haslam, D.B. and Porollo A. (2017). A fast and robust protocol for metataxonomic analysis using RNAseq data. *Microbiome* 5: 7. doi:10.1186/s40168-016-0219-5.

Cremonesi, P., Luzzana, M., Brasca, M., Morandi, S., Lodi, R., Vimercati, C. and Castiglioni, B. (2005). Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. *Molecular and Cellular Probes* 19(5): 299-305.

Czech, L. and Stamatakis, A. (2019). Scalable methods for analyzing and visualizing phylogenetic placement of metagenomic samples. *PloS ONE* 14(5): e0217050–e0217050.doi.org/10.1371/journal.pone.0217050

Dang Nghia, N., Si Trung, T. and Van Dat, P. (2017). "*Nuoc Mam*" Fish Sauce in Vietnam: A Long History from Popular Seasoning to Health Benefit Bioactive Peptides. *Annals of Food Processing and Preservation* 2(2): 1017.

Daroonpunt, R., Itoh, T., Kudo, T., Ohkuma, M. and Tanasupawat, S. (2016). Bacillus piscicola sp. nov., isolated from Thai fish sauce (Nam-pla). *International Journal of Systematic and Evolutionary Microbiology* 66(3): 1151-1155.

Das, O., Kumar, S. and Nayak, B. (2020). Relative abundance of halophilic archaea and bacteria in diverse salt-fermented fish products. *LWT-Food Science and Technology* 117: 108688. doi.10.1016/j.lwt.2019.108688.

Davin-Regli, A., Lavigne, J.P. and Pagès, J.M. (2019). *Enterobacter* spp.: Update on taxonomy, clinical aspects, and emerging antimicrobial resistance. *Clinical Microbiology Reviews* 32(4): 2-19.

Davis, G.S. and Price, L.B. (2016). Recent research examining links among *Klebsiella pneumoniae* from food, food animals, and human extraintestinal infections. *Current Environmental Health Reports* 3(2): 128-135.

De Filippis, F., Parente, E. and Ercolini, D. (2017). Metagenomics insights into food fermentations. *Microbial Biotechnology* 10(1): 91–102.

De Mandal, S., Singh, S.S., Bose Muthukumaran, R., Thanzami, K., Kumar, V. and Senthil Kumar, N. (2018). Metagenomic analysis and the functional profiles of traditional fermented pork fat '*sa-um*' of Northeast India. *AMB Express* 8:163.doi.org/10.1186/s13568-018-0695-z

De Mey, E., De Klerck, K., De Maere, H., Dewulf, L., Derdelinckx, G., Peeters, M.C. and Paelinck, H. (2014). The occurrence of N-nitrosamines, residual nitrite and biogenic amines in commercial dry fermented sausages and evaluation of their occasional relation. *Meat Science* 96(2): 821-828.

De Vuyst, L., Falony, G. and Leroy, F. (2008). Probiotics in fermented sausages. *Meat Science* 80(1): 75-78.

Deatraksa, J., Sunthornthummas, S., Rangsiruji, A., Sarawaneeyaruk, S., Suwannasai, N. and Pringsulaka, O. (2018). Isolation of folate-producing *Weissella* spp. from Thai fermented fish (Plaa Som Fug). *LWT Food Science and Technology* 89: 388-391.

dela Cruz, T.E.E. and Torres, J.M.O. (2012). Gelatin Hydrolysis Test Protocol. In: *American Society for Microbiology*. Washington, DC. Available at: https://www.asmscience.org/ content/education/protocol/protocol.3776.

Delpech, G., Pourcel, G., Schell, C., De Luca, M., Basualdo, J., Bernstein, J. and Sparo, M. (2012). Antimicrobial resistance profiles of *Enterococcus faecalis* and *Enterococcus faecium* isolated from artisanal food of animal origin in Argentina. *Foodborne Pathogens and Disease* 9(10): 939-944.

Devi, K.R., Deka, M. and Jeyaram, K. (2015). Bacterial dynamics during yearlong spontaneous fermentation for production of *ngari*, a dry fermented fish product of Northeast India. *International Journal of Food Microbiology* 199: 62–71.

Di Luccia, A., Tremonte, P., Trani, A., Loizzo, P., La Gatta, B., Succi, M. and Coppola, R. (2016). Influence of starter cultures and KCl on some biochemical, microbiological and sensory features of *soppressata molisana*, an Italian fermented sausage. *European Food Research and Technology* 242(6): 855-867.

Dias, F.S., Santos, M.R.R.M. and Schwan, R.F. (2015). Enumeration, identification and safety proprieties of lactic acid bacteria isolated from pork sausage. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* 67*:* 918-926.

Djordjevic, J., Pecanac, B., Todorovic, M., Dokmanovic, M., Glamoclija, N., Tadic, V. and Baltic, M. (2015). Fermented sausage casings. *Procedia Food Science* 5: 69-72.

Dodd, C.E., Aldsworth, T.G. and Stein, R.A. (2017). Food-borne Diseases. (third

edition), Elsevier Science Publication Ltd, San Diego.

dos Santos, R.R., Xavier, R.G.C., de Oliveira, T.F., Leite, R.C., Figueiredo, H.C.P. and Leal, C.A.G. (2019). Occurrence, genetic diversity, and control of *Salmonella enterica* in native Brazilian farmed fish. *Aquaculture* 501: 304-312.

Douglas, G.M., Maffei, V. J., Zaneveld, J., Yurgel, S.N., Brown, J.R., Taylor, C.M. and Langille, M.G. (2019). PICRUSt2: An improved and extensible approach for metagenome inference. *BioRxiv* 672295. doi.org/10.1101/672295.

Du, F., Zhang, X., Gu, H., Song, J. and Gao, X. (2019). Dynamic changes in the bacterial community during the fermentation of traditional Chinese fish sauce (TCFS) and their correlation with TCFS quality. *Microorganisms* 7(9): 371. doi:10.3390/microorganisms7090371

Ehling-Schulz, M., Guinebretiere, M.H., Monthán, A., Berge, O., Fricker, M. and Svensson, B. (2006). Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. *FEMS Microbiology Letters* 260 (2): 232–240.

Elegado, F.B., Colegio, S.M.T., Lim, V.M.T., Gervasio, A.T.R., Perez, M.T.M., Balolong, M.P. and Mendoza, B.C. (2016). Ethnic fermented foods of the Philippines with reference to lactic acid bacteria and yeasts. In: *Ethnic Fermented Foods and Alcoholic Beverages of Asia,* pp. 323-340. Springer, New Delhi.

El-Shorbagy, M.M., Reda Lamyaa, M. and Mona, H. (2012). Prevalence of Clostridium perfringens Alpha toxin in processed and unprocessed fish. *International Journal of Microbiological Research*, *3*(3), 195-199.

Eng, S.K., Pusparajah, P., Ab Mutalib, N.S., Ser, H.L., Chan, K.G. and Lee, L.H. (2015) *Salmonella*: a review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science* 8(3): 284-293.

Erb-Downward, J.R., Sadighi Akha, A.A., Wang, J., Shen, N., He, B., Martinez, F.J., Gyetko, M.R., Curtis, J.L. and Huffnagle, G.B. (2012). Use of direct gradient analysis to uncover biological hypotheses in 16s survey data and beyond. *Scientific Reports* 2: 774. doi.org/10.1038/srep00774.

Ercolini, D. (2013). High-throughput sequencing and metagenomics: moving forward in the culture-independent analysis of food microbial ecology. *Applied and Environmental Microbiology* 79(10): 3148-3155.

Faith, D.P. (1992). Conservation evaluation and phylogenetic diversity. *Biological Conservation* 61: 1-10.

FAO (2012). *Code Of Practice For Fish And Fishery Products* (second edition). Food and Agriculture Organization and World Health Organization, Rome.

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

Feldhusen, F. (2000). The role of seafood in bacterial foodborne diseases. *Microbes and Infection* 2(13): 1651-1660.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39(4): 783-791.

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

Fernández-Ginés, J.M., Fernández-López, J., Sayas-Barberá, E. and Pérez-Alvarez, J. A. (2005). Meat products as functional foods: A review. *Journal of Food Science* 70(2): R37-R43.

Ferreira, V., Barbosa, J., Vendeiro, S., Mota, A., Silva, Fátima, S., Monteiro, M., Hogg, T., Gibbs, P. and Teixeira, P. (2006). Chemical and microbiological characterization of alheira: A typical Portuguese fermented sausage with particular reference to factors relating to food safety. *Meat Science* 73: 570-575.

Ferrocino, I., Bellio, A., Giordano, M., Macori, G., Romano, A., Rantsiou, K., Decastelli, L. and Cocolin, L. (2018). Shotgun metagenomics and volatilome profile of the microbiota of fermented sausages. *Applied and Environmental Microbiology* 84(3): e02120-17. doi:10.1128/AEM.02120-17.

Fisher, E. L., Otto, M. and Cheung, G. Y. (2018). Basis of virulence in enterotoxin-mediated staphylococcal food poisoning. *Frontiers in Microbiology* 9: 436.doi: 10.3389/fmicb.2018.00436

Flores, M. (2018). Understanding the implications of current health trends on the aroma of wet and dry cured meat products. *Meat Science* 144: 53-61.

Fontán, M.C.G., Lorenzo, J.M., Martínez, S., Franco, I. and Carballo, J. (2007). Microbiological characteristics of *Botillo*, a Spanish traditional pork sausage. *LWT-Food Science and Technology* 40: 1610-1622.

Fontán, M.C.G., Lorenzo, J.M., Parada, A., Franco, I. and Carballo, J. (2007). Microbiological characteristics of "*androlla*", a Spanish traditional pork sausage. *Food Microbiology* 24(1): 52-58.

Fontana, C., Bassi, D., López, C., Pisacane, V., Otero, M. C., Puglisi, E. and Vignolo, G. (2016). Microbial ecology involved in the ripening of naturally fermented *llama* meat sausages. A focus on lactobacilli diversity. *International Journal of Food*

Microbiology 236: 17-25.

Franciosa, I., Alessandria, V., Dolci, P., Rantsiou, K. and Cocolin, L. (2018). Sausage fermentation and starter cultures in the era of molecular biology methods. *International Journal of Food Microbiology* 279. 10.1016/j.ijfoodmicro.2018.04.038.

Fratamico, P.M., Bhunia, A.K. and Smith, J.L. (2005). *Foodborne Pathogens: Microbiology and Molecular Biology*. CRC Press, Taylor & Francis Group, New York.

Fryer, P.J. and Robbins, P.T. (2005). Heat transfer in food processing: ensuring product quality and safety. *Applied Thermal Engineering* 25(16): 2499-2510.

Fuertes-Perez, S., Hauschild, P., Hilgarth, M. and Vogel, R.F. (2019). Biodiversity of *Photobacterium* spp. isolated from meats. *Frontiers in Microbiology* 10: 2399.doi: 10.3389/fmicb.2019.02399.

Fujii, T., Kyoui, D., Takahashi, H., Kuda, T., Kimura, B., Washizu, Y. and Hiramoto, T. (2016). Pyrosequencing analysis of the microbiota of *kusaya* gravy obtained from Izu Islands. *International Journal of Food Microbiology* 238: 320-325.

Fuller, D.Q. (2015). Harappan seeds and agriculture: some considerations. *Antiquity* 75: 410-414.

Gagaoua, M. and Boudechicha, H.R. (2018). Ethnic meat products of the North African and Mediterranean countries: an overview. *Journal of Ethnic Foods* 5(2): 83-98.

García, M.T., Cañamero, M.M., Lucas, R., Omar, N.B., Pulido, R.P. and Gálvez, A. (2004). Inhibition of *Listeria monocytogenes* by enterocin EJ97 produced by *Enterococcus faecalis* EJ97. *International Journal of Food Microbiology* 90(2): 161-170.

Gardini, F.; Özogul, Y.; Suzzi, G.; Tabanelli, G. and Özogul, F. (2016). Technological factors affecting biogenic amine content in foods: a review. *Frontiers in Microbiology* 7: 1218. doi: 10.3389/fmicb.2016.01218.

Garedew, L., Hagos, Z., Zegeye, B. and Addis, Z. (2016). The detection and antimicrobial susceptibility profile of *Shigella* isolates from meat and swab samples at butchers' shops in Gondar town, Northwest Ethiopia. *Journal of Infection and Public Health* 9(3): 348-355.

Garnjanagoonchorn, Wunwiboon. (2016). "Thai Fish Sauce: A Traditional Fermented Sauce." In: *Modernization of Traditional Food Processes and Products* (Ed: McElhattan, A; Misbah El ldrissi, M) pp. 115-123. Springer, Boston, MA.

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

Gassem, M. A. (2019). Microbiological and chemical quality of a traditional salted-fermented fish (Hout-Kasef) product of Jazan region, Saudi Arabia. *Saudi Journal of Biological Sciences* 26(1): 137-140.

Gdoura-Ben Amor, M., Jan, S., Baron, F., Grosset, N., Culot, A., Gdoura, R., Gautier, M. and Techer, C. (2019). Toxigenic potential and antimicrobial susceptibility of *Bacillus cereus* group bacteria isolated from Tunisian foodstuffs. *BMC Microbiology* 19(1): 196. doi.org/10.1186/s12866-019-1571-y.

Gelbíc˘ová, T., Baráková, A., Florianová, M., Jamborová, I., Zelendová, M., Pospíšilová, L., Kolác˘ková, I. and Karpíšková, R. (2019). Dissemination and comparison of genetic determinants of mcr-mediated colistin resistance in enterobacteriaceae via retailed raw meat products. *Frontiers in Microbiology* 10:2824. doi: 10.3389/fmicb.2019.02824.

Giletto, A. and Fyffe, J.G. (1998). A novel ELISA format for the rapid and sensitive detection of Staphylococcal eEnterotoxin A. *Bioscience, Biotechnology, and Biochemistry* 62 (11): 2217-2222.

Gill, A. (2017). The Importance of bacterial culture to food microbiology in the age of genomics. *Frontiers in Microbiology* 8:777. doi: 10.3389/fmicb.2017.00777

Gille, D. and Schmid, A. (2015). Vitamin B12 in meat and dairy products. *Nutrition Reviews* 73(2): 106-115.

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.

Goodrich, J.K., Di Rienzi, S.C., Poole, A.C., Koren, O., Walters, W.A. and Caporaso, J.G. (2014). Conducting a microbiome study. *Cell* 158(2): 250-262.

Govari, M. and Pexara, A. (2018). Nitrates and nitrites in meat products. *Journal of the Hellenic Veterinary Medical Society* 66: 127. doi.10.12681/jhvms.15856

Gram, L. and Huss, H.H. (1996). Microbiological spoilage of fish and fish products. *International Journal of Food Microbiology* 33(1): 121-137.

Grützke, J., Malorny, B., Hammerl, J.A., Busch, A., Tausch, S.H., Tomaso, H. and Deneke, C. (2019). Fishing in the soup–pathogen detection in food safety using metabarcoding and metagenomic sequencing. *Frontiers in Microbiology* 10: 1805.doi: 10.3389/fmicb.2019.01805

Guinebretière, M.H. and Broussolle, V. (2002). Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. *Journal of Clinical Microbiology* 40(8): 3053-3056.

Gundogan, N., Ataol, O. and Ozturk, F. (2013). Determination of virulence factors in *Staphylococcus aureus, Enterococcus faecalis* and *Enterococcus faecium* isolated from meat and milk products. *Journal of Food Safety* 33: 387–393.

Gupta, B., Ghatak, S. and Gill, J.P.S. (2013). Incidence and virulence properties of *E. coli* isolated from fresh fish and ready-to-eat fish products. *Veterinary World* 6(1): 5-9.

Gwak, H.J., and Eun, J.B. (2010). Changes in the chemical characteristics of *Gulbi*, salted and dried yellow corvenia, during drying at different temperatures. *Journal of Aquatic Food Product Technology* 19(3-4): 274-283.

Hall, J.A., Goulding, J.S., Bean, N.H., Tauxe, R.V. and Hedberg, C.W. (2001). Epidemiologic profiling: evaluating foodborne outbreaks for which no pathogen was isolated by routine laboratory testing: United States, 1982–9. *Epidemiology and Infection* 127(3): 381-387.

Hammer, Ø., Harper, D.A. and Ryan, P.D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* 4(1): 9.http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Hathwar, S.C., Rai, A.K., Modi, V.K. and Narayan, B. (2012). Characteristics and consumer acceptance of healthier meat and meat product formulations—a review. *Journal of Food Science and Technology* 49(6): 653-664.

He, Z.F., Zhen, Z.Y., LI, H.J., ZHOU, G.H. and Zhang, J.H. (2008). Microorganisms Flora Study on Jinhua Ham Fermentation. *Food Science* 1.

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

Hendriksen, R.S., Vieira, A.R., Karlsmose, S., Lo Fo Wong, D.M., Jensen, A.B., Wegener, H.C. and Aarestrup, F.M. (2011). Global monitoring of Salmonella

serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: results of quality assured laboratories from 2001 to 2007. *Foodborne Pathogens and Disease* 8(8): 887-900.

Hennekinne, J.A., De Buyser, M.K. and Dragacci, S. (2012). *Staphylococcus aureus* and its food poisoning toxins: characterization and outbreak investigation. *FEMS Microbiology Reviews* 36(4): 815–836.

Herranz, C., Casaus, P., Mukhopadhyay, S., Martınez, J.M., Rodrıguez, J.M., Nes, I.F. and Cintas, L.M. (2001). *Enterococcus faecium* P21: a strain occurring naturally in dry-fermented sausages producing the class II bacteriocins enterocin A and enterocin B. *Food Microbiology* 18(2): 115-131.

Herrera, F.C., Santos, J.A., Otero, A. and García-López, M.L. (2006). Occurrence of foodborne pathogenic bacteria in retail prepackaged portions of marine fish in Spain. *Journal of Applied Microbiology* 100(3): 527-536.

Ho, T.N.T., Nguyen, N.T, Deschamps, A., Hadj Sassi, A., Urdaci, M. and Caubet R. (2009). The impact of *Lactobacillus brevis* and *Pediococcus pentosaceus* on the sensorial quality of *"nem chua*"—a Vietnamese fermented meat product. *International Food Research Journal* 16: 71–81.

Holzapfel, W.H., Geisen, R. and Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology* 24(3): 343-362.

Howard Hughes Medical Institute HMMER 3.2.1. (2018). Available online at: www.hmmer.org. 2018. https://doi.org/.1037//0033-2909.I26.1.78.

Huda, N. (2012). Indonesian fermented fish products. In: *Handbook of Animal-Based Fermented Food and Beverage* Technology 2: 717-738.

Hussein, M.A., Merwad, A.M., Elabbasy, M.T., Suelam, I.I., Abdelwahab, A.M. and Taha, M.A. (2019). Prevalence of Enterotoxigenic *Staphylococcus aureus* and Shiga Toxin Producing *Escherichia coli* in Fish in Egypt: Quality Parameters and Public Health Hazard. *Vector-Borne and Zoonotic Diseases* 19(4): 255-264.

Hussey, M.A. and Zayaitz, A. (2007). Endospore Stain Protocol. In: *American Society for Microbiology*. Washington D.C. Available at: https://www.asmscience.org/content/education/protocol/protocol.3112.

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

Ijong, F. G. and Ohta, Y. (1996). Physicochemical and microbiological changes associated with Bakasang processing—a traditional Indonesian fermented fish sauce. *Journal of the Science of Food and Agriculture* 71(1): 69-74.

Illikoud, N., Rossero, A., Chauvet, R., Courcoux, P., Pilet, M.F., Charrier, T. and Zagorec, M. (2019). Genotypic and phenotypic characterization of the food spoilage bacterium *Brochothrix thermosphacta*. *Food Microbiology* 81: 22-31.

Ingham, S.C., Searls, G., Mohanan, S. and Buege, D.R. (2006). Survival *of Staphylococcus aureus* and *Listeria monocytogenes* on vacuum-packaged beef *jerky* and related products stored at 21℃. *Journal of Food Protection* 69(9): 2263-2267.

Inusa, S.K. and Said, I.S. (2017). Evaluation of the chemical and microbiological properties of *kilishi* sold in Kano metropolis. *Journal of Dryland Agriculture* 3(1): 59-69.

Jamali, H., Paydar, M., Radmehr, B., Ismail, S. and Dadrasnia, A. (2015). Prevalence and antimicrobial resistance of Staphylococcus aureus isolated from raw milk and dairy products. *Food Control* 54: 383-388.

Je, J.Y., Park, P.J., Byun, H.G., Jung, W.K. and Kim, S.K. (2005). Angiotensin I converting enzyme (ACE) inhibitory peptide derived from the sauce of fermented blue mussel, Mytilus edulis. *Bioresource Technology* 96(14): 1624-1629.

Jiang, S., Ma, C., Peng, Q., Huo, D., Li, W. and Zhang, J. (2019). Microbial profile and genetic polymorphism of predominant species in some traditional fermented seafoods of the Hainan area in China. *Frontiers in Microbiology* 10:564. doi: 10.3389/fmicb.2019.00564.

Junpadit, P., Suksaroj, T.T. and Boonsawang, P. (2017). Transformation of palm oil mill effluent to terpolymer polyhydroxyalkanoate and biodiesel using *Rummeliibacillus pycnus* strain TS 8. *Waste and Biomass Valorization* 8(4): 1247-1256.

Kaban, G. (2013). *Sucuk* and *pastirma*: microbiological changes and formation of volatile compounds. *Meat Science* 95(4): 912-918.

Kakati, B.K. and Goswami, U.C. (2013a). Microorganisms and the nutritive value of traditional fermented fish products of Northeast India. *Global Journal of Bio-science and Biotechnology* 2(1): 124-127.

Kakati, B.K. and Goswami, U.C. (2013b). Characterization of the traditional fermented fish product *Shidol* of Northeast India prepared from *Puntius sophore* and S*etipinna phas*a. *Indian Journal of Traditional Knowledge* 12: 85-90.

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

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y. and Morishima, K. (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Research* 45(D1): D353-D361.

Kanjan, P. and Sakpetch, P. (2020). Functional and safety assessment of Staphylococcus simulans PMRS35 with high lipase activity isolated from high salt-fermented fish (*Budu*) for starter development. *Food Science and Technology* 109183. doi.org/10.1016/j.lwt.2020.109183.

Kanno, T., Kuda, T., An, C., Takahashi, H. and Kimura, B. (2012). Radical scavenging capacities of *saba-narezushi*, Japanese fermented chub mackerel, and its lactic acid bacteria. *Food Science and Technology* 47(1): 25-30.

Karlsen, E., Schulz, C. and Almaas, E. (2018). Automated generation of genome-scale metabolic draft reconstructions based on KEGG. *BMC Bioinformatics* 19(1): 467.doi.org/10.1186/s12859-018-2472-z

Karoki, W.H., Karanja, D.N., Bebora, L.C. and Njagi, L.W. (2018). Isolation, characterization, and quantification of bacteria from African sausages sold in Nairobi county, Kenya. *International Journal of Food Science* 3861265. doi.org/10.1155/2018/3861265.

Kateete, D. P., Kimani, C.N., Katabazi, F.A., Okeng, A., Okee, M.S., Nanteza, A. and Najjuka, F.C. (2010). Identification of *Staphylococcus aureus*: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. *Annals of Clinical Microbiology and Antimicrobials* 9:23.doi.org/10.1186/1476-0711-9-23

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

Kaysner, C.A. and DePaola, A.J. (2004). Chapter 9. *Vibrio.* In: *Bacteriological Analytical Manual*. U.S. Food and Drug Administration. Maryland, United States.

Keisam, S., Romi, W., Ahmed, G. and Jeyaram, K. (2016). Quantifying the biases in metagenome mining for realistic assessment of microbial ecology of naturally fermented foods. *Scientific Reports* 6: 34155.doi: 10.1038/srep34155.

Keisam, S., Tuikhar, N., Ahmed, G., and Jeyaram, K. (2019). Toxigenic and pathogenic potential of enteric bacterial pathogens prevalent in the traditional fermented foods marketed in the Northeast region of India. *International Journal of Food Microbiology* 296: 21-30.

Kérouanton, A., Hennekinne, J.A., Letertre, C., Petit, L., Chesneau, O., Brisabois, A. and De Buyser, M.L. (2007). Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. *International Journal of Food Microbiology* 115(3): 369-375.

Kerr, W., Wang, X. and Choi, S. (2005). Physical and sensory characteristics of low-fat Italian sausage prepared with hydrated oat. *Journal of Food Quality* 28: 62-77.

Khan, Z.A., Siddiqui, M.F. and Park, S. (2019). Current and emerging methods of antibiotic susceptibility testing. *Diagnostics* 9(2): 49. doi.org/10.3390/diagnostics9020049.

Kharel, N., Palni, U. and Tamang, J.P. (2016). Microbiological assessment of ethnic street foods of the Himalayas. *Journal of Ethnic Foods* 3(3): 235-241.

Kim, B., Byun, B.Y. and Mah, J.H. (2012). Biogenic amine formation and bacterial contribution in *Natto* products. *Food Chemistry* 135(3): 2005-2011.

Kim, B.R. (2017). Deciphering diversity indices for a better understanding of microbial communities. *Journal of Microbial Biotechnology.* 27(12): 2089–2093.

Kingcha, Y., Tosukhowong, A., Zendo, T., Roytrakul, S., Luxananil, P., Chareonpornsook, K., and Visessanguan, W. (2012). Anti-listeria activity of

Pediococcus pentosaceus BCC 3772 and application as starter culture for *Nham*, a traditional fermented pork sausage. *Food* Control 25(1): 190-196.

Kitamura, Y., Kusumoto, K.I., Oguma, T., Nagai, T., Furukawa, S., Suzuki, C. and Tamaki, H. (2016). Ethnic fermented foods and alcoholic beverages of Japan. In: *Ethnic Fermented Foods and Alcoholic Beverages of Asia* (Ed: Tamang, J.P), pp. 193-236. Springer, New Delhi. ISBN: 978-81-322-2800-4.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research* 41(1): e1.doi: 10.1093/nar/gks808

Klingberg, T.D., Axelsson, L., Naterstad, K., Elsser, D. and Budde, B.B. (2005). Identification of potential probiotic starter cultures for Scandinavian-type fermented sausages. *International Journal of Food Microbiology* 105(3): 419-431.

Kobayashi, T., Taguchi, C., Kida, K., Matsuda, H., Terahara, T., Imada, C. and Thwe, S.M. (2016). Diversity of the bacterial community in Myanmar traditional salted fish *yegyo ngapi*. *World Journal of Microbiology and Biotechnology* 32(10):166.

Kołożyn-Krajewska, D. and Dolatowski, Z.J. (2009). Probiotics in fermented meat products. *Acta Scientiarum Polonorum Technologia Alimentaria* 8(2): 61-74.

Kołożyn-Krajewska, D. and Dolatowski, Z.J. (2012). Probiotic meat products and human nutrition. *Process Biochemistry* 47(12): 1761-1772.

Konieczny, P., Stangierski, J. and Kijowski, J. (2007). Physical and chemical characteristics and acceptability of home style beef jerky. *Meat Science* 76(2): 253-257.

Koo, O.K., Lee, S.J., Chung, K.R., Jang, D.J., Yang, H.J. and Kwon, D.Y. (2016). Korean traditional fermented fish products: jeotgal. *Journal of Ethnic Foods* 3(2): 107-116.

Kotiranta, A., Lounatmaa, K. and Haapasalo, M. (2000). Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes and Infection* 2(2): 189-198.

Kramer, J.M., Gilbert, R.J. and Doyle, M.P. (1989). In: Foodborne bacterial pathogens. (Eds: Doyle, M.P and Dekker, M.), pp. 22-70. Inc., New York.

Krueger, F. (2017). Trim Galore!, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore.

Kuda, T. (2015). Quality improvement and fermentation control in fish products. *Advances in Fermented Foods and Beverages* (Ed: Holzapfel, W) pp. 377-390. Woodhead Publishing Series in Food Science, Technology and Nutrition. United Kingdom.

Kuda, T., Tomomi, K., Kawahara, M., Takahashi, H. and Kimura, B. (2014). Inhibitory effects of *Leuconostoc mesenteroides* 1RM3 isolated from *narezushi* on lipopolysaccharide-induced inflammation in RAW264. 7 mouse macrophage cells and dextran sodium sulphate-induced inflammatory bowel disease in mice. *Journal of Functional Foods* 6: 631-636.

Kumar, P., Chatli, M.K., Verma, A.K., Mehta, N., Malav, O.P., Kumar, D. and Sharma, N. (2017). Quality, functionality, and shelf life of fermented meat and meat products: A review. *Critical Reviews in Food Science and Nutrition* 57(13): 2844-2856.

Kumar, S., Stecher, G. and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33(7):1870-1874.

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: 1-14. doi:10.1371/journal.pone.0129864.

Labbe, R.G. and Juneja, V.K. (2017). *Clostridium perfringens*. In: *Foodborne Diseases*, pp. 235-242. Academic Press.

Ladero, V., Calles-Enríquez, M., Fernández, M. and Alvarez, M. (2010). Toxicological effects of dietary biogenic amines. *Current Nutrition and Food Science* 6(2): 145-156.

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

Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A. and Beiko, R.G. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology* 31(9): 814-821.

Laranjo, M., Elias, M. and Fraqueza, M.J. (2017). The use of starter cultures in traditional meat products. *Journal of Food Quality* Article ID 9546026, doi.org/10.1155/2017/9546026.

Laranjo, M., Potes, M.E. and Elias, M. (2019). Role of starter cultures on the safety of fermented meat products. *Frontiers in Microbiology* 10: 853.doi: 10.3389/fmicb.2019.00853

Latorre-Moratalla, M., Bover-Cid, S., Veciana-Nogués, M.T. and Vidal-Carou, M.C. (2012). Control of biogenic amines in fermented sausages: role of starter cultures. *Frontiers in Microbiology* 3: 169.

Lee, H.J., Baek, J.H., Yang, M., Han, H.E., Ko, Y.D. and Kim, H.J. (1993). Characteristics of lactic acid bacterial community during *kimchi* fermentation by temperature downshift. *Korean Journal of Microbiology* 31: 346-353.

Lee, N., Kim, M.D., Chang, H.J., Choi, S.W. and Chun, H.S. (2017). Genetic diversity, antimicrobial resistance, toxin gene profiles, and toxin production ability of *Bacillus cereus* isolates from *doenjang*, a Korean fermented soybean paste. *Journal of Food Safety* 37(4). doi: org/10.1111/jfs.12363.

Lee, S.H., Jung, J.Y. and Jeon, C.O. (2014). Microbial successions and metabolite changes during fermentation of salted shrimp (*Saeu-Jeot*) with different salt concentrations. *PLoS One* 9(2): e90115. doi:10.1371/journal.pone.0090115.

Lee, S.Y., Sekhon, S.S., Ko, J.H., Kim, H.C., Kim, S.Y., Won, K. and Kim, Y.H. (2018). Lactic acid bacteria isolated from Kimchi to evaluate anti-obesity effect in high fat diet-induced obese mice. *Toxicology and Environmental Health Sciences* 10(1): 11-16.

Leisner, J.J., Laursen, B.G., Prévost, H., Drider, D. and Dalgaard, P. (2007). *Carnobacterium*: positive and negative effects in the environment and in foods. *FEMS Microbiology Reviews* 31(5): 592-613.

Leistner, L. (1992). Food preservation by combined methods. *Food Research International* 25(2): 151-158.

Leroy, F. and L. De Vuyst. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology* 15 (2):67–78.

Leroy, S., Lebert, I. and Talon, R. (2015). Microorganisms in traditional fermented meats. In: *Handbook of Fermented Meat and Poultry* 2nd edition) (Eds: Toldrá, F., Hui, Y.H., Astiasarán, I., Sebranek, J.G., and Talon, R.), pp. 99–105. Wiley-Blackwell, New Jersey.

Leroy, S., Vermassen, A., Ras, G. and Talon, R. (2017). Insight into the genome of *Staphylococcus xylosus*, a ubiquitous species well adapted to meat products. *Microorganisms* 5(3): 52. doi: 10.3390/microorganisms5030052.

Li, J., Yuan, X., Desta, S. T., Dong, Z., Mugabe, W. and Shao, T. (2018). Characterization of *Enterococcus faecalis* JF85 and *Enterococcus faecium* Y83 isolated from Tibetan yak (*Bos grunniens*) for ensiling *Pennisetum sinese*. *Bioresource Technology* 257: 76-83.

Li, Y., Pei, X., Yan, J., Liu, D., Zhang, H., Yu, B. and Yang, D. (2019). Prevalence of foodborne pathogens isolated from retail freshwater fish and shellfish in China. *Food Control* 99: 131-136.

Liang, D.H., Hu, Y., Cheng, J. and Chen, Y. (2020). Simultaneous sulfamethoxazole biodegradation and nitrogen conversion in low C/N ratio pharmaceutical wastewater by *Achromobacter* sp. JL9. *Science of the Total Environment* 703: 135586.

Lindbäck, T., Fagerlund, A., Rødland, M.S. and Granum, P.E. (2004). Characterization of the *Bacillus cereus* Nhe enterotoxin. *Microbiology* 150: 3959-3967.

Liu, C., Wu, H., Liu, S., Chai, S., Meng, Q. and Zhou, Z. (2019). Dynamic Alterations in Yak Rumen Bacteria Community and Metabolome Characteristics in Response to Feed Type. *Frontiers in Microbiology* 10*:* 1116*. doi: 10.3389/fmicb*.

Liu, G., Hale, G.E, and Hughes, C.L. (2000). Galactose metabolism and ovarian toxicity. *Reproductive Toxicology* 14(5): 377-384.

Liu, H., Whitehouse, C.A., and Li, B. (2018). Presence and persistence of *Salmonella* in water: the impact on microbial quality of water and food safety. *Frontiers in Public Health*, 6; 159. doi.org/10.3389/fpubh.2018.00159.

Liu, J., Ma, G., Chen, T., Hou, Y., Yang, S., Zhang, K.Q. and Yang, J. (2015). Nicotine-degrading microorganisms and their potential applications. *Applied Microbiology and Biotechnology* 99(9): 3775-3785.

Logan NA, Rodrigez-Diaz, M. (2006) *Bacillus* spp. and related genera. In: Principles and Practice of Clinical Bacteriology (Eds: Gillespie, S.H. and Hawkey, P.M.), pp. 139–158. John Wiley and Sons Ltd, West Sussex.

Lopetcharat, K., Choi, Y.J., Park, J.W. and Daeschel, M.A. (2001). Fish sauce products and manufacturing: a review. *Food Reviews International* 17(1): 65-88.

Lorenzo, J.M., Bermúdez, R., Domínguez, R., Guiotto, A., Franco, D. and Purriños, L. (2015). Physicochemical and microbial changes during the manufacturing process of dry-cured lacón salted with potassium, calcium and magnesium chloride as a partial replacement for sodium chloride. *Food Control* 50: 763–769.

Lorenzo, J.M., Munekata, P.E., Dominguez, R., Pateiro, M., Saraiva, J.A. and Franco, D. (2018). Main groups of microorganisms of relevance for food safety and stability: general aspects and overall description. *Innovative Technologies for Food Preservation* 53–107.

Lotfy, N.M., Hassanein, M.A., Abdel-Gawad, F.K.H., El-Taweel, G.E. and Bassem, S. M. (2011). Detection of *Salmonella* spp in aquatic insects, Fish and Water by MPN-PCR. *World Journal of Fish and Marine Sciences* 3(1): 58-66.

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

Lücke, F.K. (1998). Fermented sausages. In *Microbiology of Fermented Foods*, pp. 441-483. Springer, Boston, MA.

Lücke, F.K. (2015). Quality improvement and fermentation control in meat products. In: *Advances In Fermented Foods And Beverages. Improving Quality, Technologies And Health Benefi*ts (Ed: W.H. Holzapfel), pp. 357-376. Woodhead Publishing Ltd., Cambridge.

Machado-Moreira, B., Richards, K., Brennan, F., Abram, F. and Burgess, C.M. (2019). Microbial contamination of fresh produce: what, where, and how? *Comprehensive Reviews in Food Science and Food Safety* 18: 1727-1750.

MacWilliams, M.P. (2009). Citrate Test Protocol. In: *American Society for Microbiology*. Washington, DC. Available at: https://www.asmscience.org/content/education/ protocol/protocol.3203.

MacWilliams, M.P. (2012). Indole Test Protocol. In: *American Society for Microbiology*. Washington, DC. Available at: https://www.asmscience.org/content/education/ protocol/protocol.3202.

Mahenthiralingam, E., Urban, T.A. and Goldberg, J.B. (2005). The multifarious, multireplicon *Burkholderia cepacia* complex. *Nature Reviews Microbiology* 3(2): 144-156.

Mainar, M.S., Stavropoulou, D.A. and Leroy, F. (2017). Exploring the metabolic heterogeneity of coagulase-negative staphylococci to improve the quality and safety of fermented meats: a review. *International Journal of Food Microbiology* 247: 24-37.

Majumdar, R. K., Basu, S. and Nayak, B.B. (2009). Assessment of nutritional quality of *'Shidal'* a fermented fish product of Northeast India. *Journal of Indian Fisheries Association* 36: 25-34.

Majumdar, R.K. and Basu, S. (2010). Characterization of a traditional fermented fish product *Lona ilish* of Northeast India. *Indian Journal of Traditional Knowledge* 9(3): 453-458.

Majumdar, R.K., Bejjanki, S.K., Roy, D., Shitole, S., Saha, A. and Narayan, B. (2015a). Biochemical and microbial characterization of *Ngari* and *Hentaak*-traditional fermented fish products of India. *Journal of Food Science and Technology* 52: 8284-8291.

Majumdar, R.K., Roy, D., Bejjanki, S. and Bhaskar, N. (2015b). Chemical and microbial properties of shidal, a traditional fermented fish of Northeast India. *Journal of Food Science and Technology* 53(1): 401-410.

Mamuad, L.L., Kim, S.H., Biswas, A.A., Yu, Z., Cho, K.K., Kim, S.B. and Lee, S.S. (2019). Rumen fermentation and microbial community composition influenced by live *Enterococcus faecium* supplementation. *AMB Express* 9(1): 123.-83.

Mandal, P.K., Biswas, A.K., Choi, K. and Pal, U.K. (2011). Methods for rapid detection of foodborne pathogens: an overview. *American Journal of Food Technology* 6: 87–102.

Martinson, J.N.V., Pinkham, N.V., Peters, G.W., Cho, H., Heng, J., Rauch, M., Broadaway, S.C. and Walk, S.T. (2019). Rethinking gut microbiome residency and the *Enterobacteriaceae* in healthy human adults. *The Journal of ISME* 13: 2306–2318.

Marty, E., Buchs, J., Eugster-Meier, E., Lacroix, C. and Meile, L. (2011). Identification of staphylococci and dominant lactic acid bacteria in spontaneously fermented Swiss meat products using PCR–RFLP. *Food Microbiology* 29: 157–166. doi.org/10.1016/j.fm.2011.09.011.

Marui, J., Boulom, S., Panthavee, W., Momma, M., Kusumoto, K. and Saito, M.

(2015). Culture-independent bacterial community analysis of the salty-fermented fish paste products of Thailand and Laos. *Bioscience of Microbiota, Food and Health* 34(2): 45-52. McCarty, C. (1998). *CHROMASPRO 1.34: Free Program.* Available at: http://www.technelysium.com.au/chromas.html.

McCoy, C.O. and Matsen, F.A. (2013). 4th. Abundance-weighted phylogenetic diversity measures distinguish microbial community states and are robust to sampling depth. *Peer J*. 1: e157.doi: 10.7717/peerj.157

McDevitt, S. (2009). Methyl Red and Voges-Proskauer Test Protocols. *American Society for Microbiology*. In: *American Society for Microbiology*. Washington, DC. Available at: https://www.asmscience.org/content/education/protocol/ protocol. 3204.

McDonald, D. Clemente, J.C., Kuczynski, J., Rideout, J.R., Stombaugh, J., Wendel, D. and Knight, R. (2012b). The Biological Observation Matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome. *Giga Science* 1(1): 2047-217X.doi.org/10.1186/2047-217X-1-7

McDonald, D. Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A. and Hugenholtz, P. (2012a). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME Journal*. 6: 610.doi: 10.1038/ismej.2011.139.

McDowell RH, Sands EM, Friedman H. *Bacillus Cereus*. (2019). In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing, USA. Available from: https://www.ncbi.nlm.nih.gov/books/NBK459121/.

McMurdie, P.J. and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8: e61217. doi.org/10.1371/journal.pone.0061217.

McNally, C.P., Eng, A., Noecker, C., Gagne-Maynard, W.C. and Borenstein E. (2018). BURRITO: An interactive multi-omic tool for visualizing taxa–function relationships in microbiome data. *Frontiers in Microbiology* 9: 365. doi:10.3389/fmicb.2018.00365.

Mei, J., Ma, X. and Xie, J. (2019). Review on natural preservatives for extending fish shelf life. *Foods* 8(10):490. doi:10.3390/foods8100490.

Mejía, A., Luna, D., Fernández, F.J., Barrios-González, J., Gutierrez, L.H., Reyes, A.G. and Kelly, S. (2018). Improving rifamycin production in *Amycolatopsis mediterranei* by expressing a *Vitreoscilla hemoglobin* (vhb) gene fused to a cytochrome P450 monooxygenase domain. *3 Biotech* 8(11): 456.doi: 10.1007/s13205-018-1472-z

Messaoudi, A., Gtari, M., Boudabous, A. and Wagenlehner, F. (2009). Identification and susceptibility of *Klebsiella* and *Enterobacter* spp. isolated from meat products. *American Journal of Microbiological Research* 37(7): 362–369.

Mhango, M., Mpuchane, S.F. and Mpuchane, B.A. (2010). Incidence of indicator organisms, opportunistic and pathogenic bacteria in fish. *African Journal of Food, Agriculture, Nutrition and Development* 10(10): 4203-4218.

Miranda, C.D. and Zemelman, R. (2002). Antimicrobial multi resistance in bacteria isolated from freshwater Chilean salmon farms. *Science of the Total Environment* 293(1-3): 207-218.

Mokoena, M.P., Mutanda, T. and Olaniran, A.O. (2016). Perspectives on the probiotic potential of lactic acid bacteria from African traditional fermented foods and beverages. *Food and Nutrition Research* 60(1): 29630.doi: 10.3402/fnr.v60.29630.

Mokrani, T., Essid, I., Hassouna, M., Jihene, L. and Abdeljalil, G. (2018). Phenotypic and genotypic characterization of *Carnobacterium* isolated from refrigerated

Tunisian minced raw beef meat. *Gene Technology* 7:144).doi:10.4172/2329-6682.1000144.

Moon, H.J., Min, K.J., Park, N.Y., Park, H.J. and Yoon, K.S. (2017). Survival of *Staphylococcus aureus* in dried fish products as a function of temperature. *Food Science and Biotechnology* 26(3): 823-828.

Morales-López, S., Yepes, J.A., Prada-Herrera, J.C. and Torres-Jiménez, A. (2019). Enterobacteria in the 21st century: a review focused on taxonomic changes. *Journal of Infection in Developing Countries* 13: 265-273. doi: 10.3855/jidc.11216.

Møretrø, T. and Langsrud, S. (2017). Residential bacteria on surfaces in the food industry and their implications for food safety and quality. *Comprehensive Reviews in Food Science and Food Safety* 16(5): 1022-1041.

Morot-Bizot, S. C., Leroy, S., & Talon, R. (2006). Staphylococcal community of a small unit manufacturing traditional dry fermented sausages. *International Journal of Food Microbiology* 108(2): 210-217.

Moura, A.T., Saraiva, C., Miguéis, S., Esteves, A. and Fontes, M.C. (2017). Antimicrobial susceptibility of coagulase-positive and coagulase-negative staphylococci in ready-to-eat *sashimi*. *Journal of Aquatic Food Product Technology* 26(1): 95-102.

Mu, Y., Su, W., Mu, Y. and Jiang, L. (2020). Combined application of high-throughput sequencing and metabolomics reveals metabolically active microorganisms during Panxian ham processing. *Frontiers in Microbiology* 10:3 012.doi:10.3389/fmicb.2019.03012.

Mudgil, D., Baskar, S., Baskar, R., Paul, D. and Shouche, Y.S. (2018). Biomineralization potential of *Bacillus subtilis*, *Rummeliibacillus stabekisii* and *Staphylococcus epidermidis* strains in vitro isolated from Speleothems, Khasi Hill

Caves, Meghalaya, India. *Geomicrobiology Journal* 35(8): 675-694.

Murugkar, H.V., Rahman, H. and Dutta, P.K. (2003). Distribution of virulence genes in Salmonella serovars isolated from man and animals. *Indian Journal of Medical Research* 117: 66-70.

Muzaddadi, A.U. (2015). Minimisation of fermentation period of *shidal* from barbs (*Puntius* spp.). *Fishery Technology* 52: 34-41.

Muzaddadi, A.U. and Basu, S. (2003). Seedal-an indigenous fermented fishery product of North-east India. *Fishery Chemistry* 23: 30-32.

Muzaddadi, A.U. and Basu, S. (2012). An accelerated process for fermented fish (seedal) production in North-east region of India. *Indian Journal of Animal Sci*ence 82: 98-106.

Mwakinyali, S.E., Ming, Z., Xie, H., Zhang, Q. and Li, P. (2019). Investigation and characterization of *Myroides odoratimimus* strain 3J2MO aflatoxin B1 Degradation. *Journal of Agriculture and Food Chemistry* 67(16): 4595-4602.

Naidoo, K. and Lindsay, D. (2010). Survival of *Listeria monocytogenes*, and enterotoxin-producing *Staphylococcus aureus* and *Staphylococcus pasteuri*, during two types of *biltong* manufacturing processes. *Food Control* 21(7): 1042-1050.

Naik, O.A., Shashidhar, R., Rath, D., Bandekar, J.R. and Rath, A. (2018). Characterization of multiple antibiotic resistance of culturable microorganisms and metagenomic analysis of total microbial diversity of marine fish sold in retail shops in Mumbai, India. *Environmental Science and Pollution Research* 25(7): 6228-6239.

Nakamura, S., Kuda, T., An, C., Kanno, T., Takahashi, H. and Kimura, B. (2012). Inhibitory effects of *Leuconostoc mesenteroides* 1RM3 isolated from *narezushi*, a

fermented fish with rice, on *Listeria monocytogenes* infection to Caco-2 cells and A/J mice. *Anaerobe* 18(1): 19-24.

Nayak, R., Stewart, T., Wang, R.F., Lin, J., Cerniglia, C. E. and Kenney, P.B. (2004). Genetic diversity and virulence gene determinants of antibiotic-resistant *Salmonella* isolated from preharvest turkey production sources. *International Journal of Food Microbiology* 91(1): 51-62.

Nearing, J.T., Douglas, G.M., Comeau, A.M. and Langille, M. (2018). Denoising the Denoisers: an independent evaluation of microbiome sequence error-correction approaches. *Peer Journal* 6:e5364.doi:10.7717/peerj.5364.

Negus, D., Moore, C., Baker, M., Raghunathan, D., Tyson, J. and Sockett, R.E. (2017). Predator versus pathogen: how does predatory *Bdellovibrio bacteriovorus* interface with the challenges of killing Gram-negative pathogens in a host setting?. *Annual Review of Microbiology* 71: 441-457.

Nelson, M.T., LaBudde, R.A., Tomasino, S.F., Pines, R.M., Bennett, M., Dormstetter, K., Eastman, T., Hellickson, L., Hollingsworth, A., Juhnke, A., Klein, D., Mitchell, J., Otte, M., Pines, R., Schwarz, S. and Tomasino, S. (2013). Comparison of 3M™ petrifilm™ aerobic count plates to standard plating methodology for use with AOAC antimicrobial efficacy methods 955.14, 955.15, 964.02, and 966.04 as an alternative enumeration procedure: collaborative study. *Journal of AOAC International* 96(4): 717-722.

Nguyen, D.T.L., Van Hoorde, K., Cnockaert, M., De Brandt, E., De Bruyne, K., Le, B.T. and Vandamme, P. (2013). A culture-dependent and-independent approach for the identification of lactic acid bacteria associated with the production of *nem chua*, a Vietnamese fermented meat product. *Food Research International* 50(1): 232-240.

Nguyen, N.P., Warnow, T., Pop, M. and White, B. (2016). A perspective on 16S rRNA operational taxonomic unit clustering using sequence similarity. *NPJ Biofilms and Microbiomes* 2:16004.doi.org/10.1038/npjbiofilms.2016.4.

Nguyen, P.Y., Silva, A.F., Reis, A.C., Nunes, O.C., Rodrigues, A.M., Rodrigues, J.E. and Carvalho, G. (2019). Bioaugmentation of membrane bioreactor with *Achromobacter denitrificans* strain PR1 for enhanced sulfamethoxazole removal in wastewater. *Science of the Total Environment* 648: 44-55.

Ni, Y., Li, Z., Sun, Z., Zheng, P., Liu, Y., Zhu, L. and Schwaneberg, U. (2009). Expression of arginine deiminase from *Pseudomonas plecoglossicida* CGMCC2039 in *Escherichia coli* and its anti-tumor activity. *Current Microbiology* 58(6): 593-598.

Nie, X., Lin, S. and Zhang, Q. (2014). Proteolytic characterisation in grass carp sausage inoculated with *Lactobacillus plantarum* and *Pediococcus pentosaceus*. *Food Chemistry* 145: 840-844.

Nishimori, E., Kita-Tsukamoto, K. and Wakabayashi, H. (2000). *Pseudomonas plecoglossicida* sp. nov., the causative agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis*. *International Journal of Systematic and Evolutionary Microbiology* 50(1): 83-89.

Nummer, B.A., Harrison, J.A., Harrison, M.A., Kendall, P., Sofos, J.N. and Elizabeth L. Andress, E.L. (2004). Effects of preparation methods on the microbiological safety of home-dried meat jerky. *Journal of Food Protection* 67: 2337-2341.

Nummer, B.A., Shrestha, S. and Smith, J.V. (2012). Survival of Salmonella in a high sugar, low water-activity, peanut butter flavored candy fondant. *Food Control* 27(1): 184-187.

Nygaard, M.E. (2019). Swedish fermented herring as a marker of rural identity: the Alfta surströmmingsskiva. *Food, Culture and Society* 22(4): 407-422.

Ohshima, C., Takahashi, H., Insang, S., Phraephaisarn, C., Techaruvichit, P., Khumthong, R. and Keeratipibul, S. (2019). Next-generation sequencing reveals predominant bacterial communities during fermentation of Thai fish sauce in large manufacturing plants. *LWT-Food Science and Technology* 114: 108375. doi: 10.1016/j.lwt.2019.108375.

Ojha, K.S., Harrison, S.M., Brunton, N.P., Kerry, J.P. and Tiwari, B.K. (2017). Statistical approaches to access the effect of *Lactobacillus sakei* culture and ultrasound frequency on fatty acid profile of beef jerky. *Journal of Food Composition and Analysis* 57: 1-7.

Oki, K., Kudo,Y. and Watanabe, K. (2012). *Lactobacillus saniviri* sp. nov. and *Lactobacillus senioris* sp.nov., isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology* 62:601–607. doi:10.1099/ijs.0.031658-0.

Oki, K., Rai, A.K., Sato, S., Watanabe, K. and Tamang, J.P. (2011). Lactic acid bacteria isolated from ethnic preserved meat products of the Western Himalayas. *Food Microbiology* 28: 1308-1315.

Olaleye, O. and Abegunde, T. (2015). Microbiological safety assessment of selected smoked fish in Lagos Metropolis. *British Microbiology Research Journal* 9(3): 1-5.

Olgunoglu IA: *Salmonella* in Fish and Fishery Products. (2012) In: *Salmonella* - A Dangerous Foodborne Pathogen. (Ed: Mahmoud, S.M.). IntechOpen, United Kingdom.

Olympia, M., Fukuda, H., Ono, H., Kaneko, Y. and Takano, M. (1995). Characterization of starch-hydrolyzing lactic acid bacteria isolated from a

fermented fish and rice food, "*Burong Isda*", and its amylolytic enzyme. *Journal of Fermentation and Bioengineering* 80(2): 124-130.

Olympia, M., Ono, H., Shinmyo, A. and Takano, M. (1992). Lactic acid bacteria in fermented fishery product, "burong bangus". *Journal of Fermentation and Bioengineering* 73(3): 193-197.

Onmaz, N.E., Abay, S., Karadal, F., Hizlisoy, H., Telli, N and Al, S. (2015). Occurrence and antimicrobial resistance of *Staphylococcus aureus* and *Salmonella* spp. in retail fish samples in Turkey. *Marine Pollution Bulletin* 90(1-2): 242-246.

Ono, H.K., Sato'o, Y., Narita, K., Naito, I., Hirose, S., Hisatsune, J., Asano, K., Hu, D.L., Omoe, K., Sugai, M. and Nakane, A. (2015). Identification and characterization of a novel Staphylococcal emetic toxin. *Applied and Environmental Microbiology* 81(20): 7034-7040.

Opazo-Capurro, A.F., Higgins, P.G., Wille, J., Seifert, H., Cigarroa, C., González-Muñoz, P. and González-Rocha, G.E. (2019). Genetic features of antarctic *Acinetobacter radioresistens* strain A154 harbouring multiple antibiotic-resistance genes. *Frontiers in Cellular and Infection Microbiology* 9: 328. doi. 10.3389/fcimb.2019.00328.

Ordóñez, J.L., Troncoso, A.M., García-Parrilla, M.D.C. and Callejón, R.M. (2016). Recent trends in the determination of biogenic amines in fermented beverages–A review. *Analytic Chimica Acta* 939: 10-25.

Ortiz-Estrada, Á.M., Gollas-Galván, T., Martínez-Córdova, L.R. and Martínez-Porchas, M. (2019). Predictive functional profiles using metagenomic 16S rRNA data: a novel approach to understanding the microbial ecology of aquaculture systems. *Reviews in Aquaculture* 11(1): 234–235.

Osei Sekyere, J. (2018). Genomic insights into nitrofurantoin resistance mechanisms and epidemiology in clinical Enterobacteriaceae. *Future Science OA* 4(5): FSO293. doi:org/10.4155/fsoa-2017-0156.

Osimani, A., Aquilanti, L. and Clementi, F. (2018). *Bacillus cereus* foodborne outbreaks in mass catering. *International Journal of Hospitality Management* 72: 145-153.

Ottaviani, D., Pieralisi, S., Angelico, G., Mosca, F., Tiscar, P.G., Rocchegiani, E., Scuota, S., Petruzzelli, A., Fisichella, S., Blasi, G., DiRaimo, E., Leoni, F., Latini, M., Altissimi, S. and Haouet, N. (2020). *Bdellovibrio bacteriovorus* to control *Escherichia coli* on meat matrices. *International Journal of Food Science and Technology* 55: 988-994.

Ouoba, L.I.I., Thorsen, L. and Varnam, A.H. (2008). Enterotoxins and emetic toxins production by *Bacillus cereus* and other species of *Bacillu*s isolated from *Soumbala* and *Bikalga,* African alkaline fermented food condiments. *International Journal of Food Microbiology* 124(3): 224-230.

Panea, B. and Ripoll, G. (2018). Quality and safety of meat products. *Foods* 7(8): 118. doi.org/10.3390/foods7080118.

Papamanoli, E., Kotzekidou, P., Tzanetakis, N. and Litopoulou-Tzanetaki, E. (2002). Characterization of Micrococcaceae isolated from dry fermented sausage. *Food Microbiology* 19(5): 441-449.

Papamanoli, E., Tzanetakis, N., Litopoulou-Tzanetaki, E. and Kotzekidou, P. (2003). Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. *Meat Science* 65(2): 859-867.

Paparella, A., Serio, A., Rossi, C., Mazzarrino, G. and López, C.C. (2018). Food-Borne Transmission of Staphylococci. In: *Pet-To-Man Travelling Staphylococci* (Ed. Savini, V.), pp. 71-94. Academic Press, Elsevier, Cambridge.

Parente, E., Grieco, S. and Crudele, M.A. (2001). Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in Basilicata (Southern Italy). *Journal of Applied Microbiology* 90(6): 943-952.

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

Pasini, F., Soglia, F., Petracci, M., Caboni, M. F., Marziali, S., Montanari, C., Gardini, F., Grazia, L. and Tabanelli, G. (2018). Effect of fermentation with different lactic acid bacteria starter cultures on biogenic amine content and ripening patterns in dry fermented sausages. *Nutrients* 10(10): 1497. doi.org/10.3390/nu10101497.

Patel, J. B., Cockerill III, F., Eliopoulos, G., Jenkins, S., Lewis, J., Limbago, B. and Nicolau, D. (2017). M100 Performance standards for antimicrobial susceptibility testing. In: *Clinical and Laboratory Standards Institute* 240. 27th edition. United States.

Patterson, J., Kailasam, S., Giftson, H. and Immaculate, J.K. (2018). Effect of drying technologies on the biochemical properties of *Stolephorus commersonnii*. *Food Quality and Safety* 2(3): 153–158.

Pavan, A.C.L., Nakadomari, G.H., Vignoto, V.K.C., Sfaciotte, R.A.P., Melo, F.D., Ferraz, S.M. and Wosiacki, S.R. (2019). Enterotoxin and slime gene detection in *Staphylococcus coagulase* negative isolated from ground beef. *Revista De Ciência Veterinária E Saúde Pública* 6(1): 30-40.

Pederson, C.S. (1979). *Microbiology of Food Fermentations*, 2nd edition. AVI Publishing Company, Westport.

Perea-Sanz, L., Montero, R., Belloch, C. and Flores, M. (2019). Microbial changes and aroma profile of nitrate reduced dry sausages during vacuum storage. *Meat Science* 147: 100-107.

Pereira, P.M.D.C.C. and Vicente, A.F.D.R.B. (2013). Meat nutritional composition and nutritive role in the human diet. *Meat Science* 93(3): 586-592.

Perez-Montano, J. A., Gonzalez-Aguilar, D., Barba, J., Pacheco-Gallardo, C., Campos-Bravo, C. A., Garcia, S. and Cabrera-Diaz, E. (2012). Frequency and antimicrobial resistance of Salmonella serotypes on beef carcasses at small abattoirs in Jalisco State, Mexico. *Journal of Food Protection* 75(5): 867-873.

Pessione, E. and Cirrincione, S. (2016). Bioactive molecules released in food by lactic acid bacteria: encrypted peptides and biogenic amines. *Frontiers in Microbiology* 7: 876. doi: 10.3389/fmicb.2016.008762016.

Petit, T., Caro, Y., Petit, A. S., Santchurn, S. J. and Collignan, A. (2014). Physicochemical and microbiological characteristics of *biltong*, a traditional salted dried meat of South Africa. *Meat Science* 96(3): 1313-1317.

Pidcock, K., Heard, G. M. and Henriksson, A. (2002). Application of nontraditional meat starter cultures in production of Hungarian salami. *International Journal of Food Microbiology* 76(1-2): 75-81.

Pinto, M.F., Ponsano, E.H.G., Franco, B.D.G.M. and Shimokomaki, M. (2002). *Charqui* meats as fermented meat products: role of bacteria for some sensorial properties development. *Meat Science* 61: 187–191.

Plavsic, D., Okanovic, D., Gubic, J. and Njezic, Z. (2015). Microbiological and chemical evaluation of dried smoked meat product. *Procedia Food Science* 5: 239-242.

Podpečan, B., Pengov, A. and Vadnjal, S. (2007). The source of contamination of ground meat for production of meat products with bacteria *Staphylococcus aureus*. *Slovenian Veterinary Research* 44: 25-30.

Poirel, L., Figueiredo, S., Cattoir, V., Carattoli, A. and Nordmann, P. (2008). *Acinetobacter radioresistens* as a silent source of carbapenem resistance for *Acinetobacter* spp. *Antimicrobial Agents and Chemotherapy* 52(4): 1252-1256.

Poirier, S., Rué, O., Peguilhan, R., Coeuret, G., Zagorec, M., Champomier-Vergès, M.C., Loux, V. and Chaillou, S. (2018). Deciphering intra-species bacterial diversity of meat and seafood spoilage microbiota using *gyrB* amplicon sequencing: a comparative analysis with 16S rDNA V3-V4 amplicon sequencing. *PLoS ONE* 13(9): e0204629. doi.org/10.1371/journal.pone.0204629.

Połka, J., Rebecchi, A., Pisacane, V., Morelli, L. and Puglisi, E. (2015). Bacterial diversity in typical Italian salami at different ripening stages as revealed by high-throughput sequencing of 16S rRNA amplicons. *Food Microbiology* 46: 342-356.

Price, M.N., Dehal, P.S. and Arkin, A.P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PloS ONE* 5(3): e9490. doi.org/10.1371/journal.pone.0009490.

Pringsulaka, O., Thongngam, N., Suwannasai, N., Atthakor, W., Pothivejkul, K. and Rangsiruji, A. (2012). Partial characterization of bacteriocins produced by lactic acid bacteria isolated from Thai fermented meat and fish products. *Food Control* 23(2):547-551.

Priyanka, B., Patil, R.K. and Dwarakanath, S. (2016). A review on detection methods used for foodborne pathogens. *Indian Journal of Medical Research* 144(3): 327–338.

Quaglia, N.C., Storelli, M., Ioanna, F., Celano, G., Celano, G.V., Conversano, C. and Dambrosio, A. (2019). *Listeria monocytogenes* and enterotoxigenic *Staphylococcus aureus* in dry fermented sausages belonging to "Traditional Agri-Food Product" produced in Southern Italy. *Journal of Food Safety* 39(6): e12685.doi.10.1111/jfs.12685.

Quijada, N.M., De Filippis, F., Sanz, J.J., del Camino Garcia-Fernandez, M., Rodríguez-Lázaro, D., Ercolini, D. and Hernández, M. (2018). Different *Lactobacillus* populations dominate in "*Chorizo de León*" manufacturing performed in different production plants. *Food Microbiology* 70: 94-102.

Quijada, N.M., De Filippis, F., Sanz, J.J., del Camino Garcia-Fernandez, M., Rodríguez-Lázaro, D., Ercolini, D. and Hernández, M. (2018). Different *Lactobacillus* populations dominate in "*Chorizo de León*" manufacturing performed in different production plants. *Food Microbiology* 70: 94-102.

Rahmati, T. and Labbe, R. (2008). Levels and toxigenicity of *Bacillus cereus* and *Clostridium perfringens* from retail seafood. *Journal of Food Protection* 71(6): 1178-1185.

Rai, A.K., Palni, U. and Tamang, J.P. (2009). Traditional knowledge of the Himalayan people on production of indigenous meat products. *Indian Journal of Traditional Knowledge* 8 (1): 104-109.

Rai, A.K., Tamang, J.P. and Palni, U. (2010a). Microbiological studies of ethnic meat products of the Eastern Himalayas. *Meat Science* 85(3): 560-567.

Rai, A.K., Tamang, J.P. and Palni, U. (2010b). Nutritional value of lesser-known ethnic meat products of the Himalayas. *Journal of Hill Research* 23 (1&2): 22-25.

Ramkumar, R., Ravi, M., Jayaseelan, C., Rahuman, A.A., Anandhi, M., Rajthilak, C. and Perumal, P. (2014). Description of *Providencia vermicola* isolated from

diseased Indian major carp, *Labeo rohita* (Hamilton, 1822). *Aquaculture* 420: 193-197.

Rapsang, G.F. and Joshi, S.R. (2012). Bacterial diversity associated with *tungtap*, an ethnic traditionally fermented fish product of Meghalaya. *Indian Journal of Traditional Knowledge* 11: 134-138.

Rapsang, G.F., Kumar, R. and Joshi, S.R. (2011). Identification of *Lactobacillus puhozihii* from tungtap: A traditionally fermented fish food, and analysis of its bacteriocinogenic potential. *African Journal of Biotechnology* 10: 12237-12243.

Rather, M.A., Aulakh, R.S., Gill, J.P.S. and Ghatak, S. (2012). Enterotoxin gene profile and antibiogram of *Bacillus cereus* strains isolated from raw meats and meat products. *Journal of Food Safety* 32(1): 22-28.

Ratsimba, A., Leroy, S., Chacornac, J.P., Rakoto, D., Arnaud, E., Jeannoda, V. and Talon, R. (2017). Staphylococcal ecosystem of *kitoza*, a traditional Malagasy meat product. *International Journal of Food Microbiology* 246: 20-24.

Ray, A.K., Bairagi, A., Ghosh, K.S. and Sen, S.K. (2007). Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. *Acta Ichthyologica et Piscatoria* 1(37): 47-53.

Ray, B. and Bhunia, A. (2007). *Fundamental Food Microbiology*. CRC Press, Taylor & Francis Group, New York. ISBN:13:978-1-4200-0774-9.

Reiner, K. (2010). Catalase Test Protocol. In: *American Society for Microbiology*. Washington, D.C. Available at: https://www.asmscience.org/content/ education/ protocol/protocol.3226.

Reiner, K. (2012). Carbohydrate Fermentation Protocol. In: *American Society for Microbiology*. Washington, DC. Available at: https://www.asmscience.org/ content/education/protocol/protocol.3779.

Reller, L.B., Weinstein, M., Jorgensen, J.H. and Ferraro, M.J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases* 49(11): 1749-1755.

Resch, M., Nagel, V. and Hertel, C. (2008). Antibiotic resistance of coagulase-negative staphylococci associated with food and used in starter cultures. *International Journal of Food Microbiology* 127(1-2): 99-104.

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.

Ricke, S.C., Diaz, I.Z. and Keeton, J.T. (2007). Fermented meat, poultry, and fish products. In: *Food Microbiology: Fundamentals and Frontiers,* pp. 795-815. *American Society of Microbiology*. Washington D.C.

Riddervold, A. and Meling, L. (1990). The Importance of Herring in the Daily Life 187 of the Coastal Population of Norway. In *Oxford Symposium on Food & Cookery, 1989: Staplefoods: Proceedings*, pp. 187. Oxford Symposium.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *Peer J* 4: e2584.doi: 10.7717/peerj.2584.

Roh, S., Kim, K., Nam, Y., Chang, H.W., Park, E.J. and Bae, J.W. (2010). Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. *The ISME Journal* 4: 1–16.

Ryan, K.J. and Ray, C.G. (2004). *Sherris Medical Microbiology: An Introduction To Infectious Diseases* 4th Edition. McGraw- Hill. New York.

Sabry, M., Abd El-Moein, K., Hamza, E. and Abdel Kader, F. (2016). Occurrence of *Clostridium perfringens* types A, E, and C in fresh fish and its public health significance. *Journal of Food Protection* 79(6): 994-1000.

Said, L.B., Gaudreau, H., Dallaire, L., Tessier, M. and Fliss, I. (2019). Bioprotective culture: a new generation of food additives for the preservation of food quality and safety. *Industrial Biotechnology* 15(3): 138-147.

Saithong, P., Panthavee, W., Boonyaratanakornkit, M. and Sikkhamondhol, C. (2010). Use of a starter culture of lactic acid bacteria in *plaa-som*, a Thai fermented fish. *Journal of Bioscience and Bioengineering* 110: 553–557.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4(4): 406-425.

Salampessy, J., Kailasapathy, K., and Thapa, N. (2010). Fermented fish products. *Fermented Foods and Beverages of the World* 289-307.

Saleh-Lakha, S., Leon-Velarde, C.G., Chen, S., Lee, S., Shannon, K., Fabri, M. and Keown, B. (2017). A study to assess the numbers and prevalence of Bacillus cereus and its toxins in pasteurized fluid milk. *Journal of Food Protection* 80(7): 1085-1089.

Salem, A., Mostafa, E. M. and Hassan, E.A. (2015). Incidence of Some Food Poisoning Microorganisms in Salted fish. *Benha Veterinary Medical Journal* 29(2): 225-229.

Salter, A.M. (2017). Improving the sustainability of global meat and milk production. *Proceedings of the Nutrition Society* 76(1): 22-27.

Sánchez, M.M., Stavropoulou, D.A. and Leroy, F. (2017). Exploring the metabolic heterogeneity of coagulase-negative staphylococci to improve the quality and

safety of fermented meats: a review. *International Journal of Food Microbiology* 247: 24-37. doi: 10.1016/j.ijfoodmicro.2016.05.021.

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

Sanpa, S., Sanpa, S. and Suttajit, M. (2019). Lactic acid bacteria isolates from Pla-som, their antimicrobial activities and fermentation properties in *Pla-som*. *Journal of Food Health and Bioenvironmental Science* 12(1): 57-71.

Santiyanont, P., Chantarasakha, K., Tepkasikul, P., Srimarut, Y., Mhuantong, W., Tangphatsornruang, S. and Chokesajjawatee, N. (2019). Dynamics of biogenic amines and bacterial communities in a Thai fermented pork product *Nham*. *Food Research International* 119: 110-118.

Sarkadi, L.S. (2017). Biogenic amines in fermented foods and health implications. In: *Fermented foods in Health and Disease Prevention,* pp. 625-651. Academic Press.

Satomi, M., Kimura, B., Takahashi, G. and Fujii, T. (1997). Microbial diversity in Kusaya gravy. *Fisheries Science* 63(6): 1019-1023.

Sautour, M., Mary, P., Chihib, N.E. and Hornez, J. (2003). The effects of temperature, water activity and ph on the growth of *Aeromonas* hydrophila and on its subsequent survival in microcosm water. *Journal of Applied Microbiology* 95: 807-13.

Scala, G., Serra, A., Marwah, V.S., Saarimäki, L.A. and Greco, D. (2019). FunMappOne: a tool to hierarchically organize and visually navigate functional gene annotations in multiple experiments. *BMC Bioinformatics* 20: 79. doi.org/10.1186/s12859-019-2639-2.

Schierack, P., Walk, N., Reiter, K., Weyrauch, K. and Wieler, L. (2007). Composition of intestinal Enterobacteriaceae populations of healthy domestic pigs. *Microbiology* 153:3830-7. doi.10.1099/mic.0.2007/010173-0.

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

Sebastian-Leon, P., Vidal, E., Minguez, P., Conesa, A., Tarazona, S., Amadoz, A. and Dopazo, J. (2014). Understanding disease mechanisms with models of signaling pathway activities. *BMC Systems Biology* 8(1): 121. doi:10.1186/s12918-014-0121-3.

Sekhar, M.S., Sharif, N.M., and Rao, T.S. (2017). Serotypes of sorbitol-positive shiga toxigenic *Escherichia coli* (SP-STEC) isolated from freshwater fish. *International Journal of Fisheries and Aquatic Studies* 5(3): 503-505.

Shangpliang, H.N.K., 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.

Shatzkes, K., Tang, C., Singleton, E., Shukla, S., Zuena, M., Gupta, S. and Kadouri, D.E. (2017). Effect of predatory bacteria on the gut bacterial microbiota in rats. *Scientific Reports* 7(1): 1-12.

Shields, P. and Cathcart, L. (2011). Motility Test Medium Protocol. In: *American Society for Microbiology*. Washington, D.C. Available at: https://www.asmscience.org/content/education/protocol/protocol.3658.

Shimokomaki, M., Garcia, C.E.R., Pedrão, M.R. and Coró, F.A.G. (2016). Brazilian Charqui Meats. In: *Traditional Foods*, pp. 291-294. Springer, Boston, MA.

Silva, B.N., Cadavez, V., Pires, P., Dias, T., and Gonzales-Barron, U. (2018). Microbiological safety of Portuguese dry-fermented chouriço sausages as affected by processing and physicochemical factors. *Madridge Journal of Food Technology* 3(2): 138-149.

Simon, S.S. and Sanjeev, S. (2007). Prevalence of enterotoxigenic *Staphylococcus aureus* in fishery products and fish processing factory workers. *Food Control* 18(12): 1565-1568.

Sims, D., Sudbery, I., Ilott, N.E., Heger, A. and Ponting, C.P. (2014). Sequencing depth and coverage: key considerations in genomic analyses. *Nature Reviews Genetics* 15(2): 121-132.

Singh, S.S., Mandal, S.E., Lalnunmawii, E. and Kumar, N.S. (2018). Antimicrobial, antioxidant and probiotics characterization of dominant bacterial isolates from traditional fermented fish of Manipur, North-East India. *Journal of Food Science and Technology* 55:1870-1879.

Skåra, T., Axelsson, L., Stefánsson, G., Ekstrand, B. and Hagen, H. (2015). Fermented and ripened fish products in the northern European countries. *Journal of Ethnic Foods* 2(1): 18–24.

Smith, A.C. and Hussey, M.A. (2005). Gram Stain Protocols. In: *American Society for Microbiology.* Washington D.C. Available at: https://www.asmscience.org/ content/education/ protocol/protocol.2886.

Soleimani, M., Hosseini, H., Pilevar, Z., Mehdizadeh, M. and Carlin, F. (2018). Prevalence, molecular identification and characterization of *Bacillus cereus* isolated from beef burgers. *Journal of Food Safety* 38(1): doi:10.1111/jfs.12414.

Solidum, J.N., Chua, R.A., Apostol, K.I., Belmonte, J.K., Pedron, R. and Pura, A.N. (2013). Quantitative Determination of Cadmium and Chromium Levels in Selected

Meat and Meat Products from Paco Wet Market, Manila, Philippines. *International Journal* of *Chemical and Environmental Engineering* 4(3): 174-176.

Soltani, M., Ghosh, K., Hoseinifar, S.H., Kumar, V., Lymbery, A.J., Roy, S. and Ringo, E. (2019). Genus *Bacillus*, promising probiotics in aquaculture: aquatic animal origin, bio-active components, bioremediation and efficacy in fish and shellfish. *Reviews in Fisheries Sciences and Aquaculture* 27(3): 331-379.

Soncu, E. D., Arslan, B., Ertürk, D., Küçükkaya, S., Özdemir, N. and Soyer, A. (2018). Microbiological, physicochemical and sensory characteristics of Turkish fermented sausages (*sucuk*) coated with chitosan-essential oils. *Food Science and Technology* 97: 198-204. doi:10.1016/j.lwt.2018.06.049.

Song, E.J., Lee, E.S., Park, S.L., Choi, H.J., Roh, S.W. and Nam, Y.D. (2018). Bacterial community analysis in three types of the fermented seafood, *jeotgal*, produced in South Korea. *Bioscience, Biotechnology and Biochemistry* 82 (8): 1444-1454.

Soon, W.W., Hariharan, M. and Snyder, M.P. (2013). High-throughput sequencing for biology and medicine. *Molecular Systems Biology* 9: 640.doi: 10.1038/msb.2012.61.

Sørensen, S., Markedal, K.E. and Sørensen, J.C. (2018). Food, Nutrition, and Health in Denmark (Including Greenland and Faroe Islands). In: *Nutritional and Health Aspects of Food in Nordic Countries*, pp. 99-125. Academic Press.

Souza, C.D.F., Baldissera, M.D., Verdi, C.M., Santos, R.C., Da Rocha, M.I.U., da Veiga, M.L., and Baldisserotto, B. (2019). Oxidative stress and antioxidant responses in Nile tilapia *Oreochromis niloticus* experimentally infected by *Providencia rettgeri. Microbial Pathogenesis* 131: 164-169.

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

Stellato, G., La Storia, A., De Filippis, F., Borriello, G., Villani, F. and Ercolini, D. (2016). Overlap of spoilage-associated microbiota between meat and the meat processing environment in small-scale and large-scale retail distributions. *Applied and Environmental Microbiology* 82(13): 4045-4054.

Stellato, G., Utter, D.R., Voorhis, A., De Angelis, M., Eren, A.M. and Ercolini, D. (2017). A few *Pseudomonas* oligotypes dominate in the meat and dairy processing environment. *Frontiers in Microbiology* 8:264. doi:10.3389/fmicb.2017.00264.

Stromberg, Z.R., Van Goor, A., Redweik, G.A., Brand, M.J.W., Wannemuehler, M.J. and Mellata, M. (2018). Pathogenic and non-pathogenic *Escherichia coli* colonization and host inflammatory response in a defined microbiota mouse model. *Disease Models and Mechanisms* 11(11). doi:10.1242/dmm.035063.

Su, Y.C., and Liu, C. (2007). Vibrio parahaemolyticus: a concern of seafood safety. *Food microbiology* 24(6): 549-558.

Tallent, S., Hait, J. and Bennett, R. (2015), Analysis of *Bacillus cereus* toxicity using PCR, ELISA and a lateral flow device. *Journal of Applied Microbiology* 118: 1068-1075.

Tallent, S., Hait, Jennifer., Bennett R.W. and Lancette G.A. (2016). Chapter 12. *Staphylococcus aureus.* In: *Bacteriological Analytical Manual*. U.S. Food and Drug Administration. Maryland, United States.

Talon, R. and Leroy, S. (2006). Latest developments in meat bacterial starters. In: *Advanced Technologies for Meat Processing* (Eds: Nollet, L.M.L. and Toldra, F), pp. 401–418. CRC Press, Taylor & Francis Group, New York.

Talon, R., Leroy-Setrin, S. and Fadda, S. (2004). Dry fermented sausages. Chapter 23. In: Handbook of Food and Beverage Fermentation Technology (Eds: Hui, Y.H.L. Meunier-Goddik, A. S. Hansen et al.) pp. 397–416. New York: Marcel Dekker Inc.

Talon, R., Leroy-Sétrin, S. and Fadda, S. (2003). Dry fermented sausages. In: Hui, Y.H., et al. (Ed.), Handbook of Food and Beverage Fermentation Technology. Marcel Dekker Inc., New York, pp. 397–416.

Tamang, J.P. (2005). *Food Culture of Sikkim*. Sikkim Study Series volume IV. Information and Public Relations Department, Government of Sikkim, Gangtok, p. 120.

Tamang, J.P. (2010a). Diversity of fermented foods. In: *Fermented Foods and Beverages of the World* (Eds: Tamang, J.P. and Kailasapathy, K), pp. 41-84. CRC Press, Taylor & Francis Group, New York.

Tamang, J.P. (2010b). *Himalayan Fermented Foods: Microbiology, Nutrition, and Ethnic Values*. CRC Press, Taylor & Francis Group, New York, pages 295. ISBN: 9781420093247.

Tamang, J.P. (2014). Biochemical and modern identification techniques - microfloras of fermented foods. In: *Encyclopaedia of Food Microbiology,* 2nd edition (Eds: Batt, C. and Tortorello, M.A.). Elsevier Ltd., Oxford, pp. 250-258.

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

Tamang, J.P. (2020). History and Culture of Indian Ethnic Fermented Foods and Beverages. In: *Ethnic Fermented Foods and Alcoholic Beverages of India: Science*

History and Culture (Ed: Tamang, J.P.), pp. 1-40. Springer Nature, Singapore. ISBN: 978-981-15-1486-9.

Tamang, J.P. and Samuel, D. (2010). Dietary culture and antiquity of fermented foods and beverages. In: *Fermented Foods and Beverages of the World* (Eds: Tamang, J.P. and Kailasapathy, K), pp.1–40. CRC Press, Taylor & Francis Group, New York.

Tamang, J.P., Cotter, P., Endo, A., Han, N.S., Kort, R., Liu, S.Q., Mayo, B., Westerik, N., and Hutkins, R. (2020). Fermented foods in a global age: east meets west. *Comprehensive Reviews in Food Science and Food Safety* 19: 184-217. doi: 10.1111/1541-4337.12520.

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

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

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., Thapa, N., Rai, B., Thapa, S., Yonzan, H., Dewan, S., Tamang, B., Sharma, R.M., Rai, A.K., Chettri, R., Mukhopadhyay, B. and Pal, B. (2007). Food consumption in Sikkim with special reference to traditional fermented foods and beverages: a micro-level survey**.** *Journal of Hill Research* 20 (1): 1-37.

Tamang, J.P., Thapa, N., Savitri and Bhalla, T.C. (2016c). Chapter 2. 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. ISBN: 978-81-322-2798-4.

Tamang, P. (2002). Brief note on fish diversity of Teesta and Rangit river system in Sikkim and their conservation. *A Report of State Council of Science and Technology for Sikkim*, Gangtok, pp. 4.

Tamura, K., Nei, M. and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* 101(30): 11030-11035.

Tan, H.Y., Chen, S.W. and Hu, S.Y. (2019). Improvements in the growth performance, immunity, disease resistance, and gut microbiota by the probiotic *Rummeliibacillus stabekisii* in Nile tilapia (*Oreochromis niloticus*). *Fish and Shellfish Immunology* 92: 265-275.

Tan, S., Chen, X., Cui, C., Hou, Y., Li, W. and You, H. (2017). Biodegradation of saline phenolic wastewater in a biological contact oxidation reactor with immobilized cells of *Oceanimonas* sp. *Biotechnology Letters* 39(1): 91-96.

Tang, K.L., Caffrey, N.P., Nóbrega, D.B., Cork, S C., Ronksley, P E., Barkema, H.W. and Ghali, W.A. (2017). Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. *The Lancet Planetary Health* 1(8): 316-327.

Taylor, M., Cheng, J., Sharma, D., Bitzikos, O., Gustafson R, Fyfe, M. and Mah, V. (2018). Outbreak of *Vibrio parahaemolyticus* associated with consumption of raw oysters in Canada, 2015. *Foodborne Pathogens and Disease* 15(9): 554-559.

Tekale, A., Savalia, C.V., Kshirsagar, D.P., Brahmbhatt, M.N. and Chatur, Y.A. (2015). Detection and virulence gene characterization of *Salmonella* isolates from

fish by conventional and molecular methods. *Journal of Veterinary Public Health* 13(1): 43-6.

Teklemariam, A.D., Tessema, F. and Abayneh, T. (2015). Review on evaluation of safety of fish and fish products. *International Journal of Fisheries and Aquatic Studies* 3(2): 111-117.

Tew, L.S., She, L.Y. and Chew, C.H. (2016). Isolation, antimicrobial susceptibility profile and detection of Sul1, blaTEM, and blaSHV in amoxicillin-clavulanate-resistant bacteria isolated from retail sausages in Kampar, Malaysia. *Jundishapur Journal of Microbiology* 9(10): 37897-37897.

Tewari, A. and Abdullah, S. (2015). *Bacillus cereus* food poisoning: international and Indian perspective. *Journal of Food Science and Technology* 52(5): 2500-2511.

Thapa N and Pal J. (2007). Proximate composition of traditionally processed fish products of the Eastern Himalayas. *Journal of Hill Research* 20:75e7.

Thapa N, Pal J and Tamang JP. (2006). Phenotypic identification and technological properties of lactic acid bacteria isolated from traditionally processed fish products of the Eastern Himalayas. *International Journal of Food Microbiology* 107:33e8.

Thapa, N. (2016). Ethnic fermented and preserved fish products of India and Nepal. *Journal of Ethnic Foods* 3(1): 69-77. doi:10.1016/j.jef.2016.02.003.

Thapa, N. (2016a). Ethnic fermented and preserved fish products of India and Nepal. *Journal of Ethnic Foods* 3(1): 69–77.

Thapa, N. (2016b). *Microbiology and Nutrition of Ethnic Fermented and Preserved Fish Products of the Eastern Himalayas***.** Published by Today and Tomorrow's Printers & Publishers, New Delhi.

Thapa, N. and Pal. J. (2007). Proximate composition of traditionally processed fish products of the Eastern Himalayas. *Journal of Hill Research* 20(2): 75-77

Thapa, N. and Tamang, J.P. (2020). Ethnic Fermented Foods and Beverages of Sikkim and Darjeeling Hills (Gorkhaland Territorial Administration). In: *Ethnic Fermented Foods and Alcoholic Beverages of India: Science History and Culture* (Ed: Tamang, J.P.), pp. 479-537. Springer Nature, Singapore. ISBN: 978-981-15-1486-9.

Thapa, N., Pal, J. and Tamang, J.P. (2004). Microbial diversity in *ngari, hentak* and *tungtap*, fermented fish products of Northeast India. *World Journal of Microbiology and Biotechnology* 20 (6): 599-607.

Thapa, N., Pal, J. and Tamang, J.P. (2006). Phenotypic identification and technological properties of lactic acid bacteria isolated from traditionally processed fish products of the Eastern Himalayas. *International Journal of Food Microbiology* 107(1): 33-38.

Thapa, N., Pal, J. and Tamang, J.P. (2007). Microbiological profile of dried fish products of Assam. *Indian Journal of Fisheries* 54 (1): 121-125.

Theobald, A., Arcella, D., Carere, A., Croera, C., Engel, K.H., Gott, D., Gurtler, R., Meier, D., Pratt, I., Rietjens, I.M.C.M, Simon, R. and Walker, R. (2012). Safety assessment of smoke flavouring primary products by the European Food Safety Authority. *Trends in Food Science & Technology* 27(2): 97–108.

Thijs, S., Op De Beeck, M., Beckers, B., Truyens, S., Stevens, V., Van Hamme, J.D. and Vangronsveld, J. (2017). Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. *Frontiers in Microbiology* 8: 494.doi: 10.3389/fmicb.2017.00494

Thillai Sekar, V., Santiago, T.C., Vijayan, K.K., Alavandi, S.V., Stalin Raj, V., Rajan, J. J.S. and Kalaimani, N. (2008). Involvement of *Enterobacter cloacae* in the mortality of the fish, *Mugil cephalus*. *Letters in Applied Microbiology* 46(6): 667-672.

Thirkell, C.E., Sloan-Gardner, T.S., Kacmarek, M.C. and Polkinghorne, B. (2019). An outbreak of *Bacillus cereus* toxin-mediated emetic and diarrhoeal syndromes at a restaurant in Canberra, Australia 2018. *Communicable Diseases Intelligence* 43. doi.10.33321/cdi.2019.43.40.

Thongsanit, J., S. Tanasupawat, S. Keeratipibul, and S. Jatikavanich. 2002. Characterization and identification of *Tetragenococcus halophilus* and Tetragenococcus muriaticus strains from fish sauce (*Nampla*). *Japanese Journal of Lactic Acid Bacteria* 13 (1):46–52. doi: 10.4109/jslab1997.13.46.

Thung, T.Y., Radu, S., Mahyudin, N.A., Rukayadi, Y., Zakaria, Z., Mazlan, N. and Tan, C.W. (2018). Prevalence, virulence genes and antimicrobial resistance profiles of *Salmonella* serovars from retail beef in Selangor, Malaysia. *Frontiers in Microbiology* 8: 2697. doi.org/10.3389/fmicb.2017.02697.

Tirado, M.C., Clarke, R., Jaykus, L.A., McQuatters-Gollop, A.and Frank, J.M. (2010). Climate change and food safety: A review. *Food Research International* 43(7): 1745-1765.

Toldrá, F. (2004). Fermented meats. In: *Food processing: Principles and applications* (Eds. Hui, Y.H. and Smith, J.S.), pp. 399–415. Blackwell Publishing, Ames, IA.

Tran, K.T.M, May B.K., Smooker, P.M., Van, T.T.H. and Coloe P.J. (2011). Distribution and genetic diversity of lactic acid bacteria from traditional fermented sausage. *Food Research International* 44: 338–344.

Traoré, O., Nyholm, O., Siitonen, A., Bonkoungou, I.J.O., Traoré, A. S., Barro, N. and Haukka, K. (2015). Prevalence and diversity of *Salmonella enterica* in water, fish and lettuce in Ouagadougou, Burkina Faso. *BMC Microbiology* 15(1): 151.

Työppönen, S., Petäjä, E. and Mattila-Sandholm, T. (2003). Bioprotectives and probiotics for dry sausages. *International Journal of Food Microbiology* 83(3): 233-244.

Uğuz, Ş., Soyer, A. and Dalmiş, Ü. (2011). Effects of different salt contents on some quality characteristics during processing of dry-cured Turkish Pastirma. *Journal of Food Quality* 34: 204-211. doi:10.1111/J.1745-4557.2011.00382.X.

Valyasevi, R. and Rolle, R.S. (2002). An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: scope for their improvement. *International Journal of Food Microbiology* 75(3): 231-239.

van Schothorst, M., Zwietering, M.H., Ross, T., Buchanan, R.L. and Cole, M.B. (2009). Relating microbiological criteria to food safety objectives and performance objectives. *Food Control* 20: 967-979.

Veseli, I.A., dos Santos, A.C.M., Juárez, O., Stark, B.C. and Pombert, J.F. (2018). Complete genome sequence of *Vitreoscilla* sp. strain C1, source of the first bacterial hemoglobin. *Microbiology Resource Announcements* 7(5):e00922-18. doi: 10.1128/MRA.00922-18

Vignolo, G., Fontana, C. and Fadda, S. (2010). Semidry and dry fermented sausages. In: *Handbook of Meat Processing* (Ed: Toldra, F.), pp. 379-398. John Wiley & Sons, New Jersey.

Visciano, P., Schirone, M., Tofalo, R. and Suzzi, G. (2012). Biogenic amines in raw and processed seafood. *Frontiers in Microbiology* 3: 188.doi: 10.3389/fmicb.2012.00188

Visessanguan, W., Benjakul, S., Panya, A., Kittikun, C. and Assavanig, A. (2005). Influence of minced pork and rind ratios on physico-chemical and sensory quality of *Nham*–a Thai fermented pork sausage. *Meat Science* 69(2): 355-362.

Waché, Y., Do, T-L., Do, T-B-H., Do, T-Y., Haure, M., Ho, P-H., Kumar Anal, A., Le, V-V-M., Li, W-J., Licandro, H., Lorn, D., Ly-Chatain, M-H., Ly, S., Mahakarnchanakul, W., Mai, D-V,, Mith, H., Nguyen D-H, Nguyen T-K-C, Nguyen T-M-T, Nguyen T-T-T., Nguyen T-V-A, Pham H-V, Pham T-A, Phan T-T, Tan R, Tien T-N, Tran T, Try, S, Phi Q-T., Valentin, D., Vo-Van, Q-B., Vongkamjan, K., Vu, D-C., Vu, N-T. and Chu-Ky, S. (2018). Prospects for food fermentation in South-East Asia, Topics from the tropical fermentation and biotechnology network at the end of the AsiFood Erasmus+Project. *Frontiers in Microbiology* 9:2278. doi: 10.3389/fmicb.2018.02278

Wallin-Carlquist, N., Márta, D., Borch, E. and Rådström, P. (2010). Prolonged expression and production of *Staphylococcus aureus* enterotoxin A in processed pork meat. *International Journal of Food Microbiology* 141: 69-74.

Wanangkarn, A., Liu, D.C., Swetwiwathana, A., Jindaprasert, A., Phraephaisarn, C., Chumnqoen, W. and Tan, F.J. (2014). Lactic acid bacterial population dynamics during fermentation and storage of Thai fermented sausage according to restriction fragment length polymorphism analysis. *International Journal of Food Microbiology* 186: 61-67.

Wang, T., Costa, V., Jenkins, S.G., Hartman, B.J. and Westblade, L.F. (2019). *Acinetobacter radioresistens* infection with bacteraemia and pneumonia. *ID Cases* 15: e00495. doi:10.1016/j.idcr.2019.e00495.

Wang, W., Xia, W., Gao, P., Xu, Y. and Jiang, Q. (2017). Proteolysis during fermentation of Suanyu as a traditional fermented fish product of China. *International Journal of Food Properties* 20 (Sup1): 166-176.

Wang, X., Ren, H. and Zhan, Y. (2018a). Characterization of microbial community composition and pathogens risk assessment in typical Italian-style salami by high-throughput sequencing technology. *Food Science and Biotechnology* 27(1): 241–249.

Wang, X., Zhang, Y., Ren, H. and Zhan, Y. (2018b). Comparison of bacterial diversity profiles and microbial safety assessment of *salami*, Chinese dry-cured sausage and Chinese smoked-cured sausage by high-throughput sequencing. *LWT- Food Science and Technology* 90: 108-115.

Wani, N., Wani, S.A., Munshi, Z.H., Ahmad, S., Shah, M.R., Hussain, A. and Nabi, N. (2018). Isolation and virulence gene profiling of *Clostridium perfringens* from freshwater fish. *Journal of Entomology and Zoological Studies* 6(3): 176–181.

White, A.R., Kaye, C., Poupard, J., Pypstra, R., Woodnutt, G. and Wynne, B. (2004). Augmentin®(amoxicillin/clavulanate) in the treatment of community-acquired respiratory tract infection: a review of the continuing development of an innovative antimicrobial agent. *Journal of Antimicrobial Chemotherapy* 53(1): 3-20.

Wilkinson, T.J., Huws, S.A., Edwards, J.E., Kingston-Smith, A.H., Siu-Ting, K., Hughes, M. and Creevey, C.J. (2018). CowPI: A rumen microbiome focused version of the PICRUSt functional inference software. *Frontiers in Microbiology* 9: 1095. doi: 10.3389/fmicb.2018.01095.

Wongkhalaung, C. (2004). Industrialization of Thai fish sauce (Nam pla). *Food Science and Technology*647-699. *New York-Marcel Dekker*

World Food Summit (1996). World Food Summit Plan of Action, paragraph 1. In: Rome Declaration on World Food Security and World Food Summit Plan of Action; World Food Summit, 13-17 November 1996, Rome, Italy. Rome, FAO. pp. 43.

World Health Organisation. (2020). WHO strategic plan for food safety 2013-2022. Available at: https://www.who.int/news-room/fact-sheets/detail/food-safety.

Wu, G., Wu, G., Zhan, T., Shao, Z. and Liu, Z. (2013). Characterization of a cold-adapted and salt-tolerant esterase from a psychrotrophic bacterium *Psychrobacter pacificensis*. *Extremophiles* 17(5): 809-819.

Wu, Y.C., Kimura, B. and Fujii, T. (2000). Comparison of three culture methods for the identification of *Micrococcus* and *Staphylococcus* in fermented squid shiokara. *Fisheries Science* 66: 142–146.

Wu, Y.T., Zhao, M.M., Wu, N., Sun, W.Z. and Cao, B.S. (2010). Effect of *Staphylococcus condimenti* on the volatile compounds of Cantonese sausage. *Food and Fermentation Industries* (China) 36: 156–160.

Yang, X., Zhu, L., Zhang, Y., Liang, R. and Luo, X. (2018). Microbial community dynamics analysis by high-throughput sequencing in chilled beef *longissimus* steaks packaged under modified atmospheres. *Meat Science* 141: 94-102.

Ye, Y. and Doak, T.G. (2009). A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. *PLoS Computational Biology* 5(8): e1000465. doi.10.1371/journal.pcbi.1000465.

Yin, L.J., Pan, C.L. and Jiang, S.T. (2002). Effect of lactic acid bacterial fermentation on the characteristics of minced mackerel. *Journal of Food Science* 67 (2): 786–92.

Yoh, M., Matsuyama, J., Ohnishi, M., Takagi, K., Miyagi, H., Mori, K. and Honda, T. (2005). Importance of *Providencia* species as a major cause of travellers' diarrhoea. *Journal of Medical Microbiology* 54(11): 1077-1082.

Yongsawatdigul, J., Rodtong, S. and Raksakulthai, N. (2007). Acceleration of Thai fish sauce fermentation using proteinases and bacterial starter cultures. *Journal of Food Science* 72(9): M382-M390.

Yu, S., Yu, P., Wang, J., Li, C., Guo, H., Liu, C., Kong, L., Yu, L., Wu, S., Lei, T., Chen, M., Zeng, H., Pang, R., Zhang, Y., Wei, X., Zhang, J., Wu, Q. and Ding, Y. (2020). A Study on prevalence and characterization of *Bacillus cereus* in ready-to-eat foods in China. *Frontiers in Microbiology* 10:3043.doi: 10.3389/fmicb.2019.03043.

Yun, J.H. and Bae, J.W. (2018). Complete genome sequence of the halophile bacterium Kushneria marisflavi KCCM 80003T, isolated from seawater in Korea. *Marine Genomics* 37: 35-38.

Yun, J.H., Park, S.K., Lee, J.Y., Jung, M.J., and Bae, J.W. (2017). *Kushneria konosiri* sp. nov., isolated from the Korean salt-fermented seafood Daemi-jeot. *International Journal of Systematic and Evolutionary Microbiology* 67(9): 3576-3582.

Zagorec, M. and Champomier-Vergès, M.C. (2017). *Lactobacillus sakei*: a starter for sausage fermentation, a protective culture for meat products. *Microorganisms* 5(3): 56.doi: 10.3390/microorganisms5030056.

Zang, J., Xu, Y., Xia, W. and Regenstein, J. (2019). Quality, functionality, and microbiology of fermented fish: a review. *Critical Review in Food Science and Nutrition* 1: 15. 10.1080/10408398.2019.1565491.

Zang, J., Xu, Y., Xia, W., Yu, D., Gao, P., Jiang, Q. and Yang, F. (2018). Dynamics and diversity of microbial community succession during fermentation of Suan yu, a

Chinese traditional fermented fish, determined by high throughput sequencing. *Food Research International* 111: 565-573.

Zeaki, N., Johler, S., Skandamis, P.N. and Schelin, J. (2019). The role of regulatory mechanisms and environmental parameters in staphylococcal food poisoning and resulting challenges to risk assessment. *Frontiers in Microbiology* 10: 1307. doi.10.3389/fmicb.2019.01307.

Zhang, J., Kobert, K., Flouri, T. and Stamatakis, A. (2013). PEAR: a fast and accurate Illumina paired-end reAd mergeR. *Bioinformatics* 30: 614-620.

Zhang, J., Wang, X., Huo, D., Li, W., Hu, Q., Xu, C. and Li, C. (2016). Metagenomic approach reveals microbial diversity and predictive microbial metabolic pathways in Yucha, a traditional Li fermented food. *Scientific Reports* 6: 32524. doi: 10.1038/srep32524.

Zhang, P., Gänzle, M. amd Yang, X. (2019). Complementary antibacterial effects of bacteriocins and organic acids as revealed by comparative analysis of *Carnobacterium* spp. from meat. *Applied and Environmental Microbiology* 85(20):01227-19.doi: 10.1128/AEM.01227-19.

Zhang, Q.Q., Han, Y.Q., Cao, J.X., Xu, X.L., Zhou, G.H. and Zhang, W.Y. (2012). The spoilage of air-packaged broiler meat during storage at normal and fluctuating storage temperatures. *Poultry Science* 91(1): 208-214.

Zhao, C.C. and Eun, J.B. (2020). Shotgun metagenomics approach reveals the bacterial community and metabolic pathways in commercial *hongeo* product, a traditional Korean fermented skate product. *Food Research International* 131: 109030. doi.org/10.1016/j.foodres.2020.109030.

Zhao, D., Lu, F., Qiu, M., Ding, Y. and Zhou, X. (2016). Dynamics and diversity of microbial community succession of surimi during fermentation with next-generation sequencing. *Journal of Food Safety* 36: 308-316.

Zhao, W., Liu, Y., Latta, M., Ma, W., Wu, Z. and Chen, P. (2019). Probiotics database: a potential source of fermented foods. *International Journal of Food Properties* 22(1): 198-217.

Zhao, Y., Xia, D., Ma, P., Gao, X., Kang, W. and Wei, J. (2020). Advances in the detection of virulence genes of *Staphylococcus aureus* originate from food. *Food Science and Human Wellness* 9(1): 40-44.

Zheng, B., Liu, Y., He, X., Hu, S., Li, S., Chen, M. and Jiang, W. (2017). Quality improvement on half-fin anchovy (*Setipinna taty*) fish sauce by *Psychrobacter* sp. SP-1 fermentation. *Journal of the Science of Food and Agriculture* 97(13): 4484-4493.

CURRICULUM-VITAE

Name : Meera Ongmu Bhutia

Email : ongmu.10@gmail.com

Address : Sajong, Rumtek

East Sikkim, Gangtok

Mobile No : 8597635925

PhD Thesis Title: Assessment of Microbiological Safety of Some Traditionally processed Meat and Fish products of Sikkim (Supervisors: Professor Dr. Jyoti Prakash Tamang and Dr. Namrata Thapa).

QUALIFICATION

Degree	University	Month and year of joining	Month and year of passing	Marks (%)
MPhil (Microbiology)	Sikkim University	7/2012	12/2014	66
MSc (Medical Microbiology)	HNB Garhwal University	07/2009	05/2012	68
BSc MLT	Mizoram University	06/2005	02/2009	65

Research Experience: Expertise in food safety related to food-borne bacterial pathogens; Bacterial identification from Culture-dependent method, PCR analysis, Phylogeny Tree Constructions, ELISA Technique, Enterotoxin Gene Detection, Antimicrobial Resistance Patterns and Bacterial Ecology using Culture-independent method (High-throughput Amplicon Sequencing), and Predictive Functionalities; Bioinformatics Analysis of 16S rRNA Gene Sequencing Data.

Awards

1) Maulana Azad National Fellowship by University Grants Commission (UGC), Govt. of India (April 2013 to March 2018).
2) National Eligibility Test (NET) for Lecturership/Assistant Professor conducted by Agricultural Scientists Recruitment Board (ICAR) on 23/05/2016.

Oral presentations at International Conferences

1) Sakura Science SUNERISE PROGRAM on "Basic and Advanced Biomedical Approaches for enhancing QOL in Aging Societies" at AIST- Tsukuba in Japan: 14-21 Oct 2018.

2) International Conference on "Ethnic Fermented Foods and Beverages: Microbiology and Health Benefits" at Sikkim University, Gangtok: 20-21 Nov, 2015.

3) International Conference on "Chemical Ecology, Environment and Human Health: emerging frontiers and synthesis (ICCEEHH)" at Sikkim University, Gangtok: 9-10 Aug, 2019.

4) International Conference on "Nutraceuticals and Chronic Diseases (INCD)" at Indian Institute of Technology, Guwahati: 22-24 Sep 2019.

Workshop/Conferences Attended

1) Three days workshop on "International Symposium on Biodiversity and Biobanking (Biodiverse 2018)" organized by Indian Institute of Technology, Guwahati and Association for the Promotion of DNA Fingerprinting and others DNA Technologies held from 27th January to 29 January, 2018.

2) Two Days Sensitization Workshop on Cellular Biotechnology: Tissue Culture and Stem Cells" Department of Biochemistry, Sikkim Manipal University, 27th to 28th May 2016.

3) Applications of Medical Biotechnology at the outreach programme on Applications of Biotechnology, Sikkim Government College, Tadong, 19th May 2016.

4) Five Days Training on "Advanced Methods for Molecular Typing of Microbes" conducted by the State Biotech Hub (Assam), C.V.Sc., A.A.U., Khanapara, Guwahati, 9th to 13th May 2016.

Publications

1. Chettri, R., **Bhutia, M.** and Tamang, J.P. (2016). Poly-γ-glutamic acid (PGA)-producing *Bacillus* species isolated from *Kinema*, Indian fermented soybean food. *Frontiers in Microbiology* 7:971.doi: 10.3389/fmicb.2016.00971. (Impact Factor: 4.259)

2. **Bhutia, M.O.,** Thapa, N. and Tamang, J. P. (2020). Khyopeh, a traditional fermented yak meat product of Sikkim. *Indian Journal of Traditional Knowledge* 19(1): 187-191. (Impact Factor: 0.920).

3. **Bhutia, M.O**., Thapa, N., Shangpliang, H.N.J. and Tamang, J.P. (2020). Metataxonomic of bacterial communities and their gene functionality in traditional meat products of Sikkim in India. *Scientific Reports* (accepted).**(Impact Factor: 4.525).**

4. **Bhutia, M.O.,** Thapa, N., Shangpliang, H.N.J. and Tamang, J.P. (2020). Bacterial communities and their predictive metabolic pathways revealed by high-throughput sequencing method in traditionally preserved fish products of Sikkim, India. *Frontiers in Microbiology* (communicated) (Impact Factor: 4.259).

5. **Bhutia, M.O**., Thapa, N., and Tamang, J.P. (2020). Studies on enterotoxin producing bacteria in some traditionally processed meat products of Sikkim. *Meat Science* (communicated). (Impact Factor: 3.483).

6. **Bhutia, M.O.,** Thapa, N., and Tamang, J.P. (2020). Food safety aspects of some traditionally processed fish products of Sikkim with reference to ELISA test and virulence genes. *Food Control* (communicated). (Impact Factor: 4.248).

Indian Journal of Traditional Knowledge
Vol 19(1), January 2020, pp 187-191

Khyopeh, a traditional fermented yak meat product of Sikkim

Meera Ongmu Bhutia[1], Namrata Thapa[2] & Jyoti Prakash Tamang*[,1,+]

[1]DAICENTER (DBT-AIST International Center for Translational and Environmental Research) and Bioinformatics Centre, Department of Microbiology, School of Life Sciences, Sikkim University, Tadong 737 102, Gangtok, Sikkim, India

[2]Biotech-Hub, Department of Zoology, Nar Bahadur Bhandari Degree College, Tadong 737 102, Gangtok, Sikkim, India

*Department of Microbiology, Sikkim University, Tadong 737 102, Sikkim, India

E-mail: [+]jyoti_tamang@hotmail.com

Received 26 February 2019; revised 06 August 2019

The preparation of naturally fermented meat product is an integral part of socio-cultural practice of different ethnic groups of people dwelling in the Himalayan regions of India, Nepal, Bhutan and Tibet in China. This study is aimed at documenting the traditional preparation of *khyopeh*, a naturally fermented meat product of yak prepared by ethnic people of Sikkim and its food safety. This is the first report on *khyopeh* with emphasis on its traditional method of preparation and food safety

Keywords: *Khyopeh*, Sikkim, ELISA, *Staphylococcus*, Yak

IPC Code: Int. Cl.[20]: A61K 39/085, A61K 36/00

Meat is considered as highly nutritious and has become an essential component of human diet being a rich source of valuable proteins, minerals, vitamins, fats and micronutrients[1]. The consumption of meat in daily meal is common dietary culture of some ethnic people residing in the Himalayan regions of India, Nepal, Bhutan and China (Tibet)[2]. Due to high content of moisture and protein in the meat, it is easily susceptible to microbial spoilage[3], hence to prevent the spoilage and to prolong the shelf life of perishable raw meat, it is either dried or fermented or smoked[4]. The domestic livestock of Sikkim in India mostly includes cattle, pig, goat, yak etc. which are commonly used for milk, milk products, and meat. Among these livestock, yak (*Bos grunniens*) is reared in alpine and subalpine scrub lands between 2,100 to 4,500 m altitude in the Himalayas for milk products and meat[5]. In Sikkim, 88.3% of people are non-vegetarian and 11.7% are vegetarian, which depicts an increase in demand of meat and its product[6]. Some ethnic meat products of Sikkim have been documented earlier such as are *kargyong, satchu, sukakomasu* and *chilu*[7]. However, the unlisted naturally fermented meat product called *khyopeh* has not been documented yet. This paper aims to give information on the indigenous knowledge of preparation of *khyopeh* in North district of Sikkim.

*Corresponding author

Materials and methods

Documentation and data collection

Field survey was carried out at Lachung village of North Sikkim for a period of three months from October 2017 till December 2017. Data collection was done based on structured questionnaire, interviewing the people involved in traditional preparation of *khyopeh* and personally analyzing the preparation procedures. The interviewees were local elders of respective village who have had proper traditional knowledge of preparation, their culinary skills and socio-economy of the products.

Sample collection

A total of 5 samples of *khyopey* were collected from North Sikkim and taken into the laboratory in a sterile polythene bags and stored at -20°C for microbial analysis. The pH was determined directly by a digital pH meter (Thermo Scientific Instruments, Waltham, Massachusetts, USA) and moisture content was measured using OHAUS MB45 Moisture Analyzer (OHAUS, Parsippany, New Jersey, USA).

Microbiological analysis

Sample (25 g) was homogenized with 225 mL buffered peptone-water (CM 509 Oxoid Ltd., Basingstoke, UK) in a Stomacher (Seward, Thetford, UK) and serial dilutions of homogenate (10^1 to 10^9) were prepared for microbiological analysis. Decimal dilutions (1 mL) of the sample homogenate were inoculated in 3 M Petrifilm TM Aerobic Count Plate (3 M Company, Maplewood, Minnesota, US), Baird–Parker agar (Hi Media, India) plates supplemented with potassium tellurite and egg yolk emulsion for *Staphylococcus* species[8], Violet Red Bile Glucose agar (VRBGA, Hi Media Ltd.) for *Escherichia coli* and Coliform bacteria[9], (*Bacillus cereus* agar (Hi Media, Mumbai, India) for *Bacillus cereus*[4] and bile esculin azide (BEA) agar for *Enterococcus* species. The plates were incubated at 35°C for 24-48 h. On Baird Parker agar, convex, black, shiny colonies with narrow white margin surrounded by clear zone were regarded as *Staphylococcus* species and on VRBGA, Coliform bacteria formed small red colonies (~1 mm diameter), with or without a red precipitate. Colonies that showed black pigmentation on the BEA agar were regarded as *Enterococcus*[10]. The results were expressed as colony-forming units per gram (cfu/g). The isolates were preliminary identified based on Gram stain, cell morphology, catalase test, IMViC (Indole, Methyl Red, Voges-Proskauer and Citrate Utilization tests, carbohydrate fermentation and other tests[11].

Enzyme Linked Immuno Sorbent Assay (ELISA) test

ELISA tests were performed for *Staphylococcal* enterotoxins, *Bacillus diarrhoeal* enterotoxins and *Salmonella* in *khyopeh* samples using *Staphylococcus* Enterotoxin Assay (SET Total) (r-biopharm, Germany)[12], *Bacillus* diarrhoeal enterotoxin visual immunoassay (3 M Microbiology, USA)[13]and *Salmonella* visual immunoassay (3 M Microbiology, USA)[14]according to manufacturer's instructions.

Antibiotic Susceptibility Test

Antibiotic susceptibility tests of isolates grown on Mueller Hinton agar plates (Hi Media, Mumbai, India) were performed with 24 antibioticsusing the Kirby–Bauer disk diffusion method[15] following guidelines of Clinical Laboratory Standards Institute[16]. Isolates with the standard strain *Staphylococcus aureus* MTCC 96 were incubated at 37°C for 24 h and the diameters of the zones of inhibition were measured (CLSI 2017).

Results and discussion

Method of traditional preparation

Khyopeh is an ethnic fermented yak meat product, which is prepared in the North district of Sikkim. The ethnic *Lachungpa* community of Sikkim who resides in the northern parts of Sikkim mostly practices the preparation and consumption of *khyopeh*. The preparation of *khyopeh* is seasonal which is prepared only in the month of December every year as the yaks are being slaughtered for the Buddhist festivals. The main ingredient used for preparation of *khyopeh* is parts of liver, lungs, fats, intestines and innards. During traditional method of preparation of *khyopeh*, yak meat with its fat are chopped finely, and mixed with required amount of salt. The meat mixtures are stuffed into the rumen (stomach) of yak, and are tied up with rope. It is then hanged in a bamboo stripes above the kitchen oven or at attic for smoking and drying for 4 to 6 months or even for a year to make *khyopeh* (Fig. 1). *Khyopeh* is soft or hard and brownish in colour (Fig. 2). It is eaten as raw or cooked with nettle leaves, locally called *sishnu* (*Urtica dioica* L.) in the main meal with boiled rice in North Sikkim.

Socioeconomic importance

The practice of preparing *khyopeh* is quite rare and confined to Lachung village of north Sikkim which are quite far from the urban localities. Hence, it is not found in the local markets of Sikkim. It is usually prepared for home consumption and festivals. It is believed by the villagers that yak meat products have an immense medicinal potential. Yaks graze on herbs especially *Cordyceps sinensis*, locally called as *yarsagumba*, which is found only in high altitude of mountains and is believed to be an excellent potent for strengthening the immune system.

Food safety

Microbial load of five samples of *khyopeh* was

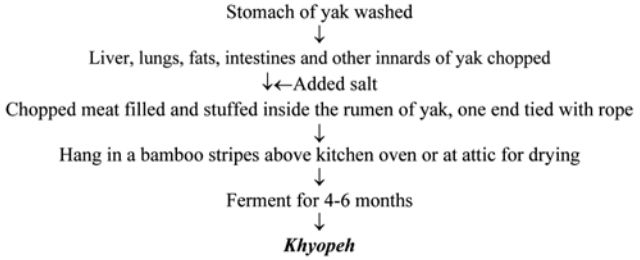


Fig. 1 — Traditional method of preparation of *khyopeh* in North Sikkim.

Fig. 2 — *Khyopeh*, an end product of fermented yak meat inside the rumen of yak.

Staphylococcaceae count (10^4-10^5 cfu/g) and Enterobactericeae count (<10^4 cfu/g). The pH and moisture content of *khyopeh* was 5.8 to 6.1 and 1.6-3.5%, respectively. Based on phenotypic characterization (Table 1), *Staphylococcus*, *Escherichia* and *Enterococcus* were tentatively identified. *Bacillus cereus* was not detected in any sample. ELISA tests were found to be negative for all bacterial toxins tested. Antibiotic sensitivity tests were performed on representative strains of *Staphylococcus*, *Enterococcus* and *Escherichia* (Fig.3). *Enterococcus* was resistant to six antibiotics, *Staphylococcus* was resistant to Oxacillin and *Escherichia* was found to be sensitive to all antibiotics tested except amoxicillin/clavulanate.

Microbiological analysis of samples *khyopeh* tested for food safety revealed presence of low population of *Staphylococcus* species and Enterobacteriaceae by plating method supported by the ELISA tests which also showed negative test for Staphylococcal enterotoxin, *Bacillus* diarrhoeal enterotoxin and *Salmonella.* Similar results were reported in traditional Greek fermented sausage[17]. Antimicrobial susceptibility test indicated that *Staphylococcus* strain KHST1was sensitive to all the antibiotics except oxacillin. However, the isolate may be considered as an

Table 1 — Phenotypic characterization of bacteria isolated from khyopeh

Isolate code	Pigment production	Catalase	Motility	Urease reaction	DNase	Sucrose	Voges - proskauer	Methyl red	Indole	Xylose	Raffinose	Urease	Rhamnose	Tentative identification
KHSTI	Golden yellow	+	-	+	+	+	+	-	-	-	-	+	-	*Staphylococcus*sp.
KHST2	Cream	+	-	+	-	+	+	-	-	-	-	+	-	*Staphylococcus*sp.
KHST3	Cream	+	-	+	-	+	+	-	-	-	-	+	-	*Staphylococcus*sp.
KHST4	Golden yellow	+	-	+	-	+	+	-	-	-	-	+	-	*Staphylococcus* sp.
KHST5	Cream	+	-	+	-	+	+	-	-	-	-	+	-	*Staphylococcus* sp.
KHENI	Black	-	-	-	-	+	+	-	-	-	-	-	-	*Enterococcus* sp.
KHENII	Black	-	-	-	-	+	+	-	-	-	-	-	-	*Enterococcus* sp.
KHENIII	Black	-	-	-	-	+	+	-	-	-	-	-	-	*Enterococcus* sp.
KHE1	Pink red	+	+	-	-	-	-	+	+	+	+		+	*Escherichia* sp.
KHE2	Pink red	+	+	-	-	-	-	+	+	+	+		+	*Escherichia* sp.
KHE3	Pink red	+	+	-	-	-	-	+	+	+	+		+	*Escherichia* sp.
KHE4	Pink red	+	+	-	-	-	-	+	+	+	+		+	*Escherichia* sp.
KHE5	Pink red	+	+	-	-	-	-	+	+	+	+		+	*Escherichia* sp.

All isolates were Gram +ve, fermented glucose, mannitol, maltose, trehalose, lactose and galactose.
None of the isolates fermented adonitol; coagulase and nitrate reductions were positive and citrate reduction was negative for all isolates, respectively.

analyzed- mesophilic aerobic count (10^6-10^7 cfu/g),

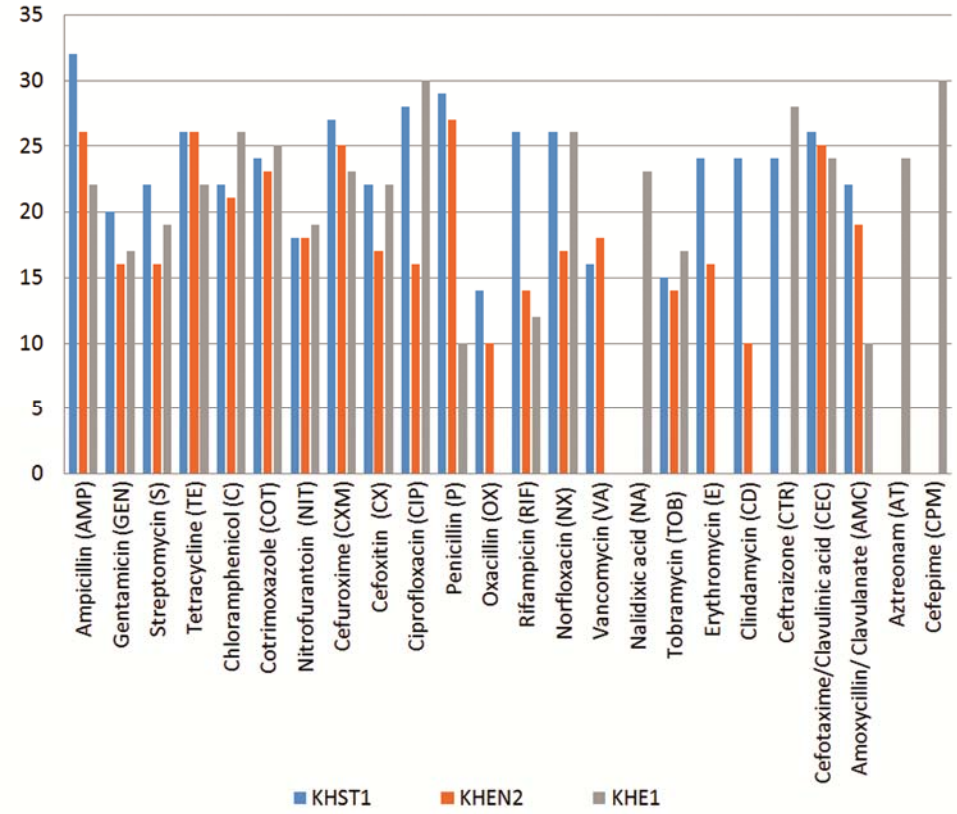


Fig. 3 — Antibiotic sensitivity tests of bacteria isolated from *khyopeh*

ORSA (Oxacillin resistant *Staphylococcus aureus*) if the isolate is resistant to multiple agents like trimethoprim-sulfamethoxazole combination, clindamycin, erythromycin, quinolones, tetracycline, and aminoglycosides[18]. *Escherichia* strain KHE1 was also found to be sensitive to most of the antibiotics except amoxicillin-clavulanate. The amoxicillin-clavulanate resistant isolates were also found in sausages in Malaysia[19]. *Enterococcus* strain KHEN1 was resistant to many antibiotics like oxacillin, cotrimoxazole, ciprofloxacin, cefoxitin, rifampicin and clindamycin. The results showed that consumption of *khyopeh* is safe by ethnic people of Sikkim.

Conclusion

Consumption of traditionally processed dried, smoked and fermented meat products without using starter cultures and chemicals by the ethnic people in Himalaya region is a common practise since centuries. *Khyopeh* holds the importance in preserving the traditional knowledge of preparation of rare and minor naturally fermented yak-meat product in Sikkim. There has been no report of food poisoning in Sikkim by consuming *khyopeh*. This is the first report on unique ethnic meat product *khyopeh* from the Himalayas.

References

1 Pereira PM & Vicente AF, Meat nutritional composition and nutritive role in the human diet. *Meat Sci*, 93(3) (2013) 586-592.

2 Tamang JP, *Himalayan Fermented Foods: Microbiology, Nutrition and Ethnic value*, (CRC Press, Taylor and Francis Group, USA, New York), 2010, 295.

3 Baccus JN & Brown WL, Use of microbial cultures: meat products. *Food Technol*, 35 (1981) 74-83.

4 Rai AK, Tamang JP & Palni U, Microbiological studies of ethnic meat products of the Eastern Himalayas. *Meat Sci*, 85 (2010) 560–567.

5 Sharma DK, Ghosh K, Raquib M & Bhattacharya M, Yak products' profile: an overview. *J Food Sci Technol* 43(5) (2006) 447-452.

6 Tamang JP, Thapa N, Rai B, Thapa S, Yonzan H, Dewan S, Tamang B, Sharma RM, Rai AK, Chettri R, Mukhopadhyay B & Pal B, Food consumption in Sikkim with special reference to traditional fermented foods and beverages: a micro-level survey. *J Hill Res*, 20(1) (2007) 1-37.

7 Rai AK, Palni U & Tamang JP, Traditional knowledge of the Himalayan people on production of indigenous meat products, *Indian J Traditional Knowledge*, 8 (1) (2009) 104-109.

8 Bennett RW, Hait JM & Tallent SM, *Staphylococcus aureus*. *Guide Foodborne Pathology*, 7 (10) (2013) 26-44.

9 Feng P, Weagant SD, Grant MA, Burkhardt W, Shellfish M & Water B, Enumeration of *Escherichia coli* and the coliform bacteria. *Bacteriological Analytical Manual* FDA/ Center for Food Safety & Applied Nutrition.

http://www.cfsan.fda.gov/~ebam/bam-4.html (2002).

10 Delpech G, Pourcel G, Schell, C, De Luca M, Basualdo J, Bernstein J, Grenovero S & Sparo M, Antimicrobial resistance profiles of *Enterococcus faecalis* and *Enterococcus faecium* isolated from artisanal food of animal origin in Argentina. *Foodborne Pathogen Dis*, 9 (2012) 939-44.

11 Lanyi B, (1988). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol*, (19) (1988) 1-67.

12 Pereira ML, Carmo LSD, Santos EJD, Pereira JL & Bergdoll MS, Enterotoxin H in Staphylococcal food poisoning. *J Food Protect*, 59(5) (1996) 559-561.

13 Rahmati T & Labbe R, Levels and toxigenic city of *Bacillus cereus* and *Clostridiumperfringens* from retail seafood. *J Food Protect*, 71(6) (2008) 1178-1185

14 Perez-Montano JA, Gonzalez-Aguilar D, Barba J, Pacheco-Gallardo C, Campos-Bravo CA, Garcia S & Cabrera-Diaz E, Frequency and antimicrobial resistance of *Salmonella* serotypes on beef carcasses at small abattoirs in Jalisco State, Mexico. *J Food Protect*, 75(5) (2012) 867-873.

15 Bauer AW, Kirby WMM, Sherris JC & Turck M, Antibiotic susceptibility testing by a standardized single disk method. *American J Clinical Pathol*, 45(4) (1966) 493-496.

16 Clinical & Laboratory Standards Institute, M100-S27. Performance Standards for Antimicrobial Susceptibility Testing. *Twenty-Seventh Informational Supplement*, Wayne, Pennsylvania (2017).

17 Drosinos EH, Mataragas M, Xiraphi N, Moschonas G, Gaitis F & Metaxopoulos J, Characterization of the microbial flora from a traditional Greek fermented sausage. *Meat Sci*, 69(2) (2005) 307-317.

18 Haberberger RL, Kallen AJ, Driscoll TJ & Wallace MR, Oxacillin-resistant phenotypes of Staphylococcus aureus. *Lab Med*, 29 (1998) 302-305.

19 Tew LS, She LY & Chew CH, Isolation, antimicrobial susceptibility profile and detection of Sul1, blaTEM, and blaSHV in amoxicillin-clavulanate-resistant bacteria isolated from retail sausages in Kampar, Malaysia. *Jundishapur J Microbiol*, 9(10) (2016) 37897-37897.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 June 2016
doi: 10.3389/fmicb.2016.00971

Poly-γ-Glutamic Acid (PGA)-Producing *Bacillus* Species Isolated from *Kinema*, Indian Fermented Soybean Food

*Rajen Chettri, Meera O. Bhutia and Jyoti P. Tamang**

Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, India

Kinema, an ethnic fermented, non-salted and sticky soybean food is consumed in the eastern part of India. The stickiness is one of the best qualities of good *kinema* preferred by consumers, which is due to the production of poly-γ-glutamic acid (PGA). Average load of *Bacillus* in *kinema* was 10^7 cfu/g and of lactic acid bacteria was 10^3 cfu/g. *Bacillus* spp. were screened for PGA-production and isolates of lactic acid bacteria were also tested for degradation of PGA. Only *Bacillus* produced PGA, none of lactic acid bacteria produced PGA. PGA-producing *Bacillus* spp. were identified by phenotypic characterization and also by 16S rRNA gene sequencing as *Bacillus subtilis, B. licheniformis* and *B. sonorensis*.

Keywords: *Kinema*, *Bacillus*, fermented soybean, poly-glutamic acid

OPEN ACCESS

Edited by:
Andrea Gomez-Zavaglia, Center for Research and Development in Food Cryotechnology, Argentina

Reviewed by:
Harold J. Schreier, University of Maryland Baltimore County, USA
Jinshui Zheng, Huazhong Agricultural University, China

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

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *15 April 2016*
Accepted: *06 June 2016*
Published: *21 June 2016*

Citation:
Chettri R, Bhutia MO and Tamang JP (2016) Poly-γ-Glutamic Acid (PGA)-Producing Bacillus Species Isolated from Kinema, Indian Fermented Soybean Food. Front. Microbiol. 7:971. doi: 10.3389/fmicb.2016.00971

INTRODUCTION

Poly-γ-polyglutamic acid (PGA), an amino acid polymer, is not synthesized by ribosomal proteins (Oppermann-Sanio and Steinbüchel, 2002); but is synthesized by Gram-positive bacteria (Yao et al., 2009) and few Gram-negative bacteria (Candela et al., 2009) produced as a polymer outside of the cell (Moraes et al., 2013). PGA-producing bacteria are mainly *Bacillus subtilis, B. anthracis, B. licheniformis, B. thuringensis, B. cereus, B. pumilus, B. amyloliquefaciens, B. mojavensis, B. atrophaeus, B. megaterium, Staphylococcus epidermidis, Natrialba aegyptiaca, Lysinibacillus sphaericus,* and *Fusobacterium nucleatum* (Kambourova et al., 2001; Cachat et al., 2008; Meerak et al., 2008; Candela et al., 2009; Cao et al., 2011). PGA is one of the functional properties of microorganisms present in fermented soybean foods (Tamang et al., 2016a). PGA is an anionic, biodegradable, water-soluble, non-toxic, and edible (Yoon et al., 2000; Zhang et al., 2011). Structurally there are two types of PGA: γ-PGA and α-PGA, which are composed of glutamic acids joined by γ or α linkages, respectively (Goto and Kunioka, 1992). γ-PGA has a structure of 5,000–10,000 units of D- and L-glutamic acids that generate a highly viscous solution when it accumulates in the culture medium (Ashiuchi et al., 2001; Tanimoto et al., 2001). PGA produced by *Bacillus* spp. has potential applications as thickener, cryoprotectant, humectant, drug carrier, biological adhesive, heavy metal absorbent, etc., with biodegradability in the fields of food, cosmetics, medicine, and water treatments (Bajaj and Singhal, 2011; Ogunleye et al., 2015).

Ethnic people of North East India consume spontaneously fermented soybean foods as side dish in meals, which include *kinema*, *tungrymbai*, *hawaijar*, *bekang*, *aakhone*, and *peruyaan* (Tamang, 2015). *Kinema* is a naturally fermented, sticky, mild-ammoniacal flavor and non-salted soybean food of Sikkim and Darjeeling in India, east Nepal and west Bhutan. It is similar to *natto* of Japan,

and *chungkokjang* of Korea. PGA is produced by *Bacillus* spp. in many Asian fermented soybean products giving the characteristic of a sticky texture to the product (Urushibata et al., 2002; Nishito et al., 2010) such as *natto* of Japan (Nagai, 2012; Kada et al., 2013), *chungkokjang* of Korea (Lee et al., 2010), *tungrymbai* and *bekang* of India (Chettri and Tamang, 2014), and *thau nao* of Thailand (Chunhachart et al., 2006). One of the criteria for good quality of *kinema* is high stickiness of the product preferred by consumers (Tamang and Nikkuni, 1996). Relative viscosity and stickiness are probably due to production of PGA by *Bacillus* spp. (Nagai et al., 1994; Tamang and Nikkuni, 1996). *B. subtilis* KK3:B4, isolated from naturally fermented *kinema* of India, produced high amount of relative viscosity of 20.1 (Tamang and Nikkuni, 1996). PGA-producing *Bacillus* strain was isolated from *kinema* of Nepal (Hara et al., 1995). Though several species of *Bacillus* such as *B. subtilis, B. licheniformis, B. cereus, B. circulans, B. thuringiensis,* and *B. sphaericus* were previously isolated from *kinema* using phenotypic characterization (Sarkar et al., 1994, 2002; Tamang, 2003; Tamang et al., 2016b); however, there has been no further report on PGA-producing strains/species of *Bacillus*, isolated from *kinema* samples of India. Hence we conducted this experiment. The present study was to screen PGA-producing species of *Bacillus* from *kinema* and to identify species of *Bacillus* by 16S rRNA sequencing.

MATERIALS AND METHODS

Sample Collection

Fresh samples of *kinema* were collected from different markets of Sikkim in India. Samples were collected aseptically in pre-sterile bottles, sealed, labeled, kept in an ice-box and were transported immediately to the laboratory. Samples were stored at 4°C for further microbial and biochemical analyses.

Isolation of Microorganisms

Ten gram of sample was homogenized in 90 mL sterile physiological saline in a stomacher lab-blender (400, Seward, UK) for 1 min and a serial dilution was made. The diluents were heated at 100°C for 2 min for inactivation of vegetative cells of endospore bacteria (Tamang and Nikkuni, 1996), were isolated and enumerated on nutrient agar (MM012, HiMedia, India), and incubated for 24 h at 37°C. Lactic acid bacteria (LAB) were isolated on plates of MRS agar (M641, HiMedia, India) supplemented with 1% $CaCO_3$ and incubated at 30°C in an anaerobic gas-jar (LE002, HiMedia, India) for 48–72 h. Total viable counts were determined on plate count agar (M091A, HiMedia, India) incubated at 30°C for 48–72 h. Isolated colonies were purified and were preserved in 15% (v/v) glycerol at −20°C for further analysis.

Phenotypic Characterization

Cell morphology and motility of isolates were observed using a phase contrast microscope (Olympus CH3-BH-PC, Japan). Isolates were Gram-stained and tested for production of catalase, carbon dioxide from glucose, ammonia from arginine, growth at different temperatures, in different concentrations of NaCl and pH in nutrient broth (M002, HiMedia, India) following the method of Schillinger and Lücke (1987). Voges–Proskauer test, nitrate reduction, starch hydrolysis, casein hydrolysis, citrate utilization test, bile salt tolerance, anaerobic growth, and sugar fermentations were determined following the method of Duc et al. (2004). Taxonomic key of Slepecky

TABLE 1 | Screening of stickiness, and PGA production at different pH and temperatures.

Organisms	Strain code	Stickiness (cm)	PGA production	
			pH 7.5	30°C
Bacillus subtilis (*n* = 13)	KAS:B5	16	++	**+++**
	KAS:B6	18	++	**+++**
	KAS:B18	6	+	+
	KAS:B29	16	++	**+++**
	KAS:B36	4	+	+
	KAS:B39	15	++	**+++**
	KLM:B68	3	+	+
	KLM:B78	3	+	+
	KLM:B86	4	+	+
	KLM:B98	4	+	+
	KAS:B102	20	++	**+++**
	KLM:B112	23	++	**+++**
	KLM:B114	2	+	+
B. licheniformis (*n* = 4)	KAS:B46	4	+	+
	KAS:B56	20	++	+++
	KLM:B92	21	++	+++
	KLM:B108	2	+	+
B. pumulis (*n* = 5)	KAS:B15	3	+	+
	KAS:B48	5	+	+
	KLM:B73	5	+	+
	KLM:B93	6	+	+
	KLM:B106	4	+	+
B. sphaericus (*n* = 8)	KAS:B9	2	+	+
	KAS:B16	4	+	+
	KAS:B19	5	+	+
	KAS:B49	6	+	+
	KLM:B66	3	+	+
	KLM:B72	2	+	+
	KLM:B82	2	+	+
	KLM:B96	2	+	+
B. cereus (*n* = 9)	KAS:B8	2	–	–
	KAS:B10	1	–	–
	KAS:B38	2	–	–
	KAS:B58	2	–	–
	KLM:B74	2	–	–
	KLM:B84	2	–	–
	KLM:B85	2	–	–
	KLM:B88	3	–	–
	KLM:B104	1	–	–

n, number of isolates in parenthesis. +++, high clumping of insoluble precipitate; ++, more clumping of precipitate; +, moderate clumping of precipitate; –, no clumping of precipitate. No precipitate was observed in pH 5 and 9, and at 45°C.

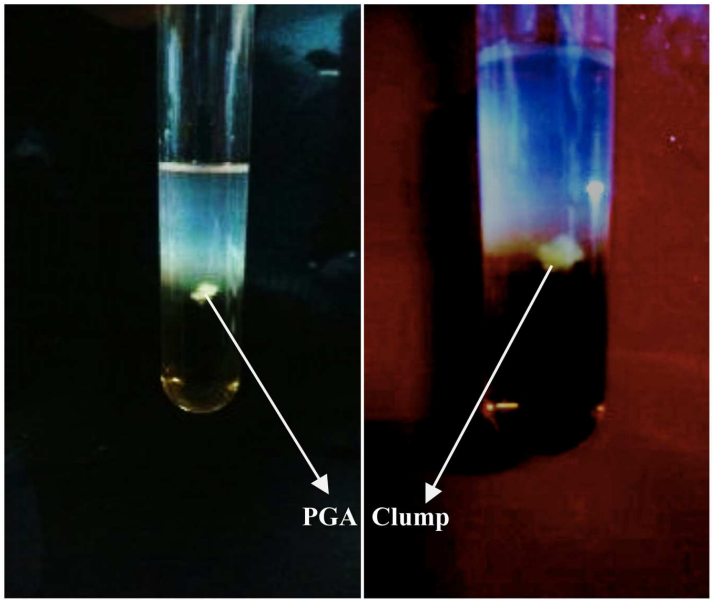


FIGURE 1 | Clumping of insoluble material presumably PGA biopolymer produced by *Bacillus subtilis* KAS:B5 after addition of ethanol into PGA medium.

and Hemphill (2006) was followed for identification of *Bacillus* spp.

Measurement of Stickiness

Cultures were grown on phytone agar (Nagai et al., 1994) at 37°C for 24 h were pulled by touching with an inoculating needle and the stickiness was measured by the length of the thread using scale in cm.

Screening of PGA

Screening of PGA by bacteria was done with a slightly modification of the method described by Nagai et al. (1997) and Meerak et al. (2007). *Bacillus* isolates were grown at 37°C for 24 h in a conical flask containing 100 ml of PGA medium that consisted of sodium glutamate 2.0%, glucose 2.0%, $(NH_4)_2SO_4$ 1.0%, Na_2HPO_4 0.1%, KH_2PO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, $Mn(Cl_2)_4.H_2O$ 0.002%, $FeCl_3{\cdot}7H_2O$ 0.005% (Kunioka and Goto, 1994). The culture after incubation was centrifuged to obtain a supernatant that contained insoluble material. An equal volume of ethanol was added to the supernatant to get fibrous precipitate presumbly the PGA (Nagai et al., 1997).

Efficiency of PGA of the isolates were tested in different pH (5, 7.5, and 9) and temperature (30 and 45°C) following the method of Meerak et al. (2007).

Degradation of PGA

Screening of LAB for degradation of PGA was performed following the method described by Tanaka et al. (1993). Strains were grown in MRS broth (M369, HiMedia, India), for 18-24 h at 30°C. The isolates were streaked on MRS agar plates containing 0.5% pure PGA (Sigma) solution (pH 4.5), and incubated at 30°C for 2–3 days. The plates were flooded with 5 ml of 18 N H_2SO_4 and allowed to stand for 30 min at room temperature. The presence of halo around the colony determines the degradation of PGA.

Genomic DNA Isolation

Genomic DNA was isolated according to the method of Wilson (2001). Amplified 16S rDNA was obtained from each strain by polymerase chain reaction (PCR) with the universal primers; forward 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse 5′-AAGGAGGTGATCCAGCCGCA-3′ (Weisburg et al., 1991). The amplicons sizes ranged from 914 BP to 1814 BP.

Gel Electrophoresis

The amplified DNA fragments were separated through gel electrophoresis by applying 10 μL of each PCR product with 1.5 μL of loading dye [(6×), DV4371, Promega, USA] into the wells of 1.5% agarose (V3125, Promega) gel containing 1.5 μL/mL ethidium bromide (H5041, Promega). DNA size markers (RMBD135, Genei; G5711, Promega) were added as standard for the calculation of size of the DNA fragments. The gel was run and photographed using gel documentation system (GelDoc FQ, Biorad, USA).

16S rDNA Sequence Analysis

The sequencing reactions were performed using ABI PRISM 3100 Genettic Analyzers (Applied Biosystems) in both direction with universal primers used for amplification. The electrophenogram data for 16S rDNA sequence was validated using Chromas 2.33

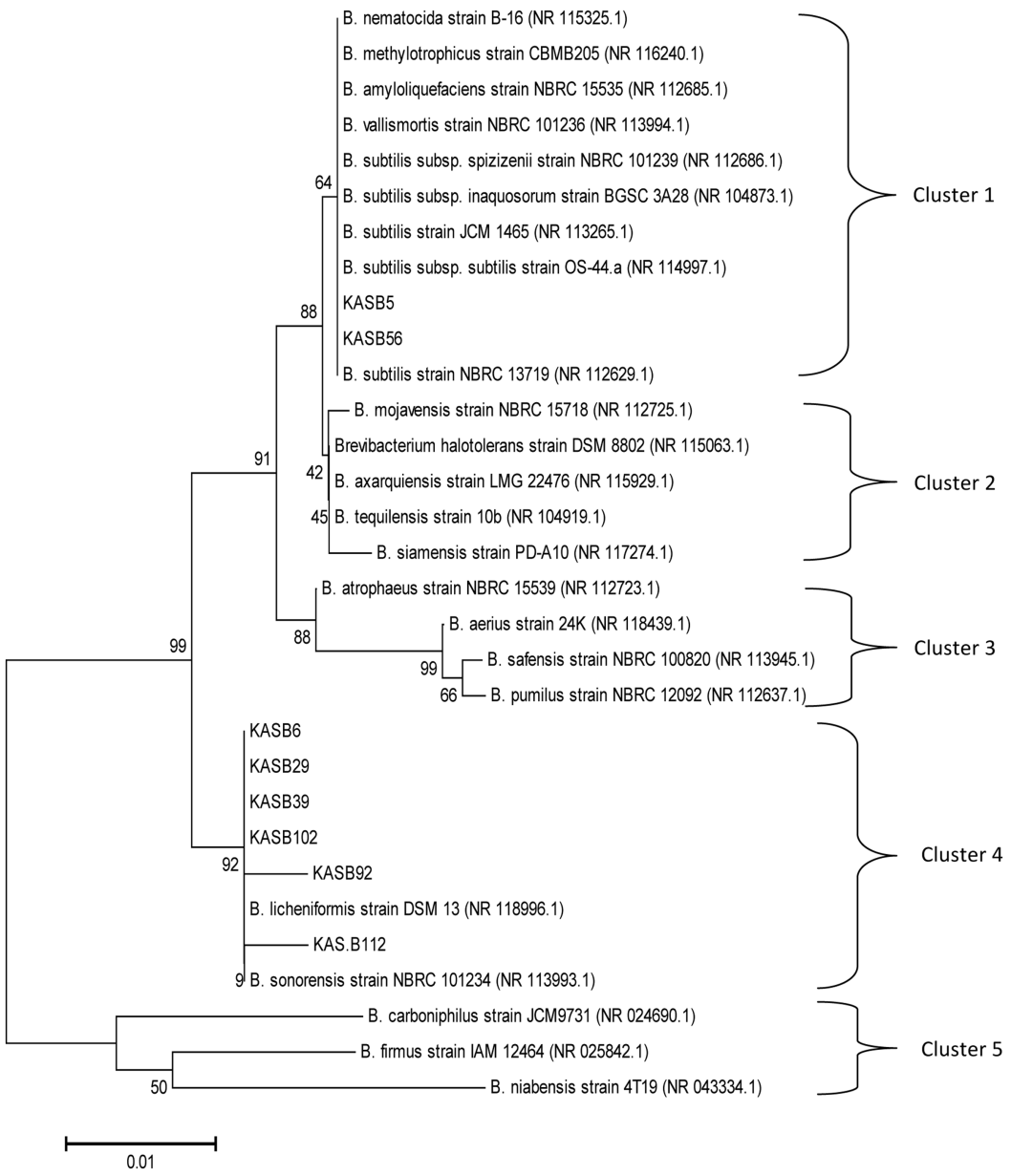


FIGURE 2 | Evolutionary relationships of the analyzed strains with their closest known taxa. The evolutionary history was inferred using the Neighbor-Joining method. The tree was constructed based on the evolutionary distance calculated from 16S rRNA gene sequences using Kimura 2-parameter method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances.

software.[1] Sequences obtained were matched with previously published bacterial 16S rDNA sequences available in the GenBank database using BLAST and the Ribosomal Database Project (RDP).

[1]www.technelysium.com.au

Phylogenetic Analysis

For phylogenetic analysis, 16S rDNA sequence of the isolates and reference sequence retrieved from NCBI-GenBank database were aligned with Clustal Omega. The resulting alignment were analysed with MEGA 6.0 to construct the phylogenetic tree. Phylogenetic tree was inferred with

TABLE 2 | Homogeny of PGA-producing *Bacillus* isolated from *kinema*.

Strain code	*Bacillus*	Accession number	Homogeny (% similarity)
KAS:B5	*Bacillus subtilis*	KX262911	96
KAS:B6	*B. licheniformis*	KX262910	98
KAS:B29	*B. licheniformis*	KX261423	94
KAS:B39	*B. licheniformis*	KX261424	97
KAS:B56	*B. subtilis*	KX262912	97
KAS:B92	*B. licheniformis*	KX261426	97
KAS:B102	*B. licheniformis*	KX261425	96
KAS:B112	*B. sonorensis*	KX262913	97

neighbor-joining (NJ) method (Saitou and Nei, 1987). Sequence divergence among the strain were quantified using Kimura-2-paramater distance model (Kimura, 1980). A total of 1,000 bootstrap replication were calculated for evaluation of the tree topology.

RESULTS AND DISCUSSION

Phenotypic Identification

The average population of *Bacillus* spp. in *kinema* was 10^7 cfu/g, LAB was 10^3 cfu/g and total viable counts were 10 cfu/g, respectively (data not shown). Thirty-nine isolates of *Bacillus* were isolated from 10 samples of *kinema*. Based on phenotypic characterization (data not shown) five species of *Bacillus* were identified from 10 samples of *kinema* as *B. subtilis, B. licheniformis, B. pumulis, B. sphaericus* and *B. cereus* (**Table 1**). About 90% of the total bacterial population found in *kinema* was *Bacillus*, indicating that *Bacillus* is the dominant bacterium in *kinema*. Sarkar and Tamang (1994) also reported that *Bacillus* is the predominant bacterium in *kinema*. *B. subtilis, B. licheniformis, B. cereus, B. circulans, B. thuringiensis,* and *B. sphaericus* were reported from *kinema* sample earlier (Sarkar et al., 1994, 2002; Nout et al., 1998; Tamang, 2003).

Screening of PGA Production

Stickiness of 39 isolates of *Bacillus* was measured (**Table 1**). The ability of 39 isolates of *Bacillus* were tested for production of PGA in PGA medium (Kunioka and Goto, 1994) in pH 5, 7.5, and 9, and at 30C and 45°C (**Table 1**). The isolates formed an insoluble material or fibrous precipitate after addition of equal volume of ethanol into the PGA medium (**Figure 1**) presumbly PGA biopolymer (Nagai et al., 1997; Ashiuchi et al., 2001). All species of *Bacillus* showed fibrous precipitate indicating the absence of PGA production except *B. cereus*.

We tested 25 isolates of LAB isolated from *kinema* for their ability to degrade poly-glutamic acid (PGA) to know whether LAB also produce PGA in *kinema* (data not shown). All LAB isolates were found to degrade PGA, indicating that they have no role in PGA production. Similar observations of degradation of PGA by LAB in fermented soybean were made earlier (Kimura and Fujimoto, 2010; Chettri and Tamang, 2014).

Molecular Characterization

On the basis of high (+++) fibrous precipitate at 30^o C and pH 7.5, and stickiness of >15 cm (**Table 1**), 8 strains of *Bacillus* viz. KAS:B5, KAS:B29, KAS:B39, KAS:B56, KAS:B102, KAS:B6, KAS:B92, and KAS:B112 were selected and were identified by 16S rRNA sequencing. Based on the similarity search with blastN and EzTaxon server the strain KAS:B5 was identified as *B. subtilis*, KAS:B6 as *B. licheniformis*, KAS:B29 as *B. licheniformis*, KAS:B39 as *B. licheniformis*, KAS:B56 as *B. subtilis*, KAS:B92 as *B. licheniformis*, KAS:B102 as *B. licheniformis* and KAS:B112 as *B. sonorensis*. Recovery of *B. sonorensis* from *kinema* is the first report.

Phylogenetic tree was constructed with neighbor joining method based on the evolutionary distance calculated from 1,000 replicates has showed 5 distinct clusters (**Figure 2**), which were separated on a scale of 0.01 nucleotide substitution. The homogeny similarity of *Bacillus* spp. and accession numbers were shown in **Table 2**. Out of 8 PGA-producing strains KAS:B5 and KAS:B56 showed similarities with *B. substilis* strain NBRC13719, *B. subtilis* subsp. *subtilis* strain OS44a and other strains of *subtilis* like JCM1465, NBRC 101236, NBRC 101239, and BGSC 3A28 with 64% of similarity percentage in cluster 1. KAS:B6, KAS:B29, KAS:B39, KAS:B102, and KAS:B92 were found in same clade of cluster 4 showing similarities with *B. licheniformis* DSM12 with 92% similarity and KAS:B112 showed similarities with *B. sonorensis* strain NBRC 101234 with 90% similarity. Strains KAS:B92 and KAS:B112 were found to show a distance gap between the other species of cluster 4 indicating the difference in nucleotide sequence and evolutionary lineage. In this paper, we could find that *B. subtilis* and *B. licheniformis* are PGA-producing bacteria in *kinema*. *B. subtilis* and *B. licheniformis* are the most widely used industrial producers of γ-PGA (Kambourova et al., 2001; Stanley and Lazazzera, 2005; Zhang et al., 2011).

CONCLUSION

Consumers prefer slimy texture of *kinema* as good quality product. Presumably slimy material in fermented soybean food is polyglutamic acid, which has been reported from several Asian fermented foods produced by *Bacillus* spp. PGA, has several applications as foods as well as non-foods. The present study revealed that some species of *Bacillus* produced PGA in *kinem*a. Further investigation is needed to characterize and purify PGA produced by *Bacillus* spp. during natural fermentation of *kinem*a.

AUTHOR CONTRIBUTIONS

RC: screening of PGA-producing *Bacillus* from *kinema*, molecular identification of *Bacillus*, screening go PGA, stickiness, and preparation of draft paper. MOB: phenotypic identification. JPT: analysis of data, compilation and finalization of paper.

REFERENCES

Ashiuchi, M., Kamei, T., Baek, D. H., Shin, S. Y., Sung, M. H., Soda, K., et al. (2001). Isolation of *Bacillus subtilis* (chungkookjang), a poly-γ-glutamate producer with high genetic competence. *Appl. Microbiol. Biotechnol.* 57, 764–769. doi: 10.1007/s00253-001-0848-9

Bajaj, I., and Singhal, R. (2011). Poly (glutamic acid) an emerging biopolymer of commercial interest. *Bioresour. Technol.* 102, 5551–5561. doi: 10.1016/j.biortech.2011.02.047

Cachat, E., Barker, M., Read, T. D., and Priest, F. G. (2008). A *Bacillus thuringiensis* strain producing a polyglutamate capsule resembling that of *Bacillus anthracis*. *FEMS Microbiol. Lett.* 285, 220–226. doi: 10.1111/j.1574-6968.2008.01231.x

Candela, T., Moya, M., Haustant, M., and Fouet, A. (2009). Fusobacterium nucleatum, the first Gram-negative bacterium demonstrated to produce polyglutamate. *Can. J. Microbiol.* 55, 627–632. doi: 10.1139/w09-003

Cao, M., Geng, W., Liu, L., Song, C., Xie, H., Guo, W., et al. (2011). Glutamic acid independent production of poly-γ-glutamic acid by *Bacillus amyloliquefaciens* LL3 and cloning of pgsBCA genes. *Bioresour. Technol.* 102, 4251–4257. doi: 10.1016/j.biortech.2010.12.065

Chettri, R., and Tamang, J. P. (2014). Functional properties of Tungrymbai and Bekang, naturally fermented soybean foods of North East India. *Int. J. Ferment. Foods* 3, 87–103. doi: 10.5958/2321-712X.2014.01311.8

Chunhachart, O., Itoh, T., Sukchotiratana, M., Tanimoto, H., and Tahara, Y. (2006). Characterization of ©-glutamyl hydrolase produced by *Bacillus* sp. isolated from Thai thua-nao. *Biosci. Biotechnol. Biochem.* 70, 2779–2782. doi: 10.1271/bbb.60280

Duc, L. H., Hong, H. A., Barbosa, T. M., Henriques, A. O., and Cutting, S. M. (2004). Characterization of Bacillus probiotics available for human use. *Appl. Environ. Microbiol.* 70, 2161–2171. doi: 10.1128/AEM.70.4.2161-2171.2004

Goto, A., and Kunioka, M. (1992). Biosynthesis and hydrolysis of poly(γ-glutamic acid) from *Bacillus subtilis* IFO3335. *Biosci. Biotechnol. Biochem.* 56, 1031–1035. doi: 10.1271/bbb.56.1031

Hara, T., Saito, H., Iwamoto, N., and Kaneko, S. (1995). Plasmid analysis in Polyglutamate producing *Bacillus* strain isolated from non-salty fermented soybean food, "Kinema" in Nepal. *J. Gen. Appl. Microbiol.* 41, 3–9. doi: 10.2323/jgam.41.3

Kada, S., Ishikawa, A., Ohshima, Y., and Yoshida, K. (2013). Alkaline serine protease AprE plays an essential role in poly-γ-glutamate production during natto fermentation. *Biosci. Biotechnol. Biochem.* 77, 802–809. doi: 10.1271/bbb.120965

Kambourova, M., Tangney, M., and Priest, F. G. (2001). Regulation of polyglutamic acid synthesis by glutamate in *Bacillus licheniformis* and *Bacillus subtilis*. *Appl. Environ. Microbiol.* 67, 1004–1007. doi: 10.1128/AEM.67.2.1004-1007.2001

Kimura, K., and Fujimoto, Z. (2010). "Enzymatic degradation of poly-gamma-glutamic acid," in *Amino-Acid Homopolymers Occurring in Nature, 95 Microbiology Monographs 15*, ed. Y. Hamano, (Berlin: Springer-Verlag), 25–116.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111–120. doi: 10.1007/BF01731581

Kunioka, M., and Goto, A. (1994). Biosynthesis of poly (γ-glutamic acid) from L-glutamic acid, citric acid, and ammonium sulfate in Bacillus subtilis IFO3335. *Appl. Microbiol. Biotechnol.* 40, 867–872. doi: 10.1007/BF00173990

Lee, H., Chang, M. J., and Kim, S. H. (2010). Effects of poly-gamma-glutamic acid on serum and brain concentrations of glutamate and GABA in diet-induced obese rats. *Nutr. Res. Pract.* 4, 23–29. doi: 10.4162/nrp.2010.4.1.23

Meerak, J., Lida, H., Watanabe, Y., Miyashita, M., Sato, H., Nakagawa, Y., et al. (2007). Phylogeny of poly-γ-glutamic acid-producing *Bacillus* strains isolated from fermented soybean foods manufactured in Asian countries. *J. Gen. Appl. Microbiol.* 53, 315–323. doi: 10.2323/jgam.53.315

Meerak, J., Yukphan, P., Miyashita, M., Sato, H., Nakagawa, Y., and Tahara, Y. (2008). Phylogeny of (-polyglutamic acid-producing Bacillus strains isolated from a fermented locust bean product manufactured in West Africa. *J. Gen. Appl. Microbiol.* 54, 159–166. doi: 10.2323/jgam.54.159

Moraes, L. P., Brito, P. N., and Alegre, R. M. (2013). The existing studies on biosynthesis of poly(γ-glutamic acid) by fermentation. *Food Public Health* 3, 28–36.

Nagai, T. (2012). Overview of studies on *Bacillus subtilis* (natto) bacteriophages and the prospects. *JARQ* 46, 305–310. doi: 10.6090/jarq.46.305

Nagai, T., Koguchi, K., and Itoh, Y. (1997). Chemical analysis of poly-(-glutamic acid produced by plasmid-free *Bacillus subtilis* (natto): evidence that plasmids are not involved in poly-glutamic acid production. *J. Gen. Appl. Microbiol.* 43, 139–143. doi: 10.2323/jgam.43.139

Nagai, T., Nishimura, K., Suzuki, H., Banba, Y., Sasaki, H., and Kiuchi, K. (1994). Isolation and characterization of Bacillus subtilis strain producing natto with strong umami-taste and high viscosity. *Nippon Shokuhin Kogyo Gakkaishi* 41, 123–128. doi: 10.3136/nskkk1962.41.123

Nishito, Y., Osana, Y., Hachiya, T., Popendorf, K., Toyoda, A., Fujiyama, A., et al. (2010). Whole genome assembly of a natto production strain *Bacillus subtilis* natto from very short read data. *BMC Genomics* 11:243. doi: 10.1186/1471-2164-11243

Nout, M. J. R., Bakshi, D., and Sarkar, P. K. (1998). Microbiological safety of Kinema, a fermented soyabean food. *Food Control* 9, 357–362. doi: 10.1016/S0956-7135(98)00126-1

Ogunleye, A., Bhat, A., Irorere, V. U., Hill, D., Williams, C., and Radecka, I. (2015). Poly-γ-glutamic acid: production, properties and applications. *Microbiology* 161, 1–17. doi: 10.1099/mic.0.081448-0

Oppermann-Sanio, F. B., and Steinbüchel, A. (2002). Occurrence, functions and biosynthesis of polyamides in microorganisms and biotechnological production. *Naturwissenschaften* 89, 11–22. doi: 10.1007/s00114-001-0280-0

Saitou, N., and Nei, M. (1987). The Neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Sarkar, P. K., Hasenack, B., and Nout, M. J. R. (2002). Diversity and functionality of Bacillus and related genera isolated from spontaneously fermented soybeans (Indian Kinema) and locust beans (African Soumbala). *Int. J. Food Microbiol.* 77, 175–186. doi: 10.1016/S0168-1605(02)00124-1

Sarkar, P. K., and Tamang, J. P. (1994). The influence of process variables and inoculum composition on the sensory quality of kinema. *Food Microbiol.* 11, 317–325. doi: 10.1006/fmic.1994.1036

Sarkar, P. K., Tamang, J. P., Cook, P. E., and Owens, J. D. (1994). Kinema-a traditional soybean fermented food: proximate composition and microflora. *Food Microbiol.* 11, 47–55. doi: 10.1006/fmic.1994.1007

Schillinger, U., and Lücke, F. K. (1987). Identification of lactobacilli from meat and meat products. *Food Microbiol.* 4, 199–208. doi: 10.1016/0740-0020(87)90002-5

Slepecky, R. A., and Hemphill, H. E. (2006). "The genus *Bacillus*-nonmedical," in *The Prokaryotes, A Handbook on the Biology of Bacteria: Bacteria, Firmicutes, Cyanobacteria*, 3rd Edn, Vol. 4, eds M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (New York, NY: Springer-Verlag), 530–562.

Stanley, N. R., and Lazazzera, B. A. (2005). Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-γ-dl-glutamic acid production and biofilm formation. *Mol. Microbiol.* 57, 1143–1158. doi: 10.1111/j.1365-2958.2005.04746.x

Tamang, J. P. (2003). Native microorganisms in fermentation of kinema. *Indian J. Microbiol.* 43, 127–130.

Tamang, J. P. (2015). Naturally fermented ethnic soybean foods of India. *J. Ethnic Foods* 2, 8–17. doi: 10.1016/j.jef.2015.02.003

Tamang, J. P., and Nikkuni, S. (1996). Selection of starter culture for production of kinema, fermented soybean food of the Himalaya. *World J. Microbiol. Biotechnol.* 12, 629–635. doi: 10.1007/BF00327727

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

Tamang, J. P., Watanabe, K., and Holzapfel, W. H. (2016b). Review: diversity of microorganisms in global fermented foods and beverages. *Front. Microbiol.* 7:377. doi: 10.3389/fmicb.2016.00377

Tanaka, T., Yaguchi, T., Hiruta, O., Futamura, T., Uotani, K., Satoh, A., et al. (1993). Screening for microorganisms having poly ((-glutamic acid) endohydrolase activity and the enzyme production by *Myrothecium* sp.TM-4222. *Biosci. Biotechnol. Biochem.* 57, 1809–1810. doi: 10.1271/bbb.57.1809

Tanimoto, H., Mori, M., Motoki, M., Torii, K., Kadowaki, M., and Noguchi, T. (2001). Natto mucilage containing poly-γ-glutamic acid increases soluble calcium in the rat small intestine. *Biosci. Biotechnol. Biochem.* 65, 516–521. doi: 10.1271/bbb.65.516

Urushibata, Y., Tokuyama, S., and Tahara, Y. (2002). Characterization of the *Bacillus subtilis* ywsC gene, involved in gamma-polyglutamic acid production. *J. Bacteriol.* 184, 337–343. doi: 10.1128/JB.184.2.337-343

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703.

Wilson, K. (2001). Preparation of genomic DNA from bacteria. *Curr. Protoc. Mol. Biol.* Chap. 2, Unit 2.4. doi: 10.1002/0471142727.mb0204s56

Yao, J., Jing, J., Xu, H., Liang, J., Wu, Q., Feng, X., et al. (2009). Investigation on enzymatic degradation of γ-polyglutamic acid from *Bacillus subtilis* NX-2. *J. Mol. Catal. B* 56, 158–164. doi: 10.1016/j.molcatb.2007.12.027

Yoon, S., Do, J., Lee, S., and Chag, H. (2000). Production of poly-δ-glutamic acid by fed-batch culture of *Bacillus licheniformis*. *Biotechnol. Lett.* 22, 585–588. doi: 10.1023/A:1005610400511

Zhang, D., Xu, Z., Xu, H., Feng, X., Li, S., Cai, H., et al. (2011). Improvement of poly(γ-glutamic acid) biosynthesis and quantitative metabolic flux analysis of a two-stage strategy for agitation speed control in the culture of *Bacillus subtilis* NX-2. *Biotechnol. Bioprocess. Eng.* 16, 1144–1151. doi: 10.1007/s12257-011-0074-y

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.