FINAL REPORT

of

ICIMOD-Mountain Chair

[December 2019 to December 2022]

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FORWARD

I have been hearing for a long that the ICIMOD Mountain Chair is very prestigious and honoured among academic fraternity. The reality came true when I was selected as the ICIMOD Mountain Chair in 2019. I extend my sincere thanks to Dr. David Molten, the then Director General of ICIMOD, Dr. Eklabya Sharma, former DDG and Dr. Shachi for my selection. I expected to visit all 8 HUC countries during my Chair tenure, unfortunately due to COVD-19, I could visit only Nepal and Myanmar, and out of HUC, I visited Cambodia in 2022. Despite of COVID-19 and lockdown, I delivered several talks and lectures to students and faculty members of Universities/Research Institutes of different countries in online mode. During the COVID-19 periods, I published more that 25 research papers, out of which 2 publications came out of the Mountain Chair involvement. The most remarkable achievements during the Mountain Chair tenure was my section as prestigious Fellow of Indian National Science Academy and National Academy of Science, India in 2022. At last I extend my gratitude to Dr. Pema Gyamtsho, Director General of ICIMOD, Dr. Shachi and Ms. Achala for their constant support. Though my tenure as Mountain Chair is expired this month, however I remain committed to promote the higher education and quality research in food science and food ecosystem in the Hindu Kush Himalayas.

प्रो. ज्योति प्रकाश तामांग

14th December 2022

Terms of Reference (ToR)

- On 5th December 2019, International Centre for Integrated Mountain Development (ICIMOD) conferred the ICIMOD-Mountain Chair to me for two years (2019-2021) at ICIMOD Headquarter at Kathmandu, Nepal by Dr. David Molten, Director General of ICIMOD (**Fig. 1**). As per the ToR, the Mountain Chair was activated from 15.12.2019 to 14.12.2021.

- Due to COVID-19, ICIMOD has extended one year from 2021 to December 2022 without any extra financial implications.

Terms of Reference (ToR) of ICIMOD Mountain Chair

DELIVERABLES

- At least 03 visits to HUC members in Bhutan, China, and Myanmar to promote inter-university cooperation through lecture series, PhD students' co-supervision and other scholarly exchange activities.
- Provision of expert inputs to the development of relevant HUC Thematic Working Groups and Task Forces.
- Provision of networking services to expand the HUC network, as evinced in inclusion of new full and associate members or initiation of Memorandum of Understanding to be signed with ICIMOD by higher education and think tank institutions within and outside of the HKH.
- Co-convene an Inception Meeting of Himalayan Food and Nutritional Security Thematic Working Group, in coordination with the HUC Secretariat and HUC participating members.

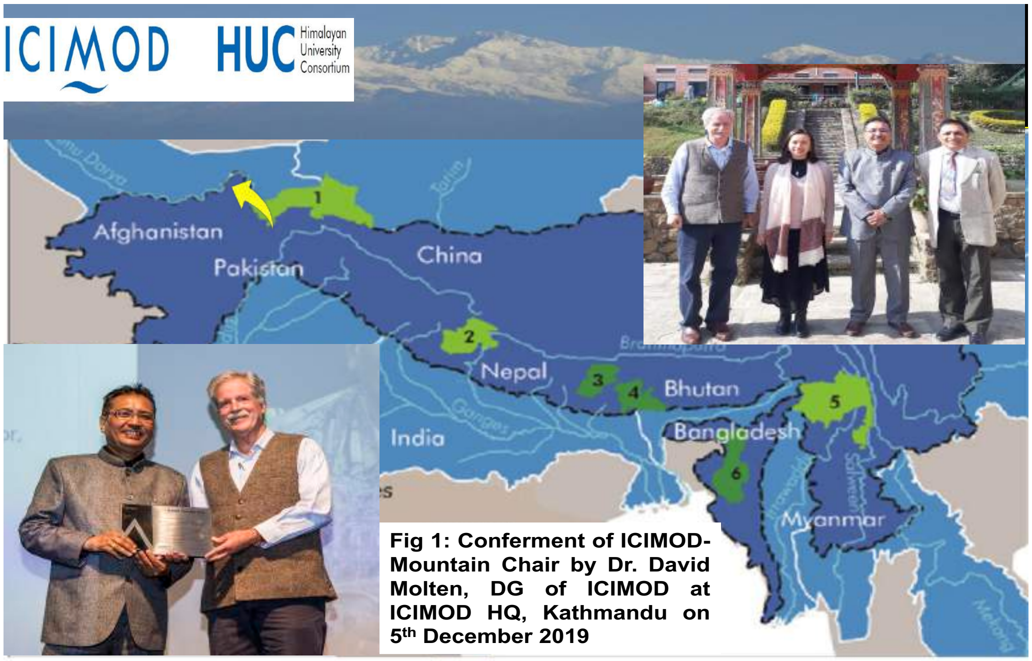

Fig 1: Conferment of ICIMOD-Mountain Chair by Dr. David Molten, DG of ICIMOD at ICIMOD HQ, Kathmandu on 5th December 2019

Progress Report of ICIMOD-Mountain Chair [December 2019 to December 2020]

Visit to Myanmar

As per the ToR, I visited Myanmar from 20.01.2020 to 25.01.2020.

- On 20th January 2020, I visited Yangon, Myanmar and went to National Education Policy Commission, Govt of Myanmar and met Prof. Myo Kwe, Chairman of National Education Policy Commission, and other officials and discussed on roles of ICIMOD-HUC and Indian Universities and Higher Education systems of India and Myanmar (**Fig. 2**). Prof. Myo briefed me about higher education systems and Universities in Myanmar, their plan to upgrade and strengthen the Universities.

- On 21st January 2020, I visited Yangon University, Yangon and delivered a talk at Department of Industrial Chemistry, on "Asian fermented foods: Culture and Metagenomics" (**Fig. 3**). Several faculty members and students participated in the talk. In the afternoon, I had personally interacted with MSc and PhD students of this University on how to prepare a research work plan for MSc dissertations, and PhD thesis.

- On 22nd January 2020, I went to Mandalay University, and met Rector Prof. Thida Win, Professor Nyo Nyo, Prof. Min and Prof. Nilar, Heads of various Departments of Mandalay University (**Fig. 4**). On 23rd January 2020, I delivered a talk at the Conference hall and highlighted the HUC activities and roles of Indian Universities and higher education & research to officials and all Heads of the Departments of University of Mandalay. In afternoon, I visited various academic Departments of the University and delivered a lecture to MSc and Ph students of Department of Industrial Chemistry and Department of Geography, University of Mandalay, Mandalay on "Asian fermented foods: Culture and Metagenomics" (**Fig. 5**). On 24th December 2020, I went to a traditional village of Myanmar at Pyinnolwin in Mandalay state with Prof. Nilar and her departmental colleagues and two MSc students. This village is known for production of *pe poke*, an ethnic fermented soybean food of Myanmar. I documented the traditional methods of preparation, tasted the product, recorded its sensory property and interacted with rural producer (**Fig. 6**), and collected samples for analysis of metagenomics and nutritional value at my lab in India.

- In afternoon of the 24th January 2020, I met Shri Nandan Singh Baisoria, Consul General of India at Consulate General of India office, Mandalay as a courtesy call. Then I visited Myanmar Institute of Information Technology which was constructed under India-Myanmar MoU and financially supported by India for academic and infrastructure

Fig 2: Meeting and discussion with Prof. Myo Kwe, Chairman and officials of National Education Policy Commission, Govt of Myanmar at Yangon on 20th January 2020

Fig 3: Department of Industrial Chemistry, University of Yangon, Myanmar

Fig 4: Office of Rector, Mandalay University

Fig 5: Department of Industrial Chemistry and Department of Geography, University of Mandalay, Mandalay

Fig 6: *Pe poke* collection: Pyinnolwin village in Mandalay state

Fig 7: Karmaveer Bhaurao Patil College, Vashi, Mumbai 2nd March 2020 at Mumbai

Proposed Visits to China and Bhutan

- Due to COVID-19 pandemic and lock down all over the world, my proposed visit to Bhutan and China could not be materialized during 2020.

- I tried several times to link with South West Minju University in China and Royal University of Bhutan, but the officials in these Universities were reluctant to organize the webinar probably die to lock down and COVID-19 pandemic.

1. Key-note speaker (Physical) in various Institutes/Universities in Webinar as ICIMOD Mountain Chair

SL	Purpose of visits/activities	Institute and Place	Country	Duration	Sponsor for my participation
1	Key note Lecture: "Sequence-based Taxonomy and Omics of Some Ethnic Fermented Foods and Beverages of India"	2nd Annual National Conference of Midnapore City College 'MCCCON 2020' on "Bridge Between Bioinformatics and Nutrigenomics" at Midnapore City College, Midnapore, West Bengal	India	5-6 February 2020	Organizer of Midnapore City College, Midnapore
2	Key note Lecture: "History and Micro-omics of Indian Fermented Foods and Beverages" **(Fig. 7)**	National Conference on "Innovative Approaches and Emerging Issues in Food and Pharmaceutical Microbiology" organized by Rayat Shikshan Sanstha's Karmaveer Bhaurao Patil College, Vashi, Mumbai	India	2nd to 3rd March 2020	Organiser of Karmaveer Bhaurao Patil College

2. Key-note speaker in various Institutes/Universities in Webinar as ICIMOD Mountain Chair

SL	Purpose of visits/activities	Institute and Place	Country	Duration	Sponsor for my participation
1	Invited Speaker in Webinar "Importance of Healthy Ethnic Foods of the Himalayas"	Webinar on "Traditional Foods in the Himalayas in view of COVID-19" organised by GBPIHED, Almora	India	5th June 2020	Online (no cost)
2	Coordinator and speaker:" Gastronomy and Health Benefits of the Hindu Kush	Webinar on "Traditional Foods and their in health and nutrition security in the HKS" organised by HUC, ICIMOD, Kathmandu	Nepal	24th June 2020	Online (no cost)

	Himalayan Ethnic Foods”				
3	Panel Discussion	National Webinar on “Fermented Foods-Current status and future prospects” organised by Dairy Microbiology Department, SMC College of Dairy Science, Anand Agricultural University in association with its Alumni Association, Gujarat State Biotechnology Mission (GSBTM) and SASNET-Fermented Foods, Anand, Gujarat	India	25th June 2020	Online (no cost)
4	Key-note lecture on: “Indian Ethnic Fermented Foods and Beverages: Past and Present”	Webinar on “Fermented Foods and Beverages” organised by Central Institute of Technology, Kokrajhar, Assam	India	20th August 2020	Online (no cost)
5	Key-note lecture on: “Development in the field of Metataxonomic and Foodomics of Indian Ethnic Fermented Foods from Past to Present”	National Webinar on “New Horizon in Omics in Microbial Research”, organised by Department of Botany, Kalimpong College, Kalimpong	India	22nd August 2020	Online (no cost)
6	Key-note Speaker: “Glutne-free finger millet-based foods of the Himalayas”	Himalaya Day Celebration: National Webinar on Himalaya and Nature, organised by Indian Institute of Millet Research, ICAR, UNDP, Hyderabad	India	9th September 2020	Online (no cost)
7	Key-note lecture on: Gastronomy of Gorkha Ethnic Foods and Beverages: History, Culture and Metagenomics	Webinar on Gorkha Cuisine, organised by Darjeeling Society for Environment, Darjeeling	India	27th September 2020	Online (no cost)
8	Invited speaker: “Dietary History, Metataxonomic and Health Benefits of Indian Ethnic Fermented Foods”	National Webinar on: “Food, Nutrition and Health”, organised by National Institute of Planet Genomic Research, New Delhi	India	27th November 2020	Online (no cost)

9	Invited speaker: "HKH Ethnic Foods to combat COVID-19"	Webinar on "South-South Cooperation Forum on Food Security and Agrobiodiversity in Times of COVID-19 and Climate Change", organized by UNEP together with UNDP, UN Women, IFAD, IFPRI，ICIMOD and the Global Alliance for the Future of Food, Nairobi	Kenya	2nd December 2020	Online (no cost)
10	Invited Panelist	International Webinar on "Fermented Functional Foods-Current status and future prospects" organized by SMC College of Dairy Science, Anand Agricultural University in association with its Alumni Association, Gujarat State Biotechnology Mission (GSBTM) and SASNET-Fermented Foods, Anand, Gujarat	India	15th December 2020	Online (no cost)
11	Invited speaker on: "Ethnic Foods for combating COVID-19"	Webinar on "International Symposium on Combating COVID-19 – Ethnopharmacology & Traditional Foods and Medicine", organized by IBSD, Imphal, Manipur	India	18th December 2020	Online (no cost)

Research Activities:

- During my visit to Mandalay University, Myanmar in January 2020, I collected samples of *pe poke*, an ethnic fermented soybean food of Myanmar from traditional village at Pyinnolwin in Mandalay state on 23rd January 2020 with help of faculty members and students of Mandalay University. My visit was sponsored under the ICIMOD Mountain Chair, and the research work at his laboratory was supported by Department of Biotechnology, Ministry of Science and Technology, Government of India. I documented the traditional methods of preparation, tasted the product, recorded its sensory property and interacted with rural producer, and collected samples and brought to Department of Microbiology, Sikkim University, Gangtok for metagenomics analysis.

- In this research work, I included my PhD students viz. Mr. Pynhunlang Kharnaior and Ms. Priyambada Pariyar from Department of Microbiology, Sikkim University; Prof. Ni Lar, Prof. Khin Si Win, Prof. Ae Mar of Department of Industrial Chemistry, University of Mandalay; Prof. Nyo Nyo of Department of Geography, University of Mandalay. The research work was also assisted by Dr. Namrata Thapa, Head, DBT-Hub of Nar Bahadur Bhandari Degree College, Sikkim University.

- We published the original research paper on metagenomics of *pe poke*, an ethnic fomented soybean food of Myanmar in *Plos One*, one of the prestigious scientific journals. This is the first publication on metagenomics of *pe poke* of Myanmar in the world.

  Tamang, J.P., Kharnaior, P., Pariyar, P., Thapa, N., Lar, N., Win, K.S., Mar, A. and Nyo, N. (2021). Shotgun sequence- based metataxonomic and predictive functional profiles of *Pe poke,* a naturally fermented soybean food of Myanmar. *PLoS ONE* 16(12): e0260777. https://doi.org/10.1371/journal.pone.0260777. (**Impact Factor: 3.752**). **(Citations: 2).**

Abstract

Pe poke is a naturally fermented sticky soybean food of Myanmar. The present study was aimed to profile the whole microbial community structure and their predictive gene functionality of *pe poke* samples prepared in different fermentation periods viz. 3 day (3ds), 4 days (4ds), 5 days (5ds) and sun-dried sample (Sds). The pH of samples was 7.6 to 8.7, microbial load was 2.1-3.9 x 10^8 cfu/g with dynamic viscosity of 4.0±1.0 to 8.0±1.0cP. Metataxonomic profile of *pe poke* samples showed different domains viz. bacteria (99.08%), viruses (0.65%), eukaryota (0.08%), archaea (0.03%) and unclassified sequences (0.16%). *Firmicutes* (63.78%) was the most abundant phylum followed by *Proteobacteria* (29.54%) and *Bacteroidetes* (5.44%). *Bacillus thermoamylovorans* was significantly abundant in 3ds and 4ds ($p<0.05$); *Ignatzschineria larvae* was significantly abundant in 5ds ($p<0.05$), whereas, *Bacillus subtilis* was significantly abundant in Sds ($p <0.05$). A total of 172 species of *Bacillus* was detected. In minor abundance, the existence of bacteriophages, archaea, and eukaryotes were also detected. Alpha diversity analysis showed the highest Simpson's diversity index in Sds comparable to other samples. Similarly, a non-parametric Shannon's diversity index was also highest in Sds. Good's coverage of 0.99 was observed in all samples. Beta diversity analysis using PCoA showed no significant clustering. Several species were shared between samples and many species were unique to each sample. In KEGG database, a total number of 33 super-pathways and 173 metabolic sub-pathways were annotated from the metagenomic Open Reading Frames. Predictive functional features of *pe poke* metagenome revealed the genes for the synthesis and metabolism of wide range of bioactive compounds including various essential amino acids, different vitamins, and enzymes. Spearman's correlation was inferred between the abundant species and functional features.

(1) We have acknowledged the ICIMOD in our publication during my Mountain Chair tenure.

Yearly Progress Report of ICIMOD-Mountain Chair w.e.f. December 2020 to December 2021 of Professor Dr. Jyoti Prakash Tamang

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Due to COVID-19 Pandemic, all above mentioned activities could not be implemented physically in 2021, however, some of them have been accomplished in online mode.

- During 2021, I have delivered several lectures/talks in different aspects of food sciences and microbiology as Key-note speaker/Invited speaker in online mode as follows:

SL	Purpose of visits/activities	Institute and Place	Country	Duration	Mode
1	ICIMOD Mountain Chair Lecture at Royal University of Bhutan, Thimphu	Developments in metagenomics and health promoting benefits of ethnic fermented and beverages of the Himalayas including of Bhutan	Bhutan	29 January 2021	Online
2	Key-note Speaker: "Revitalizing ethnic cuisine for improved nutrition, nature positive food production and equitable livelihoods, organised by ICIMOD, Kathmandu	Himalayan ethnic foods: gastronomy, cuisine and health benefits	Nepal	8 June 2021	Online
3	Invited Speaker: International Conference on Forests, trees and agroforestry for diverse sustainable landscapes, Kunming, China; Co-organized by the CGIAR Research program on Forests, Trees and Agroforestry (FTA) and the Kunming Institute of Botany, Chinese Academy of Sciences (CAS)	Indigenous crops and their roles in food and nutritional security in the Eastern Himalayas	China	23 June 2021	Online

4	Invited lecture: Department of Microbiology, Parul University, Jaipur	Indian ethnic fermented foods: ancient to metagenomics	India	20 August 2021	Online
5	Invited Speaker: Indo-Japan SAKURA Science Cafè 2 'Sunrise in Clouds', Dailab Piknikh Series Xxxviii-Xxxxii	DAICENTER-Sikkim University: Omics and Data Mining	Japan	27 August 2021	Online
6	Invited Speaker: Universitas Padjadjaran, Bandung, Indonesia	"Ethno-microbiology" to metagenomics of ethnic fermented foods of Asia	Indonesia	28 October 2021	Online
7	Invited Lecture: Asian Conference of Lactic Acid Bacteria 11 (ACLAB11), Japan	"Ethno-microbiology" of Indian fermented foods: conventional to sequence-based taxonomy	Japan	5 November 2021	Online
8	Invited Speaker: Internal Conference on Microbes in Sustainable Development, New Delhi	Integrative metagenomics and metabolomics of some Himalayan fermented foods; microbial communities and health benefits	India	17 November 2021	Online
9	Invited Speaker: Asia Soy Excellence Center – Food Protein (ASEC-FP) Soy Food And Beverage Basic Course 3 (Bc3). Module 3: Topic 3.1 Current Soy Food Products and Processing Technologies: Traditional Soy & Value-Added Soy Products	Traditional fermented soybean food products: characteristics and processing technology	Singapore	22 November 2021	Online
10	Key-note Speaker: The 10th International Conference on Fermented Foods, Health Status and Social Well-being, University of Peradeniya, Sri Lanka	Genomics- metabolites data mining of some Asian fermented foods: microbial communities and health benefits	Sri Lanka	17 December 2021	Online

Yearly Progress Report of ICIMOD-Mountain Chair w.e.f. December 2021 to December 2022 of Professor Dr. Jyoti Prakash Tamang

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Mission of my visits to Nepal as ICIMOD Mountain Chair in 5-9th June 2022

The objective of ICIMOD Mountain Chair is to connect and interact with different HUC in in the HKH regions. Due to COVID-19, my activities as Mountain Chair was totally virtual in 2020 and 2021 except my visit to Yangon University and Mandala University in Myanmar in January 2020. Now the travel restriction in Nepal is relaxed, so I visited Kathmandu and Jiri in Nepal during 4th June to 9th June 2022 to deliver the talk on importance of beneficial microorganisms in promoting health benefits and nutritional value of some ethnic fermented foods and beverages and also to advocate the the role of higher education mainly in science to rural Nepal.

5th June: I (accompanied by my wife Dr. Namrata) visited Jiri accompanied by two young scholars of ICIMOD (thanks to Dr. Shachi) and staff of Nepali Chaurasi Byanjan Pvt. Ltd Shri Shankar Jiril and interacted with Home Stay providers and mangers, delivered talk to ethnic food tourism program; Mayor and member of Jiri Municipality. The visit was organised by Nepali Chaurasi Byanjan Pvt. Ltd.

6th June: Visited Charikot and delivered talk to local hoteliers and restaurant owners.

7th June: ICIMOD meeting with DG and HUC over dinner hosted by Dr. Pema Gyamtsho, Director General of ICIMOD, joined by other guests including first Secretary of Indian Embassy.

8th June: Delivered special talk on Validation of Ethno-microbiological Knowledge of the Himalayan People for Production of Fermented Foods at NEFOSTA (Nepal Food Scientists and Technologists Association), Kathmandu and interacted with NEFOSTA members. In the afternoon, we met the Excellency President of Nepal at Rastapati Bhawan

9th June: Key note speaker and Chief Guest at All Nepal Chefs Conference, Kathmandu organised by Nepali Chaurasi Byanjan Pvt. Ltd. At Kathmandu.

Photo: Dinner hosted by Dr. Pema Gyamtsho, DG of ICIMOD at Kathmandu on 7th June 2022

Photo: With Her Excellency President of Nepal on 8th June 2022

Visit to Cambodia in 21-27th August 2022

Since my research work is focused in the ethnic fermented foods mostly the similar types of ethnic fermented soybean foods of HKH regions and other Asian countries. *Sieng*, an ethnic fermented food of Cambodia has not been scientifically studied in details mostly in terms of metagenomic and metabolomics. *Sieng* is very similar to the Burmese *pe poke*; *kinema* of India, Nepal and Bhutan, and *natto* of Japan. Hence, I visited Cambodia.

23rd August 2022: We visited Faculty of Ago-Industry, Royal University of Agriculture, Phnom Penh, Cambodia, and met the Rector and Dean. I delivered talk and interacted with students.

24th to 26th August 2022: Visited different villages and collected the samples of *sieng* and documented the traditional practice knowledge of production. I brought the dried samples to my lab for analysis.

We analyzed 6 samples of *sieng*:

1. In DPPH radical scavenging activity (antioxidant), S-4 of Battambang has the highest percentage of activity with 54.11± 0.09%, followed by S-1 of Mondulkiri with 48.59± 0.06%. In the previous report, DPPH radical scavenging percentage was found to be more than 50%.

2. However, S-6 of Kampong Cham has the lowest DPPH radical scavenging percentage activity (anti oxidant) with 32.97± 0.07%, which denotes that the sample has less reduction capacity of DPPH out of all six *sieng* samples.

3. Previous studies reported that fermented soybean has significantly higher radical scavenging (anti-oxidant) activity than those of cooked non-fermented soybean (CNF). This higher anti-oxidant activity can be attributed to the extensive hydrolysis of proteins and production of phenolic compounds with higher anti-oxidant properties through fermentation step.

4. S-4 of Battambang has the highest anti-inflammatory activity with a concentration of 126.97± 1.07 µg NDGA/g, followed by S-3 of Kampong Thom and S-6 of Kampong Cham with a concentration of 122.08± 2.39 µg NDGA/g and 117.68± 0.21 µg NDGA/g, respectively. A known LOX inhibitor, NDGA (Nordihydroguaiaretic acid) was used as standard in this assay.

5. In anti-obesity, *S-5* of Phnom Penh has the highest lipase inhibition percentage of 48.26± 1.05%. However, S-4 of Battambang has the lowest lipase inhibition activity (anti-obesity) of 11.75 ± 0.69%. The concentration of lipase inhibitory activity in rice miso increased with addition of black soybean as well as prolonging of fermentation as reported in some previous studies.

6. Additionally, in anti-diabetic properties, S-4 of Battambang has the highest amylase inhibition percentage of 77.18 ± 0.02 %, followed by S-1 of Mondulkiri with an inhibition percentage of 74.13± 0.06 %.

7. Acarbose was used as positive control for anti-diabetic assay, and it shows the inhibition of 88%. Higher % of inhibition reflects the higher anti-diabetic affect.

Photo: With Rector and Deans of Royal University of Agriculture, Phnom Penh, Cambodia on 23rd August 2022

Photo: Delivering lecture to Food Science students of Royal University of Agriculture, Phnom Penh, Cambodia on 23rd August 2022

First-ever meeting of two coterminous ICIMOD Mountain Chairs: Professor Jyoti Prakash Tamang of India and Prof. Christopher Scott of USA in 31st October to 3rd November 2022

Prof. Tamang hosted Prof. Christopher Scott of Pennsylvania State University - USA at Sikkim University for a series of interdisciplinary and applied-research discussions to address collaborative opportunities on themes including climate impacts on mountain water towers, hydro-energy development tradeoffs ranging from local communities to South Asia-wide cooperation, and ethno-microbiology of the Himalayan foods as well as Sikkim's commitment to organic agriculture.

Prof. Scott interacted with students, post-doctoral fellows, and professors in the Department of Microbiology (to discuss food systems), Department of Geology including Centre of Excellence on Water resources, Cryosphere and Climate Change, (glacial melt, GLOF outburst floods, and water security), and various other departments of Sikkim University.

For his official lecture titled "Water-energy-food nexus: Himalayan hydropower tradeoffs for resource security with carbon neutrality," Prof. Scott was warmly welcomed by Prof. Tamang and introduced by Vice-Chancellor Prof. Avinash Khare. The lecture was attended by a diverse group of academic participants ranging from anthropology to geology. The question and answer sessions raised important topics of hydropower sustainability including graduate students who queried Prof. Scott on local social and environmental impacts. Follow-up potential for Penn State – SU collaborations under Himalayan University Consortium framework was highlighted and both Prof. Scott and Prof. Tamang with SU colleagues committed for further collaboration.

The visit included time to for Prof. Scott to get exposure to Sikkim's incredible culture, cuisine, and natural beauty.

Photo 1: Prof. Scott is being welcomed by Professor Avinash Khare, Vice-Chancellor of Sikkim University (Central University) at Sikkim University, Gangtok in presence of Prof. Tamang on 2nd November 2022.

Photo 2: Two ICIMOD Mountain Chairs-Prof. Scott (USA) and Prof. Tamang (India) at Food Microbiology and Bioinformatics Centre, Department of Microbiology, Sikkim University, Gangtok on 1st November 2022.

Photo 3: Prof. Scott and Prof. Tamang with PhD and Post-doc students of Department of Microbiology, Sikkim University, Gangtok.

NASI and INSA Fellow:

I was conferred the Fellows of two highest academics in India viz. Indian National Science Academy, New Delhi in October 2022 and National Academy of Sciences, India, Prayagraj in November 2022 during my ICIMOD Mountain Chair.

During 2022, I have delivered several lectures/talks in different aspects of food sciences and microbiology as Key-note speaker/Invited speaker in online mode as follows:

SL	Purpose of visits/activities	Institute and Place	Country	Duration	Mode
1	Plenary lecture: Ethno-microbiology to genomic mining of Indian traditional fermented foods	28th Indian Convention of Food Scientists & Technologists on Virtual Platform, Mysore	Mysore, India	22 January 2022	Online
2	Key-note lecture: Use of Genomics and bioinformatics in fermented foods	15th National Seminar cum Workshop on 'Genomics and Bioinformatics in the High-throughput era', organised by North Bengal University	Siliguri, India	27th March 2022	Online
3	Keynote lecture: Foods in fermented foods	iConnect Event under ANB: "Nutriomics for Better Health", organised by National Institute of Plant Genome Research, New Delhi	New Delhi, India	30th March 2022	Online
4	Key-note lecture: Validation of Ethno-microbiological Knowledge of the Himalayan People for Production of Fermented Foods	Nepal Food Scientists and Technologist Association, Central Food Research Laboratory, Kathmandu	Kathmandu, Nepal	8th June 2022	Physical
5	Key-note lecture: The Himalayan Ethnic Foods for Culinary Tourism	The Himalayan Culinary Workshop organised by Nepali Chaurasi Bhanjan Ptd. and ICIMOD, Kathmandu, Nepal	Kathmandu, Nepal	9th June 2022	Physical
6	Key-note Talk: Integrative Approaches of Metagenomics & Metabolomics for Validation of Indigenous Knowledge of Food Fermentation	The 6th International Conference on "Current Issues in Agricultural, Biological, and Applied Sciences for Sustainable Development (CIABASSD – 2022)", organized by Himalayan	Kalimpong, India	12th June 2022	Physical

		Scientific Society for Fundamental and Applied Research (HIMSFAR), Kalimpong Science Centre, Deolo, Kalimpong			
7	Invited Speaker: Indian Fermented Dairy Products and Selection of Starter Cultures	Innovative Technologies for Industrializing Traditional Animal Products, organized by Universitas Padjadjaran, Bandung, Indonesia in Massive Open Online Course (MOOC, https://mooc.live.unpad.ac.id/) and Zoom for Online lecture	Bandung, Indonesia	3rd August 2022	Online
8	Invited Talk: Current Development in fermented foods in Asia.	Faculty of Ago-Industry, Royal University of Agriculture, Phnom Penh, Cambodia	Phnom Penh, Cambodia	23rd August 2022	Physical
9	Foundation Lecture: Impact of Ethno-Microbiology of the Himalayan Ethnic Fermented Foods on Agro-Environment and Nutritional Security	Annual Day Foundation Lecture of GB Pant of Institute of Environment and Development, Ministry of Environment and Forest, Panthnag, Sikkim	Gangtok, India	10th September 2022	Physical
10	Keynote Lecture: Domestication of some beneficial microbes for food fermentation	The 7th Annual International Conference of the Indian Network of Soil Contaminated Research (INSCR 2022) on "MODULATING THE ENVIRONMENT WITH MICROBES"	New Delhi, Inda	7th November to 11th November 2022	Online

11	Invited talk: Integrative Approaches of Metagenomics & Metabolomics Mining of Fermented Foods for Validation of Ethno-microbiological Knowledge of the Ethnic Indians	International Conference On Biotechnology, Sustainable Bioresources and Bioeconomy, Indian Institute of Technology Guwahati, India organized by Biotech Research Society of India	IIT-Guwahati, India	7th to 11th December 2022	Physical

PLOS ONE

RESEARCH ARTICLE

Shotgun sequence-based metataxonomic and predictive functional profiles of *Pe poke*, a naturally fermented soybean food of Myanmar

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1 Department of Microbiology, DAICENTER (DBT-AIST International Centre for Translational and Environmental Research) and Bioinformatics Centre, School of Life Sciences, Sikkim University, Gangtok, Sikkim, India, **2** Department of Zoology, Biotech Hub, Nar Bahadur Bhandari Degree College, Sikkim University, Tadong, Sikkim, India, **3** Department of Industrial Chemistry, University of Mandalay, Mandalay, Myanmar, **4** Department of Geography, University of Mandalay, Mandalay, Myanmar

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Abstract

Pe poke is a naturally fermented sticky soybean food of Myanmar. The present study was aimed to profile the whole microbial community structure and their predictive gene functionality of *pe poke* samples prepared in different fermentation periods viz. 3 day (3ds), 4 days (4ds), 5 days (5ds) and sun-dried sample (Sds). The pH of samples was 7.6 to 8.7, microbial load was 2.1–3.9 x 10^8 cfu/g with dynamic viscosity of 4.0±1.0 to 8.0±1.0cP. Metataxonomic profile of *pe poke* samples showed different domains viz. bacteria (99.08%), viruses (0.65%), eukaryota (0.08%), archaea (0.03%) and unclassified sequences (0.16%). *Firmicutes* (63.78%) was the most abundant phylum followed by *Proteobacteria* (29.54%) and *Bacteroidetes* (5.44%). *Bacillus thermoamylovorans* was significantly abundant in 3ds and 4ds ($p<0.05$); *Ignatzschineria larvae* was significantly abundant in 5ds ($p<0.05$), whereas, *Bacillus subtilis* was significantly abundant in Sds ($p<0.05$). A total of 172 species of *Bacillus* was detected. In minor abundance, the existence of bacteriophages, archaea, and eukaryotes were also detected. Alpha diversity analysis showed the highest Simpson's diversity index in Sds comparable to other samples. Similarly, a non-parametric Shannon's diversity index was also highest in Sds. Good's coverage of 0.99 was observed in all samples. Beta diversity analysis using PCoA showed no significant clustering. Several species were shared between samples and many species were unique to each sample. In KEGG database, a total number of 33 super-pathways and 173 metabolic sub-pathways were annotated from the metagenomic Open Reading Frames. Predictive functional features of *pe poke* metagenome revealed the genes for the synthesis and metabolism of wide range of bioactive compounds including various essential amino acids, different vitamins, and enzymes. Spearman's correlation was inferred between the abundant species and functional features.

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Citation: Tamang JP, Kharnaior P, Pariyar P, Thapa N, Lar N, Win KS, et al. (2021) Shotgun sequence-based metataxonomic and predictive functional profiles of *Pe poke*, a naturally fermented soybean food of Myanmar. PLoS ONE 16(12): e0260777. https://doi.org/10.1371/journal.pone.0260777

Editor: Ashwani Kumar, Dr. H.S. Gour Central University, INDIA

Received: April 30, 2021

Accepted: November 9, 2021

Published: December 17, 2021

Data Availability Statement: The sequences of pe poke metagenomes were submitted to National Center for Biotechnology Information (NCBI) under Bio project ID PRJNA694857 with Sequence Read Archive (SRA) Number: SRR13574418 (3ds), SRR13574417 (4ds), SRR13574416 (5ds) and SRR13574415 (Sds). The data has been released publicly on request under the following URL. Bioproject ID PRJNA694857 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA694857) SRR13574418 (3ds): https://www.ncbi.nlm.nih.gov/sra/SRR13574418 SRR13574417 (4ds): https://www.

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Funding: Jyoti Prakash Tamang is grateful to the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India for financial support through DBTDAICENTER project, sanction number: BT/B1/14/042/2017.

Competing interests: The authors have declared that no competing interests exist.

Introduction

The community-specific ethnic fermented foods have been centre of interest for their unique gastronomy as well as colossal microbial diversity [1]. Myanmar has several ethnic fermented foods and beverages including fermented soybeans, which have been traditionally prepared and consumed by more than 135 different ethnic communities [2]. Among fermented foods, traditional methods of fermentation of locally grown soybean is an ancient practice mostly seen in North-western regions of Myanmar bordering with North East states of India and North Western parts of Myanmar bordering with Northern Thailand. Both mould-fermented and bacterial-fermented soybean are prepared and consumed widely in Myanmar [3]. *Pe poke* is an ethnic fermented soybean food of northern Myanmar. There is no historical documentation of origin of *pe poke* in Myanmar, however, it is believed that soybean has been introduced to Myanmar from Yunnan province of China [4]. During traditional method of preparation of *pe poke*, soybeans are soaked in water overnight, dewatered, boiled and wrapped in leaves, and are kept in a warm place for natural fermentation of 3–5 days (Fig 1a and 1b). Sometimes, freshly prepared *pe poke* is mashed with addition of salt and hot pepper, shaped as flat wafers, and are sun dried (Fig 1c). Some people prefer to eat *pe poke* immediately after fermentation and make into a typical Burmese-style cuisine as a side dish (Fig 1d) and fried fritters (Fig 1e) with boiled rice in main meal. This is mostly observed in the North-western regions of Myanmar bordering with India, where similar types of sticky fermented soybean foods are prepared such as *hawaijar* in Manipur, *bekang* in Mizoram, *peruyaan* and *peron namsing* in Arunachal Pradesh and *axone* or *aakhone* in Nagaland states of India [5]. Whereas, in the North-eastern regions of Myanmar bordering with Thailand, freshly prepared *pe poke* is made into flat wafers, and are sun dried, which is similar to *thua nao* of Northern Thailand [6]. *Pe poke* is one of the delicacies in the

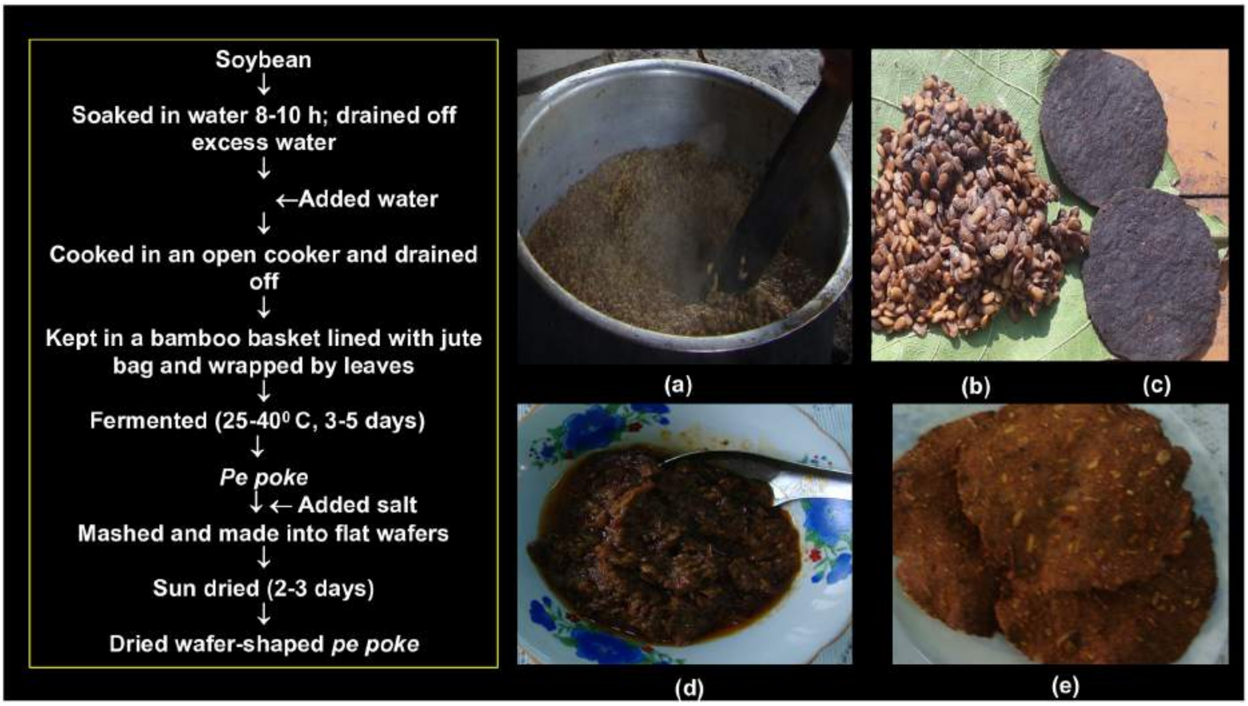

Fig 1. Traditional method of preparation of *pe poke* in Myanmar, (a) Boiling of soybeans; (b) freshly fermented *pe poke*; (c) sun-dried wafer-shaped *pe poke*; (d) *pe poke* curry; and (e) fried *pe poke* fritters.

https://doi.org/10.1371/journal.pone.0260777.g001

diets of ethnic people of Myanmar, however, the consumption of *pe poke* among younger generation is declining. Traditionally prepared *pe poke* is sold in local markets by marginal farmers in many regions of Myanmar. *Pe poke* is similar to other sticky fermented soybean foods of Asia such as *kinema* of India, Nepal and Bhutan, *natto* of Japan, *thua nao* of Thailand, *cheonggukjang* of Korea, *douchi* of Yunnan province of China and *sieng* of Laos [7, 8].

Though *pe poke* is a popular ethnic food in Burmese gastronomy, but information on microbiology and nutritional aspects of *pe poke* is very rare, except few reports on *Bacillus subtilis* as the main fermenting bacterium in *pe poke* [9, 10]. It is necessary to understand the microbial community structure in *pe poke*, which is prepared by natural fermentation, moreover, such rare ethnic product has not been studied in details to profile its microbial community structure. We choose the shotgun metagenome sequence tool to profile the entire microbial community up to species, which is considered as one of the most reliable metataxonomic tools [11], that may sequence the genomes of untargeted cells in a microbial community to decode community structures including culturable and unculturable bacteria, yeasts, fungi, virus and archaea in food samples [12]. Hence, we aimed to study the metataxonomic of abundant domains in naturally fermented *pe poke* of Myanmar, prepared in different fermentation periods, by shotgun metagenomic sequencing method, supported by machine learning tools. Functional profiles of metagenomes were also predicted using the SqueezeMeta pipeline [13] and KEGG database [14]. We believe this is the first report of microbial community structures in naturally fermented *pe poke* by shotgun metagenome sequence tool.

Materials and methods

Sample collection and analysis of pH

Samples of *pe poke*, traditionally prepared in different fermentation periods viz. 3 days (3ds), 4 days (4ds), 5 days (5ds) and sun-dried sample (Sds) were collected from Pyinnolwin village in Mandalay state of Myanmar. Samples were collected in pre-sterile containers kept in ice-box carriers and transported to the Department of Industrial Chemistry, University of Mandalay and stored at 4˚C. All samples were kept in ice-box carrier, by feeling with fresh ice in every 5–6 hours, till we reached to the Department of Microbiology, Sikkim University, Gangtok, India for immediate microbiological analysis. The pH of *pe poke* samples was determined by homogenizing 1 g of sample in physiological saline (0.85% sodium chloride, NaCl) and was measured using digital pH-meter (Orion 910003, Thermo Fisher Scientific, USA).

Total viable count

Samples were coarsely crushed by a sterile spatula, and ten grams of the sample were homogenized with 90 mL of 0.85% physiological saline in a stomacher lab blender 40 (Seward, United Kingdom) for 5 min. The homogenized samples were serially diluted in the same diluents, and 1 mL of appropriate diluents was plated in plate count agar (M091S, HiMedia, India) using pour plate method and incubated at 37˚C for 24 h. The number of colonies was counted as colony forming unit (cfu/g).

$$\text{cfu/g} = (\text{No. of colonies x dilution factor})/\text{volume of inoculum taken}$$

Measurement of viscosity

The dynamic viscosity of *pe poke* samples was determined using the method described by Ratha and Jhon [15]. Thirty grams of samples were mixed with 30 mL of distilled water and subjected to vigorous shaking in a conical flask (250mL) for 30 min. The slimy part was collected and 30 mL of its aliquot (100 rpm at 20˚C) was measured for dynamic viscosity in centipoise (cP) using a viscometer (DV1MRVTJ0, Brookfield AMETEK, MA, USA). The experiment was done in triplicate sets.

Genomic DNA extraction

Ten grams of coarsely crushed samples of *pe poke* were homogenised in Stomacher (400 Circulator, Seward, UK) with 90 mL of sterile 0.1 M phosphate buffer saline (pH 6.4) for 5 min. After homogenization, the homogenate was filtered and the filtrate was used for the extraction of genomic DNA using the Nucleospin® Food DNA kit (MACHEREY-NAGEL GmbH & Co. KG, Duran, Germany) as per the manufacturer's protocol. Concentration of DNA was then quantified using spectrophotometer (Eppendorf, USA). The quality of DNA was checked in 0.8% agarose gel electrophoresis and visualized using Gel Doc EZ imager (BioRad, USA).

Metagenomics sequencing and library preparation

Pe poke metagenome library preparation for long reads sequencing was performed by following the method of Sevim et al. [16]. The 10 μg of DNA was used to create the ONT (Oxford Nanopore Technologies) library. The generated DNA fragments was sheared using Covaris g-tubes (Covaris Inc., Woburn, MA USA) and DNA was repaired using NEBNext FFPE (Formalin-Fixed, Paraffin-Embedded) Repair Mix (New England BioLabs, Ipswich, MA USA) according to the manufacturer's instructions. AMPure XP beads (62 μl) were added to the FFPE-repair reaction and incubated at room temperature for 30 min on a Hula mixer, followed by two washes with 70% ethanol. Beads were then resuspended with 93 μl of nuclease free water and incubated for 30 min at room temperature on a Hula mixer; 90 μl of the eluate was then transferred to a clean 1.5 mL Eppendorf tube.

The fragmented and repaired DNA underwent end repair and A-tailing using the NEBNExt End Repair/dA-Tailing Module (New England BioLabs) following manufacturer's protocol: The reaction volume was doubled to 120 μl, incubation was performed at 20˚C for 20 min and at 65˚C for 20 min. AMPure XP beads (120 μl) were added to the end-prep reaction and incubated for 30 min at room temperature on a Hula mixer, followed by two washes with 70% ethanol. Beads were then resuspended in 31 μl of nuclease free water and incubated for 30 min at room temperature on a Hula mixer; 61 μl of the eluate was then transferred to a clean 1.5 mL Eppendorf tube. The resulting DNA was quantified using the Qubit HS DNA kit.

The resulting DNA ligation and clean-up were performed using the SQK-LSK108 kit (Oxford Nanopore Technologies, Oxford, United Kingdom) following manufacturer's instructions. The ligation reaction was incubated at room temperature for 10 min and then overnight at 4˚C. The ligated samples were purified using 40 μl of AMPure XP beads, incubated for 30 min at room temperature on a Hula mixer followed by two washes using the kit-provided wash buffer. The beads were resuspended in 15 μl of the kit-provided elution buffer and then incubated for 30 min at room temperature on a Hula mixer; 15 μl of the eluate was then transferred to a clean 1.5 mL tube and quantified using the Qubit HS DNA kit. The library was then sequenced on a MinION using the R9 flow cell sequencing chemistry and were processed using the MinKNOW software (v1.13.1).

Bioinformatics analysis

Metataxonomic. Raw data derived from MinION (TM) ONT (Oxford Nanopore Technologies) in fast5 format was converted into a fastq files using poretools v0.6.0 for the bioinformatics analysis of *pe poke* metagenome [17]. After conversion, the quality of the fastq files were then examined using NanoPlot [18] and generated the corrected-assembled data via canu-assembler [19]. A database derived from GenBank containing millions of protein sequences from bacteria, archaea, viruses, fungi, and other microbial eukaryotes was downloaded within Kaiju via *kaiju-makedb -s nr_euk*. Taxonomy assignment of the assembled quality sequences was performed using a taxonomical pipeline, Kaiju [20] in which a default "greedy algorithm" was used to map the sequences against the database [21]. A cut-off for a minimum required match length ($m = 11$, default), minimum match score of 80 ($s = 80$) and the E-value ($E = 0.05$) was set to filter the mismatches. Filtering of query sequences containing low-complexity regions was performed to avoid false positive taxon assignments that may cause by bogus matches or other sequencing noises [20]. Amino acid substitution model was performed with a total score for each match calculated as in amino acid sequence alignment and ranked a multiple match and taxon classification from the database. After translation of ORFs into a set of amino acid fragments, we ranked the fragments by their BLOSUM62 (BLOcks SUbstitution Matrix) score and start the database search with the highest scoring fragment [22]. The fragments were searched backwards via BWT (Burrows-Wheeler transform) algorithm against database [23] and the higher score of fragments in the search was used for classifying the reads and outputs the taxon identifier [20].

Predictive functional features. Predictive functional features of the metagenome was performed on Quality-filtered contigs using the SqueezeMeta pipeline version 1.3.0 [13]. After importing of data, the contigs of <500bp were removed using prinseq [24], followed by gene prediction of the assembled using Prodigal (v2.6.2) [25] and the predicted genes were searched for homologies against the functional databases using DIAMOND, computational tool for the alignment of sequencing reads against a protein reference database [26]. After running the DIAMOND, the method assigned as functions to each Open Reading Frames (ORF) was carried out using the fun3 method (fun3 method produced functional assignments to compare genes sequences against the functional database) for Clusters of Orthologous Groups/Non-supervised Orthologous Groups (COGs/NOGs) using evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) database [27] and Kyoto Encyclopedia of Genes and Genomes (KEGG) database [14]. In the process of analysis, the highest-scoring ORFs in the contig with an exceeding of 30% (default) were considered for annotation [13]. Best hits gene annotations were further processed for pathways prediction and enzyme classification [28]. Metabolic pathways assigned against the KEGG database was categorised in three level: high-level function (Level 1), lower-level function (Level 2) and the sub-pathways (Level-3) [29]. Enzymes involved in lysine biosynthesis, alanine, aspartate, glutamate, glycine, serine, threonine metabolism, pentose phosphate pathways, galactose metabolism and phosphotransferase system were mapped against the KEGG pathways database [30].

Statistical analysis

Inter species diversity. Significance among the abundant species (>1%) was calculated using Fisher exact test [31]. Inter species diversity of *pe poke* metagenome was performed among the samples (3ds, 4ds, 5ds and Sds) using Tukey's test in IBM SPSS v20.0 [32]. Shared and unique species was calculated using InteractiVenn: a web-based tool for the analysis [33].

Alpha and beta diversity. Differences of species distribution among the samples was measured using diversity indices, Simpson and Shannon diversity index was calculated, and principal coordinate analysis (PCoA) was plotted based on Bray-Curtis dissimilarities using PASTv4 (Paleontological Statistics Software Package) [34]. Furthermore, UPGMA (Unweighted Pair Group Method with Arithmetic mean) hierarchical clustering was performed for similarity analysis based on microbial communities which was compared between the samples and support the result observed in beta diversity [35].

Predictive functional features. The predictive functional profiles of *pe poke* were tested using Tukey's test to check the inter pathways distribution among the samples (3ds, 4ds, 5ds and Sds) using IBM SPSS v20.0 [32]. UPGMA hierarchical clustering was also performed to compare the functional distribution among the samples [35]. Heatmap visualization of functional profiles, level-1 and level-2 was carried out using a web tool: ClustVis [36]. Correlation between the major species and functional features was performed by a non-parametric Spearman's rank correlation using IBM SPSS v20.0 (Statistical Package for the Social Sciences), and a network-based visualization was generated using MetScape v3.1.3 in Cytoscape v3.8.2.

Results

The pH of *pe poke* samples was 7.6 to 8.7 with the microbial load of 2.1–3.9 x 10^8 cfu/g. The dynamic viscosity of samples was 4.0±1.0 to 8.0±1.0 cP (centipoise).

Microbial community

A total of 1085311 reads were obtained from all samples of *pe poke* with an average of 271327.7 reads per sample. Average length of the reads was found 901.5. Total number of bases recovered from the samples were 314,801,023 bases for 3ds, 176,709,193 bases for 4ds, 372,945,882 bases for 5ds and 41,455,352 bases for Sds, respectively. Shotgun metagenomic sequence analysis of *pe poke* metagenome showed different domains viz., bacteria, archaea, viruses and eukaryotes with 46 phyla, 328 families, 718 genera and 1475 species. Taxonomic classification at domain level revealed the abundance of bacteria (99.08%) followed by viruses (0.65%), eukaryota (0.08%), archaea (0.03%) and unclassified sequences (0.16%) (Fig 2a). At bacterial phylum level, *Firmicutes* was the most abundant phylum followed by *Proteobacteria*, *Bacteroidetes* and others (1.24%) (Fig 2b) including phyla with a relative abundance of <1% (S1 Table). *Bacillaceae* was the most abundant family followed by *Alcaligenaceae*, *Flavobacteriaceae*, *Sphingobacteriaceae*, *Planococcaceae*, *Enterococcaceae*, *Morganellaceae*, *Pseudomonadaceae* (Fig 2c) and others detected at <1% abundance (S2 Table). Taxonomic annotation

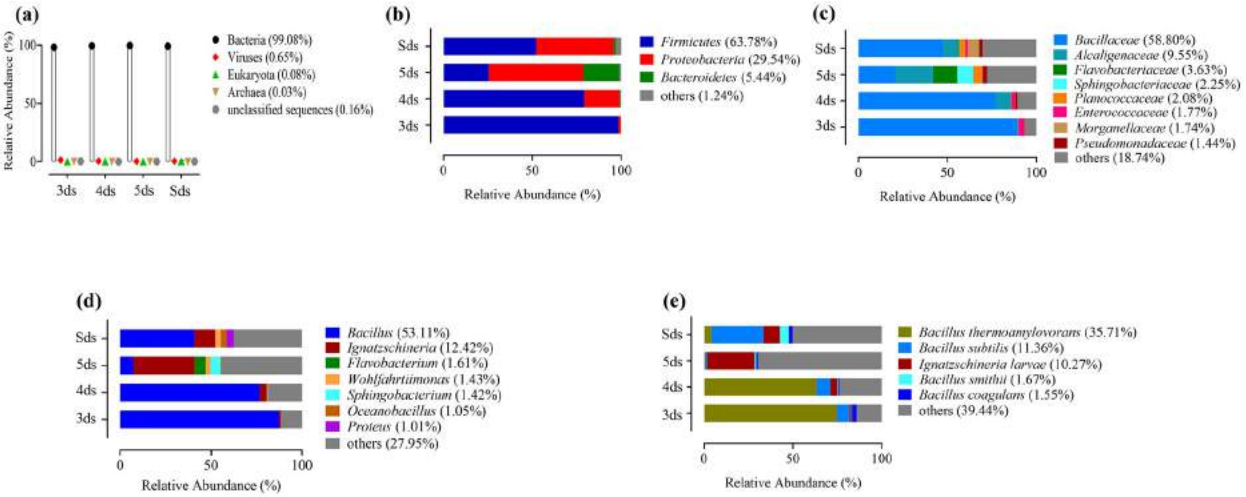

Fig 2. Relative abundance of microbial communities in *pe poke* (a) domain, (b) phyla, (c) family, (d) genera and (e) species.

https://doi.org/10.1371/journal.pone.0260777.g002

revealed the abundance of *Bacillus* (53.11%) at genus level followed by *Ignatzschineria*, *Flavobacterium*, *Wohlfahrtiimonas*, *Sphingobacterium*, *Oceanobacillus*, *Proteus* (Fig 2d) and others detected at <1% abundance (S3 Table). At species level, *Bacillus thermoamylovorans* was the most abundant species in *pe poke* samples, followed by *B. subtilis*, *Ignatzschineria larvae*, *B. smithii*, *B. coagulans* (Fig 2e) and others detected at <1% abundance (S4 Table). No phylum, family, genus and species with a relative abundance of >1% were detected from other domains viz. archaea, viruses and eukaryote in *pe poke* metagenomes.

Firmicutes was found abundant phylum in samples of 3ds, 4ds and Sds, whereas *Proteobacteria* was observed abundant in 5ds. In comparison among 3ds, 4ds and Sds, *Bacillus thermoamylovorans* was significantly ($p<0.05$) abundant in 3ds (Fig 3a and 3b) and *Ignatzschineria larvae* was significantly ($p<0.05$) abundant in 5ds (Fig 3a); whereas, *B. subtilis* was significantly ($p<0.05$) abundant in Sds (Fig 3b). Similarly, when compared among 4ds, 5ds and Sds, *B. thermoamylovorans* was significantly ($p<0.05$) abundant in the 4ds (Fig 3c and 3d), *Ignatzschineria larvae* in 5ds (Fig 3c) and *Bacillus subtilis* was significantly ($p<0.05$) abundant in Sds (Fig 3d and 3e). In *pe poke* metagenomic analysis, a total of 172 species of *Bacillus* were detected, out of which the abundant species with a relative abundance of >1% were *B. thermoamylovorans*, *B. subtilis*, *B. smithii* and *B. coagulans* (S5 Table). Besides *Bacillus*, lactic acid bacteria (LAB) were also detected with a cumulative abundance of 1.78%, which included 94 species (S6 Table). Apart from bacterial domain, taxonomic annotation also revealed 16 species of archaea (S7 Table), 31 species of eukaryotes i.e., 3 species of yeasts, 15 species of filamentous moulds and 13 species of other eukaryotes (S8 Table), and 33 species of virus (bacteriophages), out of which 21 species were *Bacillus* phages (S9 Table).

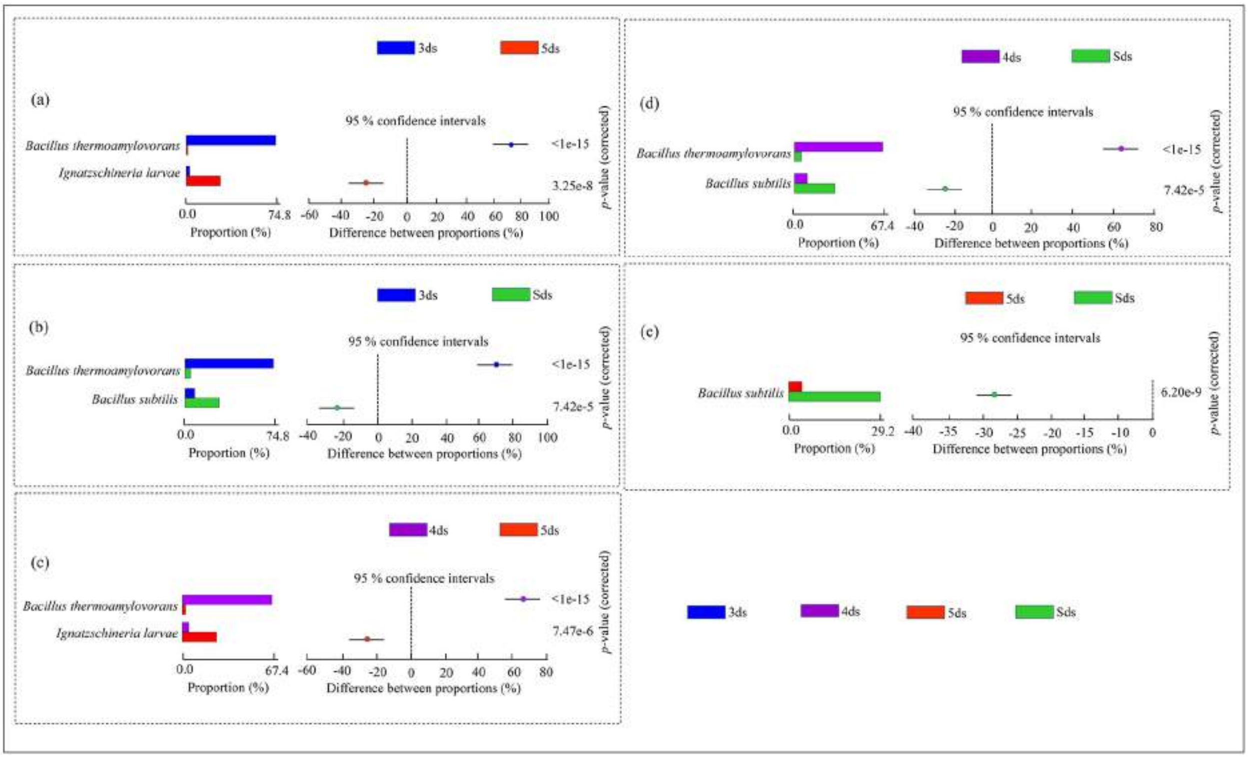

Fig 3. Fisher exact test performed at species level to observe the significant differences and similarities of predominant species comparing between the samples.

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Table 1. Indices of alpha diversity among the four samples of *pe poke*.

Indices	Samples			
	3ds	4ds	5ds	Sds
Simpson (1-D)	0.43	0.62	0.86	0.91
Shannon	2.25	3.14	5.46	5.99
Goods Coverage	0.99	0.99	0.99	0.99

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Diversity indices

Alpha diversity analysis showed the highest Simpson's diversity index in Sds comparable to other samples (Table 1). Similarly, a non-parametric Shannon's diversity index was also highest in Sds (Table 1). Good's coverage of 0.99 was observed in all the samples. Beta diversity analysis using PCoA (Fig 4a) and UPGMA (Fig 4b) showed no significant clustering. Statistically, in term of species abundance, the inter species diversity among the samples was calculated using Tukey's test (Fig 4c) and a significant difference was observed between 3ds and 4ds ($p = 0.006469$), 5ds ($p = 0.02537$) and Sds ($p = 0.01874$). Similarly, 4ds was significantly different from 5ds ($p = 0.0189$) and Sds ($p = 0.01227$), and 5ds was significantly different from Sds ($p = 0.006633$).

Shared and unique species

Metataxonomic annotation of *pe poke* metagenome revealed a huge diversity of microbial communities including shared and unique species (Fig 5a–5d). Based on different domains that have been classified via taxonomic classification, we observed about 204 bacterial core

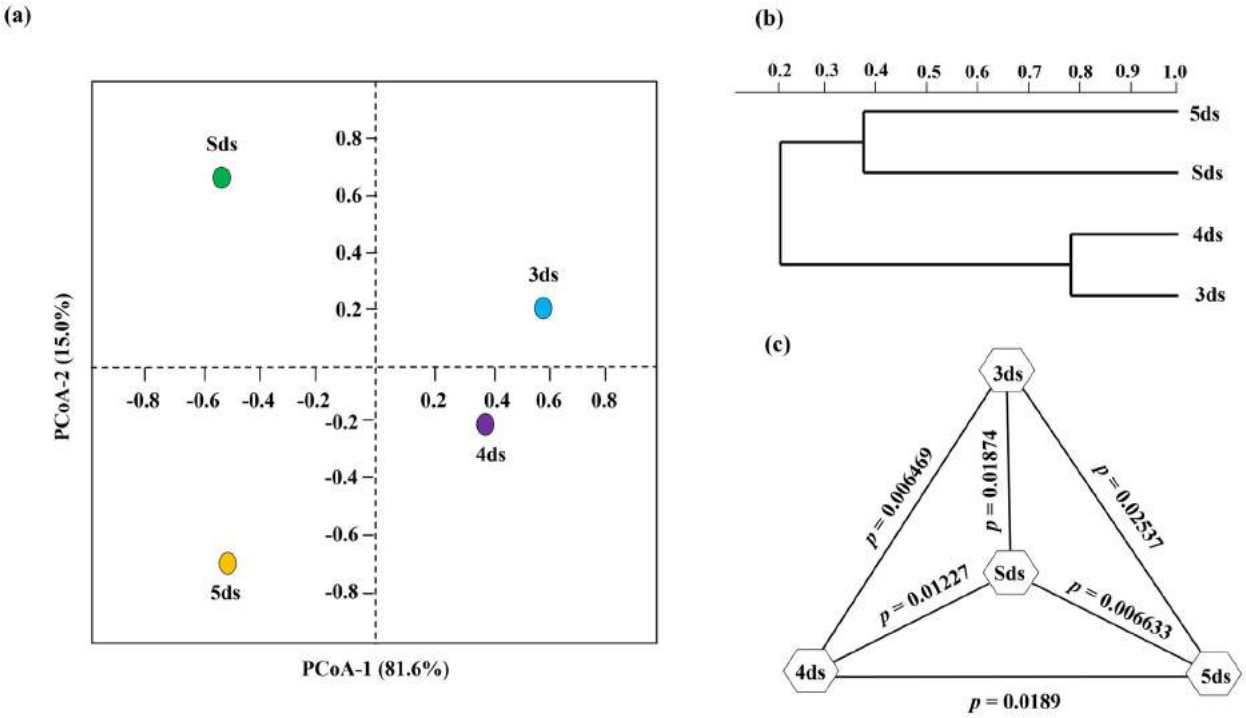

Fig 4. (a) PCoA of *pe poke* metagenomes based on microbial species. (b) UPGMA showed no clustering between samples, and (c) one-way ANOVA Tukey's test revealed the significant differences among samples ($p < 0.05$).

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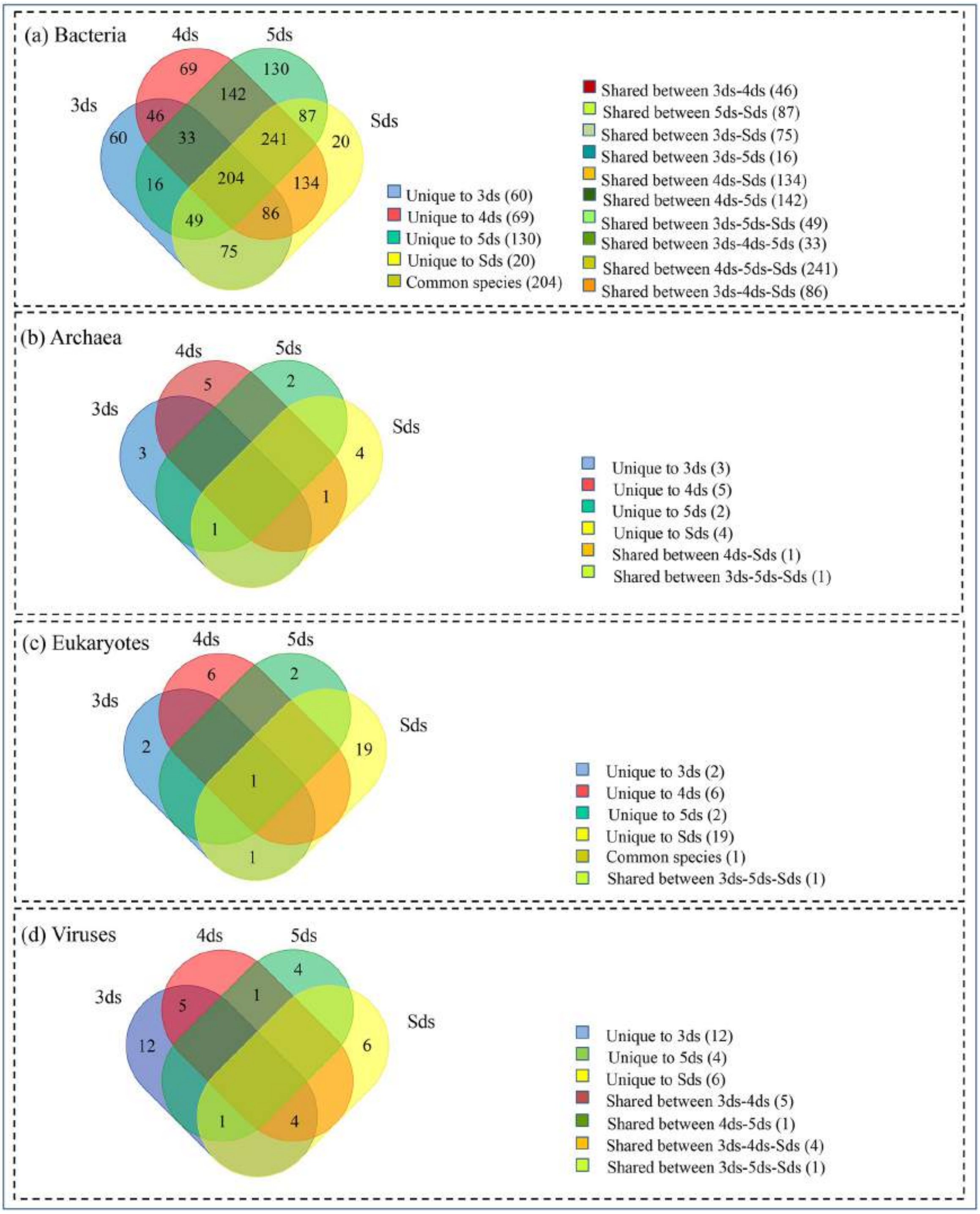

Fig 5. Shared, unique and common species in *pe poke* metagenome represented by InteractiVenn.

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species were common in all the samples (Fig 1a). Additionally, the unique species were 60, 69, 130 and 20 in 3ds, 4ds, 5ds and Sds, respectively (S10 Table). Among archaeal species, no core species was found common to all samples. *Methanobacterium formicicum* was shared between 3ds, 5ds and Sds, and *Halapricum salinum* was shared between 4ds and Sds (Fig 1b). The unique species were 3, 5, 2 and 4 in 3ds, 4ds, 5ds and Sds, respectively (S11 Table). Among eukaryota, *Mucor ambiguus* was the core species commonly found in all samples. *Batrachochytrium dendrobatidis* shared between 3ds 5ds and Sds (Fig 1c) and the unique species were 2, 6, 2, 19 in the 3ds, 4ds, 5ds and Sds, respectively (S12 Table). In the category of viruses, no common species was observed. *Aeribacillus* phage AP45 was shared between 4ds and 5ds, and *Geobacillus* virus E3 was shared between 3ds, 5ds and Sds (Fig 5d). The unique species were 12, 4, 6 in 3ds, 5ds and Sds, respectively (S13 Table).

Predictive functional features

The mapping of metagenomic sequences against the databases of orthologous gene groups (COG and KO) revealed many enriched functional features. About 56% were assigned to COG functional genes and the remaining 44% ORFs were assigned to KEGG functional pathways. In COG annotation, general function prediction only was the abundant followed by DNA replication, recombination and repair, amino acid transport and metabolism, carbohydrate transport and metabolism, transcription, translation, ribosomal structure and biogenesis, inorganic ion transport and metabolism, energy production and conversion, cell envelope biogenesis, outer membrane (S14 Table). In KEGG database, a total number of 33 super-pathways and 173 metabolic sub-pathways were annotated from the metagenomic ORFs. At KO level-1, metabolism was the most abundant followed by environmental information processing, genetic information processing, cellular processes, human diseases, organismal systems and poorly characterised (Fig 6a). At KO level—2, the abundant functional prediction was carbohydrate metabolism followed by other metabolisms (Fig 6b) and super-pathways with relative abundance of <1% mapped against KEGG (S15 Table). Furthermore, at KO level-3, super-pathways with relative abundance of <1% mapped against KEGG showed genes related to ABC transporters was the most abundant followed by other predictive metabolic pathways (S16 Table). Based on the distribution of functional features, no clustering of samples was observed by performing the UPGMA analysis (Fig 6c). Tukey's test was performed to check the significant differences of functional features between the samples (Fig 6d).

In enzyme classification, we detected genes encoding for enzymatic activity such as protease, serine protease, amylase, lipase, galactosidase, glucosidase, glutamate decarboxylase and functions involve in poly-γ-glutamic acid biosynthesis (S17 Table). Predictive metabolic pathways were mapped against the KEGG pathways database such as lysine biosynthesis (Fig 7), alanine, aspartate and glutamate metabolism (Fig 8), glycine, serine and threonine metabolism (Fig 9), pentose phosphate pathways (Fig 10) and galactose metabolism (Fig 11). The predictive enzymes involved in different pathways were observed such as lysine metabolism (S18a Table), alanine, aspartate and glutamate metabolism and 4-aminobutanoic acid (γ-aminobutyric acid, or GABA) (S18b Table), glycine, serine, threonine metabolism and ectoine biosynthesis (S18c Table), pentose phosphate pathways (S18d Table) and galactose metabolism (S18e Table).

Correlation between predominant species and predictive functions

Spearman's correlation was inferred between the abundant species and functional features (Fig 12). *Bacillus thermoamylovorans*, *B. subtilis*, *B. smithii* and *B. coagulans* were positively correlated with alanine, aspartate and glutamate metabolism, pentose phosphate pathway, and glycolysis/gluconeogenesis. Lysine biosynthesis and galactose metabolism were positively correlated

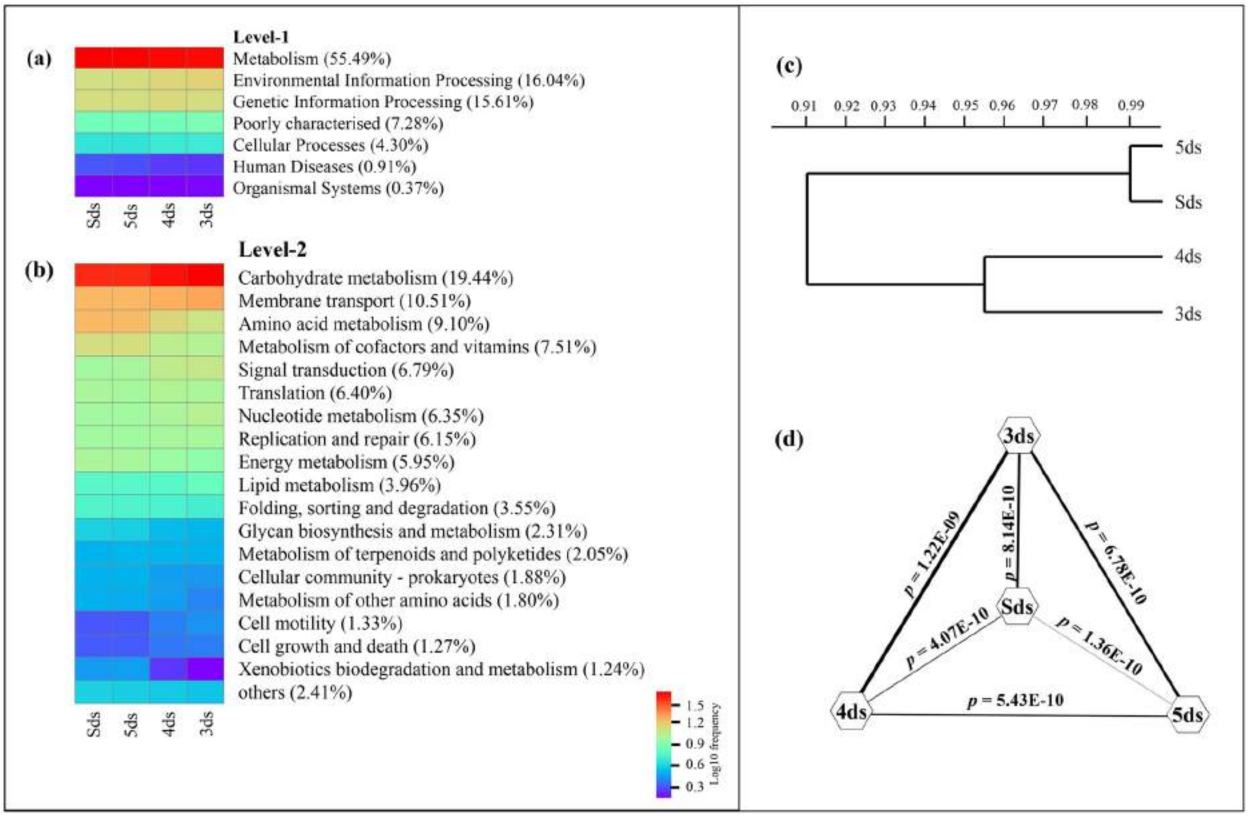

Fig 6. The abundant functional features in *pe poke* metagenome (a) Level-1 and (b) Level-2 with a relative abundance more than 1%. (c) UPGMA showed no clustering between samples and (d) significant differences among samples observed using one-way ANOVA Tukey's test.

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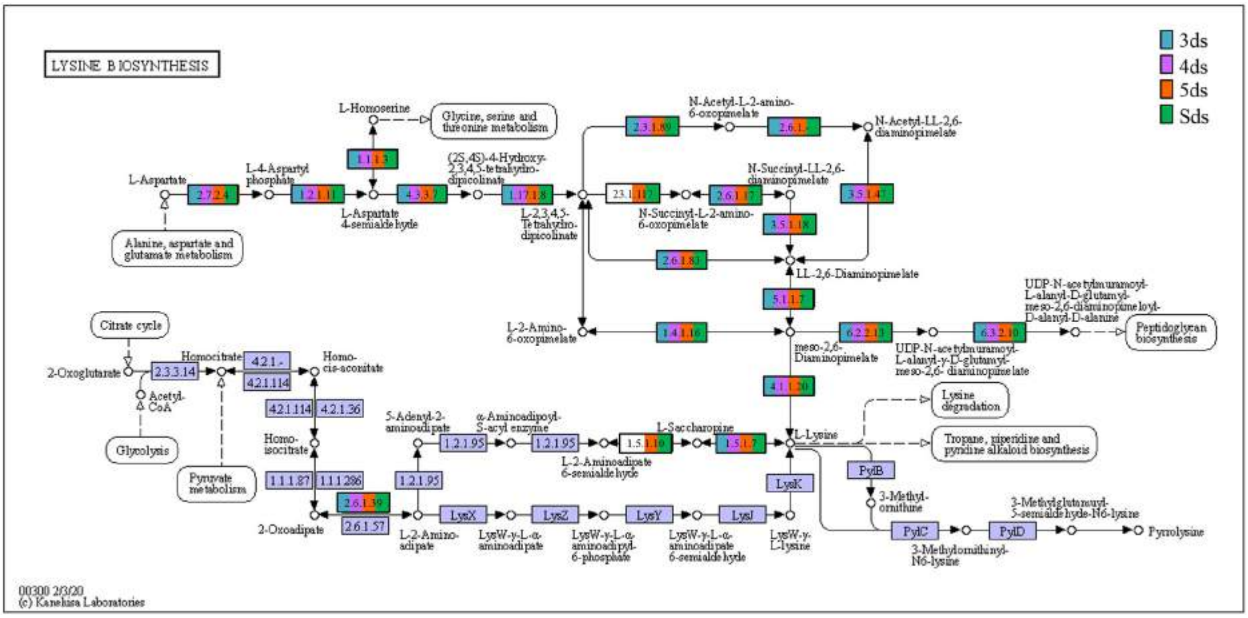

Fig 7. Enzymes involve in lysine biosynthesis detected in *pe poke* metagenome.

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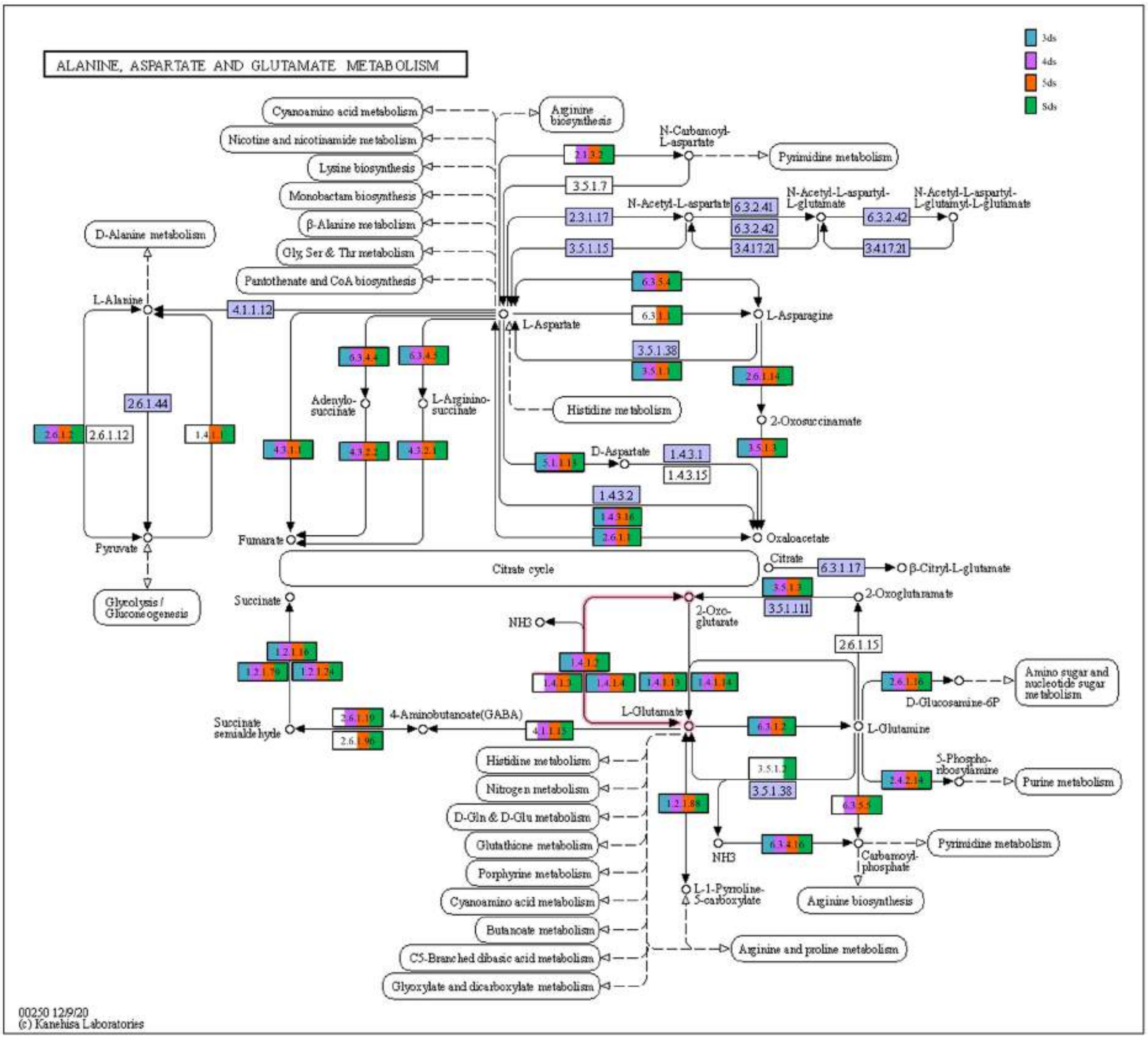

Fig 8. Enzymes involved in alanine, aspartate and glutamate metabolism and 4-aminobutanoic acid (γ-aminobutyric acid, or GABA) detected in *pe poke* metagenome.

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with *B. thermoamylovorans* and *B. coagulans*, whereas negatively correlated with *B. subtilis* and *B. smithii*. Glycine, serine and threonine metabolism and cysteine and methionine metabolism was positively correlated with *B. subtilis* and *B. smithii*, and negatively correlated with *B. thermoamylovorans* and *B. coagulans*. Among metabolism of cofactors and vitamins, *B. thermoamylovorans* and *B. coagulans* were positively correlated with thiamine metabolism; *B. smithii* with porphyrin and chlorophyll metabolism and *B. subtilis* with folate biosynthesis (Fig 12).

Discussion

Microbial community

Pe poke is an alkaline (pH 7.8–8.7) naturally fermented soybean food of Myanmar, which is prepared traditionally by ethnic Burmese people. Though *pe poke* is considered as a sticky

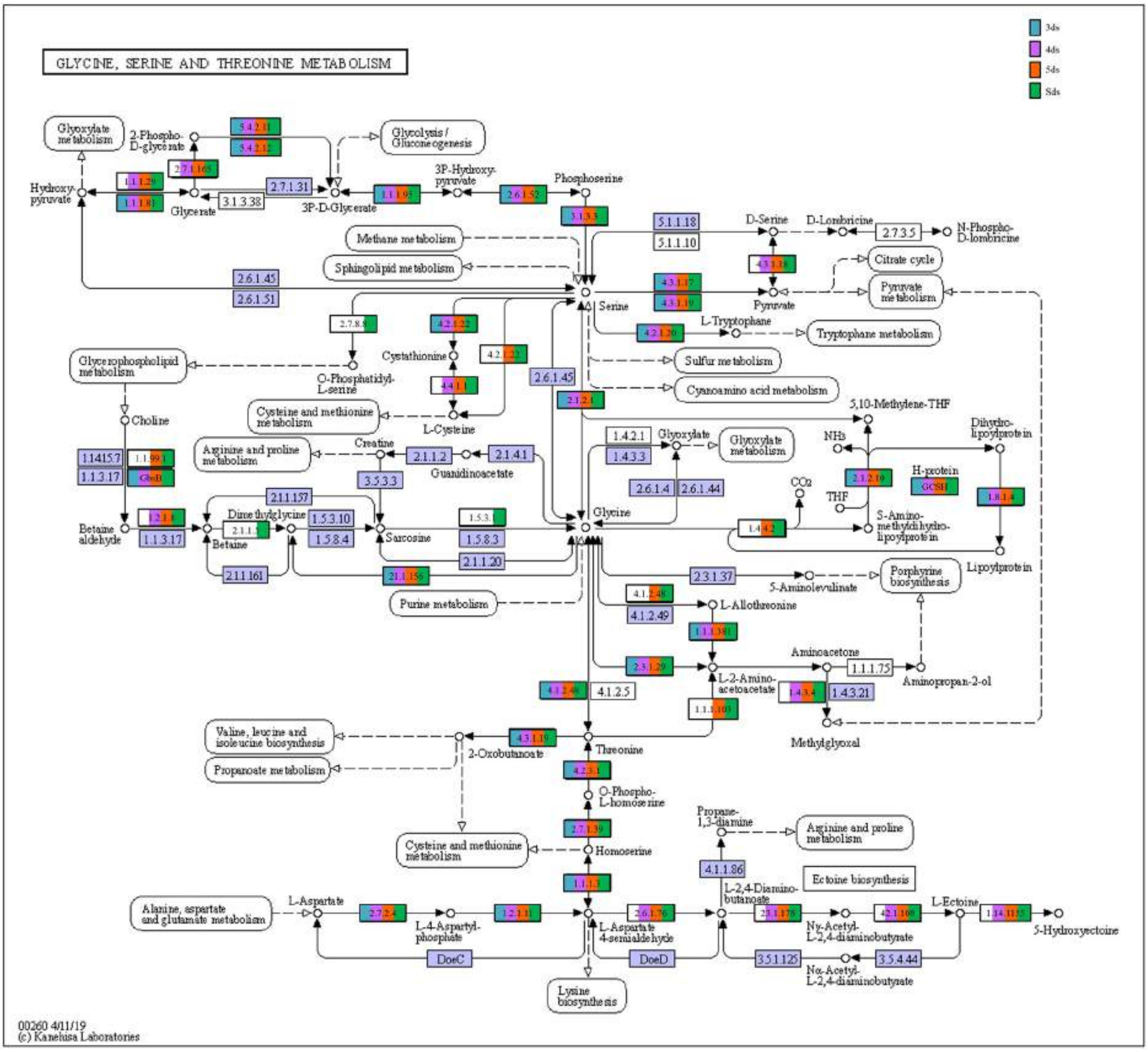

Fig 9. Enzymes involved in glycine, serine and threonine metabolism and ectoine biosynthesis detected in *pe poke* metagenome.

https://doi.org/10.1371/journal.pone.0260777.g009

fermented soybean food, however, the dynamic viscosity of samples was 8.0±1.0cP as compared with that of *natto*, a highly sticky Japanese fermented soybean with dynamic viscosity of >23cP [37]. The microbiological population of *pe poke*, as determined by cultural method, showed a viable load of 10^8 cfu/g, indicating its richness in microbial diversity. Since fermentation periods during natural fermentation of *pe poke* vary from 3 to 5 days, we collected the samples fermented for 3 days, 4 days and 5 days, and also the sun-dried samples for profiling the microbial community using the shotgun metagenome sequence tool to know the abundant microbial domains with their predictive functional features. Bacteria were detected as the most abundant domain, and the least abundant domains were archaea, eukaryotes and viruses, which reflects the comprehensive general picture of the microbial communities of *pe poke*.

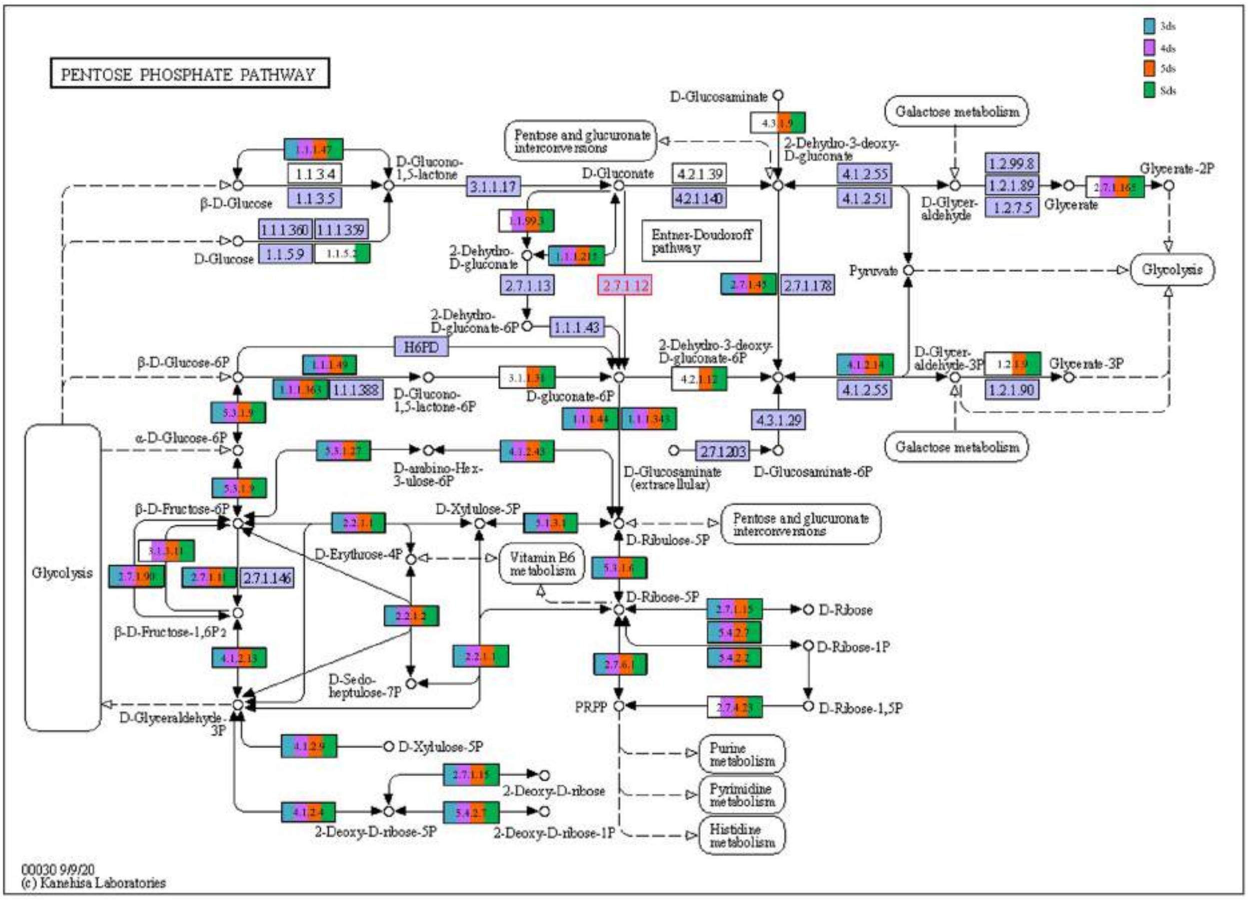

Fig 10. Enzymes involved in pentose phosphate pathways.

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The higher abundance of *Firmicutes* and the presence of *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* in the minority groups were previously reported in other fermented soybean foods such as *kinema* of India, Nepal and Bhutan [8], *douchi* of China [38] and *da-jiang* of Korea [39]. *Bacillaceae* and *Bacillus* were reported in *pe poke* as the abundant family and genus, respectively. A colossal interspecies diversity of *Bacillus* with more than 172 species was detected in *pe poke* metagenomes by shotgun sequence tool. By cultural method, only *B. subtilis* was reported in *pe poke* [9, 10]. At species level, we observed the abundance of *B. thermoamylovorans* in 3ds and 4ds, *Ignatzschineria larvae* in 5ds, and *B. subtilis* in Sds sample, respectively. *B. thermoamylovorans* is a heat resistant [40] and amylolytic bacterium [41], which is reported in *cheonggukjang* [42], *kinema* [8] and *douchi* [43], and it may also involve in producing thermo-stable enzymes during fermentation at high temperatures [44]. *B. subtilis*, the second abundant species in *pe poke*, is one of the major bacterial species in many Asian fermented soybean foods [8, 45–47]. We also observed *B. coagulans*, which is resistance to high temperatures, and produces various enzymes applicable to food industry [48]. The abundance of *B. smithii* in *pe poke* metagenome was also previously reported in fermented soybean foods such as *tungrymbai* of Meghalaya state and *bekang* of Mizoram state of North-East India [46]. Abundance of *Bacillus* species indicates high proteolytic activity, amylase activity and lipase activity [49–52]. *Ignatzschineria larvae* was also found abundant in 5 days-*pe poke*,

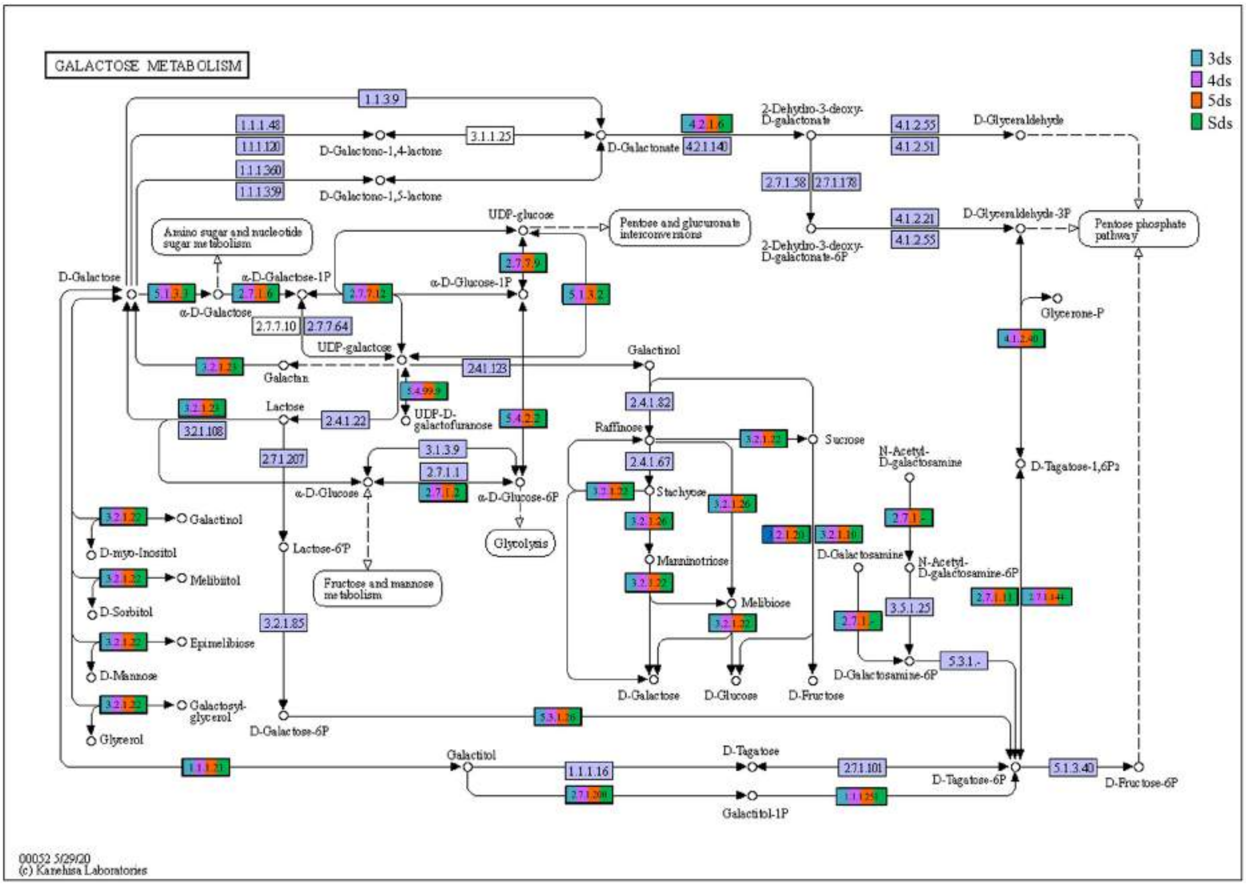

Fig 11. Enzymes involved in galactose metabolism.

https://doi.org/10.1371/journal.pone.0260777.g011

probably contaminated from flies [53], during prolonged fermentation under unhygienic condition. Some LAB were also detected in samples of *pe poke*, which may have beneficial antimicrobial activity against pathogenic bacteria [54].

Myoviridae, *Podoviridae* and *Siphoviridae* were the abundant families of viruses belonging to the order *Caudovirales* in *pe poke*. In fermented soybean food, bacteriophages have been reported to cause food spoilage [55] and the abnormal effect on products that may cause reduction of viscous poly-γ-glutamic acid in fermented soybean foods [56]. Bacteriophages may kill the beneficial starter, hamper the bacterial growth, delay fermentation process, yield low-quality, and lower down the bioactivities of the food product [57]. However, some suggested an alternative hypothesis that the presence of bacteriophages is considered to be a very useful therapy in reducing pathogenic bacteria in food products [58]. The presence of archaeal and eukaryotic species were in low abundances in *pe poke* metagenomes. Archaea contributes to development of taste, aroma flavour, dietary supplements, acetate production during fermentation, and even protect food from spoilage by yeasts [59]. Domain Eukarya consisted of yeasts, filamentous moulds, different species of algae, protozoa and parasites was detected in low abundances in *pe poke*. Filamentous moulds are known to contribute flavour in fermented soybean product [60] and possess high proteolytic activity [61].

Diversity index, which considers both number of species as well as relative abundance of each species for evaluating diversity [62], showed highest value for the Sds of *pe poke*, probably

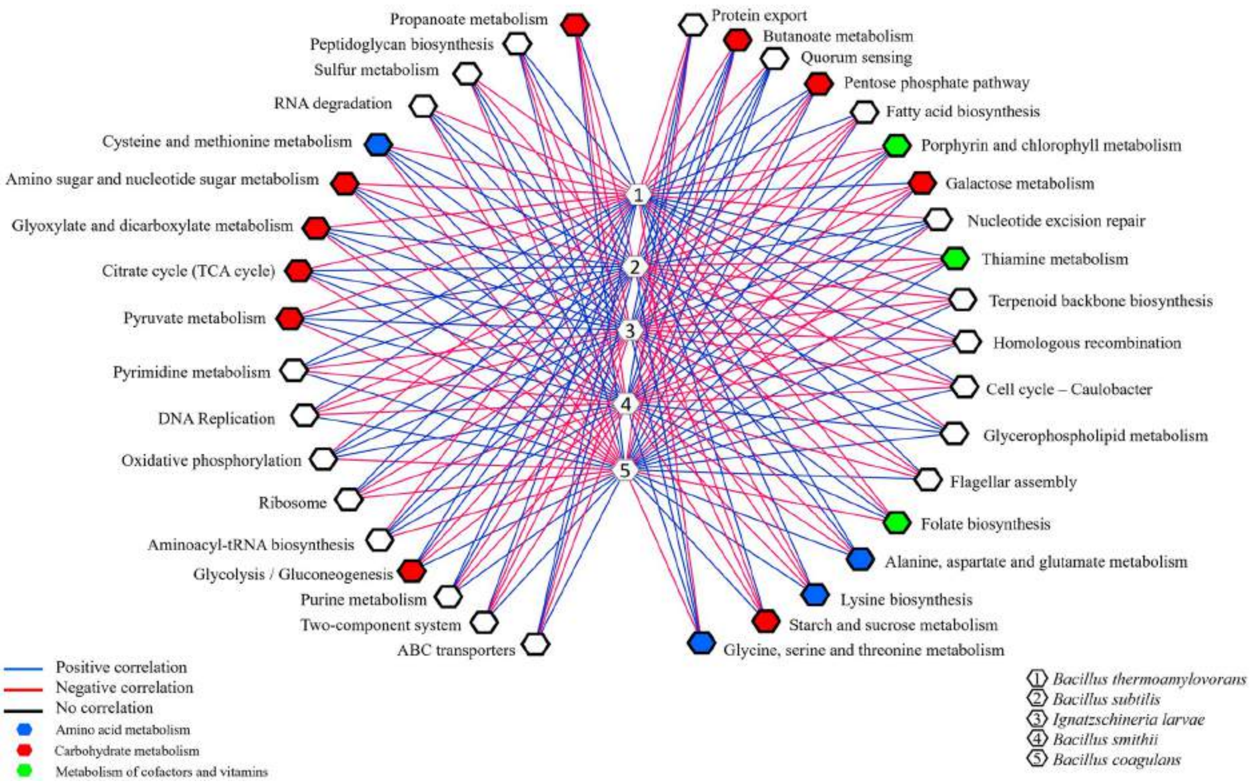

Fig 12. Spearman-s correlation was performed between the predominant species and functional features that has a relative abundance >1% using IBM SPSS (Statistical Package for the Social Sciences) Statistics v.20 and represented via correlation-based network.

https://doi.org/10.1371/journal.pone.0260777.g012

due to the duration of fermentation that may cause the changes in species abundance [63]. A goods' coverage observed in our study indicates a maximum microbial diversity [64] in the samples. In beta diversity, we observed a discrete association among metagenome samples corroborated by PCoA plot, based on their taxonomic features, which may be due to the changes with fermentation time and environmental factors [39, 65]. Several unique and shared species were observed in different samples, probably due to abiotic factors or unusual associations among species from different domains [66].

We found that natural fermentation days of 3–4 days may be suitable for consumption of *pe poke* due to abundance of *B*. *thermoamylovorans* and *B*. *subtilis*, which are considered as safe fermenting bacteria in fermented foods [8, 67] comparable to 5 days *pe poke* with abundance of *Proteobacteria*, which contains several pathogenic bacteria [68].

Predictive functional features

The predictive functional analysis of *pe poke* metagenome, mapped against KEGG database, suggested the abundance of metabolism including pathways for carbohydrate metabolism and amino acid metabolism. Abundance of genes related to carbohydrate metabolism (pentose phosphate pathway, and glycolysis) is important for microbial metabolism [69]. The genes for predictive enzymes such as α-glucosidase, α-galactosidase, and β-galactosidase were detected in galactose metabolism pathway in *pe poke*, essential for degradation of starch and oligosaccharides into simpler forms during fermentation [70]. Genes involved in the processing of lignocellulose were also detected in *pe poke* metagenome, which suggested that plants-derived carbohydrate act as the source of energy for aerobic (via tricarboxylic acid, TCA cycle) or anaerobic (fermentation) microbes [71]. It was also reported that β-glucosidase could be involved in the hydrolysis of cello-oligosaccharides [72], and biosynthesis of isoflavone glycosides [73], and also involved in digestion and hydrolysis of macromolecules present in soybean seeds during fermentation [74]. Genes related to glycine, serine and threonine metabolism detected in *pe poke*, may enhance the nutritional value of the product [8, 75]. The abundance of genes related to alanine, aspartate, glutamate metabolism in *pe poke* metagenome may contribute to the enhancement of taste and flavour of the product [76]. Folate biosynthesis, the key pathway of new therapies against infectious diseases caused by various microorganisms [77] was detected in *pe poke*. The positive correlation between *Bacillus subtilis* and folate biosynthesis was observed in *pe poke*, the key pathway of new therapies against infectious diseases [78] and also confers the protection against inflammation, cancer, anaemia, cardiovascular diseases [79].

Abundance of genes related to ABC transporters specific for peptides were detected in *pe poke* metagenomes, which may felicitate the uptake of di-/tripeptides [80]. The active role of the microbial population in the transformation of polysaccharide and short-chain carbohydrate in *pe poke* has been supported by the phosphotransferase system (PTS), the source of transport and phosphorylation of various sugar which forms mono/disaccharides, amino sugars, polyols, and other sugar derivatives [73].

In enzyme classification, we observed the presence of predictive enzymes involvement in the biosynthesis of lysine, alanine, aspartate, glutamate, glycine, serine and threonine, which enhance the nutritional value of the product [54]. Additionally, we also detected enzymes typically encoded by *ect*ABCD gene cluster of bacteria [81] that have an excellent function-preserving property [82]. Genes related to serine protease such as fibrinolytic enzymes were detected in *pe poke*, which may play as antithrombotic agents [83, 84]. Gene related to signal transduction system that regulates poly-γ-glutamic acid (PGA) synthesis [85, 86].

A positive correlation observed between *Bacillus* species and predictive amino acids metabolism indicates the ability to accumulate most of amino acids such as alanine, aspartate, glutamate, glycine, serine, and threonine that enhance the nutritional values in the product [87], and also contributes to taste perception and flavour enhancement [88]. A positive correlation between lysine biosynthesis and *B. thermoamylovorans* was detected in *pe poke*, which was also reported in *douchi* [89]. Lysine has several health promoting benefits to consumers [90]. *B. coagulans* showed positive correlation with biosynthesis of thiamine (vitamin B1), one of the major growth factors that promotes the growth of *B. coagulans* [91], similarly *B. coagulans* showed a positive correlation with galactose metabolism, where α- and β-galactosidases (detected in galactose metabolism pathway) can hydrolyse a non-digestible galactoside present in the food matrix [92]. *B. subtilis* showed positive correlation with predictive folate (vitamin B_9) biosynthesis; *B. subtilis* is reported to harbour pathways component for folate (vitamin B_9) production [79]. Though prediction of some pathways related to human disease were also observed, but their abundance were too low to make any significant impact.

Conclusion

Pe poke is a popular traditional fermented soybean cuisine in the Burmese food culture, however, its microbiology and functional properties have not been studied in details, except few reports on *Bacillus* sp. Hence, we profiled the microbial community in samples of *pe poke*, which were naturally fermented for 3 days, 4 days, 5 days, respectively and also sundried *pe poke*, by shotgun metagenomic analysis. Colossal diversity of microbial communities in *pe poke* was observed. We found that natural fermentation days of 3–4 days may be suitable for consumption of *pe poke* due to abundance of *Bacillus thermoamylovorans* and *B. subtilis*. Several predictive biosynthesis of amino acids, vitamins and other bioactive compounds have been inferred indicting the functional properties of this unique Burmese fermented soybean food, and moreover, the information obtained from this study may help to sensitise the commercial producers and consumers aware on microbial community, the health benefits, hygiene and general safety in *pe poke*.

Supporting information

S1 Table. The minor phyla with a relative abundance of <1% detected in *pe poke*.
(DOCX)

S2 Table. The minor families with a relative abundance of <1% detected in *pe poke*.
(DOCX)

S3 Table. The minor genera with a relative abundance of <1% detected in *pe poke*.
(DOCX)

S4 Table. The minor species with a relative abundance of <1% detected in *pe poke*.
(DOCX)

S5 Table. The overall species of *Bacillus* detected in *pe poke*.
(DOCX)

S6 Table. The species of lactic acid bacteria detected in *pe poke*.
(DOCX)

S7 Table. Archaeal species detected in samples of *pe poke*.
(DOCX)

S8 Table. Eukaryotic species including yeasts, moulds, algae, protozoa and parasites detected in *pe poke*.
(DOCX)

S9 Table. Viral species detected in samples of *pe poke*.
(DOCX)

S10 Table. Shared and unique bacterial species detected in *pe poke*.
(DOCX)

S11 Table. Shared and unique archaeal species detected in *pe poke*.
(DOCX)

S12 Table. Shared and unique eukaryotic species detected in *pe poke*.
(DOCX)

S13 Table. Shared and unique viral species detected in *pe poke*.
(DOCX)

S14 Table. Clusters of Orthologous Groups (COGs) detected in *pe poke*.
(DOCX)

S15 Table. The relative abundance of <1% mapped against KEGG database at level-2 (Super-pathways).
(DOCX)

S16 Table. The relative abundance of <1% mapped against KEGG database at level-3 (Sub-pathways).
(DOCX)

S17 Table. Predictive enzyme classification detected in *pe poke*.
(DOCX)

S18 Table. Predictive enzymes classification involved in different pathways in *pe poke*: (a) Lysine biosynthesis; (b) Biosynthesis of alanine, aspartate and glutamate metabolism and 4-aminobutanoic acid (GABA); (c) Glycine, serine, threonine metabolism and ectoine biosynthesis; (d) Pentose phosphate pathways and (e) Galactose metabolism.
(DOCX)

Acknowledgments

Jyoti Prakash Tamang is grateful to International Centre for Integrated Mountain Development (ICIMOD) Chair for financial support to visit Myanmar and collection of samples.

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Formal analysis: Pynhunlang Kharnaior.

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Writing – review & editing: Jyoti Prakash Tamang.

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Tamang *Journal of Ethnic Foods* (2022) 9:30
https://doi.org/10.1186/s42779-022-00146-3

Journal of Ethnic Foods

REVIEW ARTICLE

Open Access

Dietary culture and antiquity of the Himalayan fermented foods and alcoholic fermented beverages

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Abstract

One of the important dietary cultures of the Himalayan people is consumption of fermented foods and drinking of alcoholic fermented beverages, which are traditionally produced by the multiracial and multiethnic communities of the Himalayan people using their 'ethno-microbiological' knowledge of food fermentation. Besides delicacy, ethnical and cultural values, the Himalayan fermented foods are also the hubs of biological resources of beneficial and functional microorganisms which have been contributing health-promoting benefits to thousands of Himalayan people with a dearth of modern food products. There is no or rare publications on sporadic history, antiquity and origin of the Himalayan fermented foods. We designed questionnaire and sought information from different communities in different places of the Himalayas on oral history, legendries on origin and antiquity of fermented foods and alcoholic fermented beverages. Hence, the present article discusses the food culture, oral history, legendries and antiquity of some Himalayan fermented foods and alcoholic fermented beverages in the Himalayan regions, and also to validate the scientific evidence-based ethno-microbiological practices performed by the Himalayan people for food fermentation.

Keywords: Himalayas, Fermented foods, Alcoholic fermented beverages, Ethno-microbiology, Dietary culture

Introduction

Dietary culture and traditional gastronomy of the Himalayan people have evolved as a result of the sagacity, innovativeness, food preferences, availability of ago-biological resources and experiences based on trial and error over a period of time. Ethnic fermented foods are defined as foods produced by the ethnic people using their native knowledge of food fermentation from locally available raw materials of plant or animal sources either naturally/spontaneously or back-slopping or by adding starter culture(s) containing functional microorganisms which modify the substrates biochemically and organoleptically into edible products that are culturally and socially acceptable to the consumers [1]. The snow-clad Himalayas (हिमालय) are geographically divided into three regions: the Eastern Himalayas [eastern Nepal; Darjeeling hills, Sikkim, Arunachal Pradesh and states of north-east in India; Bhutan; and Tibet Autonomous Region (TAR) in China]; the Central Himalayas (central and western Nepal and TAR); and the Western Himalayas (Jammu & Kashmir, Ladakh, Himachal Pradesh and Uttarakhand in India; and TAR) (Fig. 1). The rich biological resources including the ago-resources and livestock of the Himalayas are supportive systems for the survival and livelihood of thousands of ethnic multiracial mountain communities mostly belonging to the Aryan, the Mongoloid and the Negroid, considered as original races [2] living in the sub-Himalayan regions (ranging from 400 to 6000 m). There are rare historical records of agricultural and pastoral systems in the Himalayas; however, the oldest

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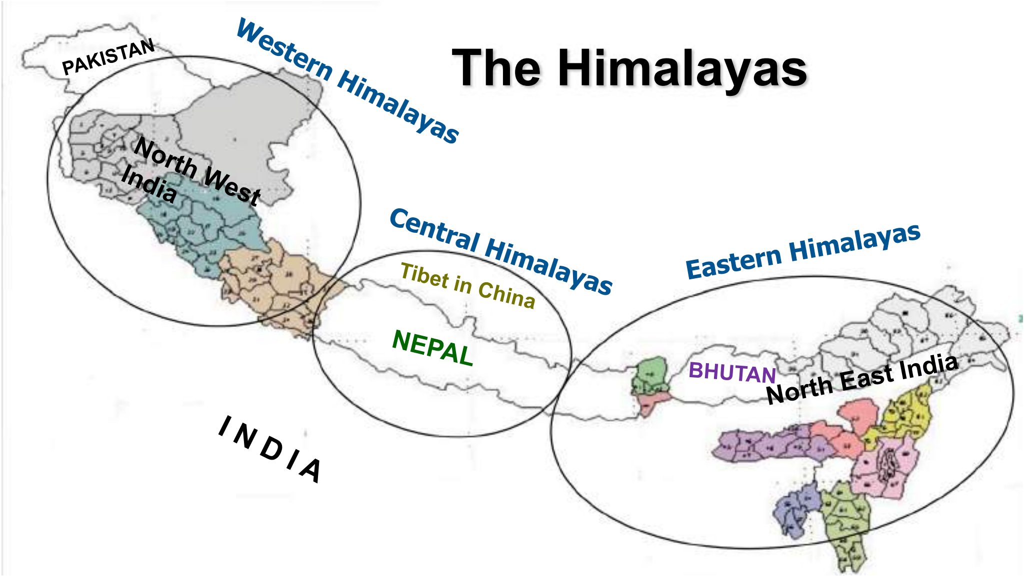

Fig. 1 Geographical divisions of the Himalayas: Eastern, Central and Western Himalayas

historical record of domestication of animals in Nepal was traced back to the Gopal dynasty (1500–1000 BCE) in Nepal and the agricultural systems during the Lichchhavi dynasty (450–750 CE) [3]. Rice or maize is a staple food in the Eastern Himalayan regions, whereas wheat or barley is a stable diet in the Western Himalayan regions. Soya food eating culture is predominant in the Eastern Himalayan regions of India, Nepal and Bhutan. Historically animal dairy products are the traditional dietary culture of the ethnic people of the Western and Central Himalayas, whereas in the Eastern Himalayas, traditional dairy products are restricted to east Nepal, Darjeeling hills, Sikkim and Arunachal Pradesh in India and Bhutan [4]. More than 400 different types of common, unique, artisan, exotic and rare ethnic fermented foods and alcoholic fermented beverages are consumed as staple foods, side dish, curry, soup, savoury, pickles, condiments and alcoholic drinks by the multiethnic Himalayan people due to diverse geographical coordinates and ago-climatic variations (Table 1). Interestingly, the Himalayan fermented foods cover all types of available substrates ranging from milk to alcohol, soybeans to cereals, vegetables to bamboo, meat to fish, etc., and are grouped as fermented soybeans, fermented non-soybean legumes, fermented vegetables and bamboo shoots, fermented milk, fermented cereals, fermented/preserved meats and fish, consortia of microorganisms in the form of uneatable dry artisan starter culture for production of various alcoholic beverages and distilled liquor. The overall number of Himalayan fermented foods looks large; however, most of the fermented foods, based on major raw substrates, are similar in nature with slight variations in traditional preparation methods during natural fermentation, culinary practices and mode of consumption. Moreover, listing of high number of the Himalayan fermented foods is due to colossal linguistic diversity and diverse ethnicity [5]. Every fermented product has a local name, coined by the particular ethnic Himalayan communities in their language/dialect, mostly derived from the Indo-European and Tibeto-Burman languages [6]. Ancient Himalayan people had to choose their food preferences for survival, wherever they settled in the sub-Himalayan regions, based on the accessibility and availability of plant or animal resources. That is how the localization of certain fermented foods is variable within the Himalayans regions (Table 1).

Mega diversity of the Himalayan fermented foods and alcoholic fermented beverages is historically contributed by the majority ethnic Nepali/Gorkha community, one of the earliest inhabitants of the Himalayas, comprising of more than 126 castes. Presently, the ethnic Nepali/Gorkha makes the large stallholders of the Himalayan fermented foods and alcoholic fermented beverages of India (Darjeeling hills, Sikkim, Assam, Uttarakhand), Nepal and Bhutan. Antiquity of food culture is linked with the cultural and political history as well as ethnicity of the region. Hence, the present paper discusses the food culture, legends and antiquity of some Himalayan fermented foods and alcoholic fermented beverages originated in ancient or medieval Nepal and other Himalayan regions, and to correlate the possible validation of some evidence-based traditional practices performed by the

Table 1 Geographical distribution of ethnic fermented foods and beverages in the Himalayas

Substrates	Product characters	Himalayas		
		Western	Central	Eastern
		Local names of fermented products (countries/states/regions)		
(A) Plant-based Himalayan fermented foods				
Soybean	Alkaline, sticky, solid	Not produced	Not produced	*Aagya* (AP), *Axone/Aakhonii* (N), *Bari* (B), *Bekang* (Mz), *Bemerthu* (A), *Bekanthu* (A), *Bezeithu* (T), *Chukchoro*, *Grep chhurpi/Lipi chhurpi* (AP), *Hakhu mata* Mn), *Hawaijar* (Mn), *Khuichang* (Mn), *Kinema* (Dj, S, EN, B), *Penam* (AP), *Peruñyaan* (AP), *Peron Piak* (AP), *Paeha/peha* (AP), *Pintop panau* (AP*)*, *Satlyangser* (S), *Thonao* (AP), *Tungrymbai* (Mg)
Non-soybean legumes	Slightly acidic, solid	*Borhe* (HP), *Dangalbari* (HP), *Maseura* (WN), *Mashbari* (HP), *Papad* (HP), *Sepubari* (HP), *Wari/bari* (HP, U)	*Maseura* (WN)	*Maseura* (Dj, S)
Cereal	Fermented dough, baked, fried	*Aska* (HP), *Babru* (HP), *Bagpinni* (HP), *Bhatooru* (HP), *Bhatabaru* (HP), *Chilra* (HP), *Chzot/Girda* (JK), *Czochwor* (JK), *Gulgule* (HP), *Jalebi* (HP, U, WN), *Khambir* (L), *Lawaas* (JK), *Marchu* (HP), *Nan* (HP), *Pakk* (HP), *Selroti* (WN), *Siddu* (HP), *Seera* (HP), *Tchog* (HP)	*Jalebi* (CN), *Selroti* (CN)	*Jalebi* (EN. Dj, S), *Selroti* (EN, Dj, S, B)
Vegetable	Acidic, sour taste, sun-dried	*Gundruk* (WN), *Sinki* (WN)	*Gundruk* (CN), *Sinki* (CN)	*Ankamthu* (Mz), *Anishi* (N), *Antramthu* (A), *Bastanga* (N), *Cutocie* (N), *Ganang tamdui*, (Mn, N), *Gundruk* (EN, Dj, S, A, B), *Goyang* (S), *Hungrii* (N), *Inziangsang* and *Inziang-dui* (Mn, N), *Kahudi* and *Kharoli* (A), *Khalpi* EN, Dj, S, B), *Panitenga* (A), *Sinki* (EN, Dj, S, B), *Ziangsang* (Mn, N)
Bamboo	Acidic, sour taste, fresh or sun-dried	Not produced	*Mesu* (CN)	*Bastanga* (N), *Ekung*, *Eup* and *Hirring* (AP), *Khorisa* (A), *Lung-siej* (Mg), *Mesu* (EN, Dj, S, B), *Miya mikhri* (A), *Moiya pangsung* or *moiya koshak* (T), *Soibum*, *Soijim* or *soijin* and *Soidon* (Mn), *Thunkhiang/*Thunkheng(A, Mn), Tuaium (Mz), *Thunbin* (Mn), *Tuaithur* (A)

Table 1 (continued)

Substrates	Product characters	Himalayas		
		Western	**Central**	**Eastern**
		Local names of fermented products (countries/states/regions)		
(B) Animal-based Himalayan fermented foods				
Milk	Acidic, savoury	*Dahi* (WH), *Chhura* (L), *Chura loenpa* (TAR), *Kalari* (JK), *Kurut* (TAR), *Lassi* (WH), *Mar* (L), *Mohi, Phuh* (JK), *Qula* (TAR), *Tara* (L), *Zamuthdod* (JK)	*Dahi* (CN), *Lassi* (CN), *Mohi* (CN)	*Dahi* (EN, Dj, S, B, A, AP), *Chhu* (Dj, S), *Chhurpi* (EN, Dj, S, B, AP), *Charka* (AP), *Chhur chirpen* (AP), *Chhur singba/chhur mingba* (AP), *Churkam* (B), *Chhurpupu/Churtang* (AP), *Dahi, Shedan* (S, B), *Datshi* (B), *Maa/MorMohi* (EN, Dj, S, B), *Marchang* (AP), *Philu* (S), *Phrung* (AP), *Shyow* (S, B), *Somar* (Dj, S),
Fish	Fermented or traditionally preserved, sun-dried, smoked, or salted	*Sidra, Suka ko maacha,* and *Sukuti* (WN)	*Sidra, Suka ko maacha,* and *Sukuti* (CN)	*Ayaiba* (Mn), *Bordia* (A), *Chucha* (A), *Gnuchi* (Dj, S), *Godak* (T), *Hidal* (A), *Hukoti* (A), *Hentak* (Mn), *Ithiitongba* (Mn), *Karati* (A), *Lashim* (A), *Lona Illis* (T), *Mio* (AP), *Naduba siyan, Nah-grain* and *Namsing* (A), *Naakangba* (Mn, N), *Ngiiyiyaan* (AP), *Ngari* (M), *Sepaa* and *Sheedal* (T), *Sidra, Suka ko maacha,* and *Sukuti* (EN, Dj, S, B), *Tungtap* (Mg)
Meat	Fermented or traditionally preserved, sun-dried, smoked, or sausage-like products	*Arjia* (U, HP), *Chartayshya* (U, HP), *Jamma* (U, HP), *Kheuri, Lang kargyong* (L), *Lang chilu* L), *Shekwa* (WN), *Suka ko masu* (WN), *Yak kargyong* and *yak kheuri* (L)	*Sukako ko masu* (CN), *Shekwa* (CN)	*Ashikumna/Thevochie* (N), *Bagjinam* (N), *Bongkarot* and *Bongthu* (Mn), *Dingkyo* (AP), *Faak kargyong* (Dj, S), *Gwag ruum* and *Guaighi kang* (Mn), *Honoheingrain* (A), *Jang kap* (N), *Kargyong, Kheuri, Khyopeh* (S), *Lang kargyong* (Dj, S, B), *Lang satchu* (Dj, S, B), *Lang chilu* and *Luk chilu* (S, B), *Lang kheuri* (S, B), *Lukter* (AP), *Pikey Pila* (AP),*Saayung, Sahro* and *Sathu* (Mn), *Sa-um* (Mz), *Shekwa* (EN), *Suka ko masu* (EN, Dj, S, B), *Yak chilu, Yak kargyong, Yak kheuri* and *Yak satchu* (S, B)
(C) Himalayan amylolytic starters and alcoholic fermented beverages				
Non-eatable, cereal-based starter culture	Artisan, amylolytic, solid, dry, ball or oval or flattened, starter cultures (consortia of mycelial moulds, yeasts and bacteria)	*Bakhar* (U, HP), *Balan* (U, HP), *Dhehli* (HP), *Keem Khai* (U, HP), *Malera/treh* (HP), *Marcha, manapu* and *mana* (WN), *Phab* (L)	*Marcha, manapu* and *mana* (CN)	*Angkur* (A), *Apop pitha* (A), *Bhekur-pitha* (A), *Chamri* (Mn), *Chang-poo* or *phab* (B) *Chowan* (T), *Dabai, Dawdim* (Mz), *Dhehli, Emao* (A), *Hamei* (Mn), *Humao* (A), *Ipoh/Siye* (AP), *Khai* (Mn, N), *Khekhrii* (T), *Marcha* (EN, Dj, S, B), *Modor pitha* (A), *Opop* (AP), *Phab* (B, AP), *Phut, Paa* and *Pee* (AP), *Thap* (A), *Thiat* (Mg), *Xaaz/Xaj pitha* (A)

Table 1 (continued)

Substrates	Product characters	Himalayas		
		Western	**Central**	**Eastern**
		Local names of fermented products (countries/states/regions)		
Alcoholic fermented beverages	Cereals (rice, finger millets, maize, barley, wheat)-based mild, sweet, alcoholic beverages	*Ayela* (WN), *Angoori* or *kinnauri* (HP), *Ark/ara* (HP), *Chyang* (L), *Buza* (L), *Chulli* (HP), *Chhind*, *Daru* (HP), *Ghanti* (HP), *Jann* (U, HP),, *Khor*, *Kodo ko jaanr* (WN), *Lugri* (HP), *Pachwai* (U, HP), *Rak* (HP), *Raksi* (WN), *Sez* (U), *Soora/Sura* (HP)	*Ayela* (CN), *Bhaati jaanr* (CN), *Kodo ko jaanr* (CN), *Makai ko jaanr* (CN), *Simal tarul ko jaanr* (CN), *Raksi* (CN)	*Acham* (Mn), *Ahom* (A), *Aarak* or *Hor-Alank* (A), *Aitanga* (Mn), *Apong* (A, AP), *Aara* (AP), *Bhaati jaanr* (EN, Dj, S, B), *Buza*, *Chakti*, *Chulli*, *Chyang* (EN, Dj, S, B, AP), *Chee* (S), *Chhind*, *Dekuijao* and *Duizou* (N), *Ennog* (AP), *Faapar ko jaanr* (EN, Dj, S), *Gahoon ko jaanr* (EN, Dj, S), *Haria* (A), *Jou* (N), *Judima* (A), *Juharo* and *Juhning* (A), *Kiad* (Mg), *Khor* (Mn), *Kodo ko jaanr* (CN, Dj, S, B), *Langi* (T), *Laopani* (A), *Madhu*(N), *Makai ko jaanr* (EN, Dj, S, B), *Mingari* (AP), *Nchiangne* and *Nduijao* (N), *Nyongin* (AP), *Oa* (AP), *Opo* (AP), *Patso* (Mn), *Pheijou* (Mn), *Pona* (AP), *Raksi* (EN, Dj, S, B), *Rakzu* (Mz), *Ruhi* (N), *Simal tarul ko jaanr* (EN, Dj, S), *Sira-Oa* (AP), *Themsing* (AP), *Timpui* (Mn), *Tin-zu* (Mz), *Toddy* (A), *Xaj pani/koloh pani* (A), *Waiyu* (Mn), *Yu* (Mn), *Yu angouba* (Mn), *Zoungao* (Mn, N), *Zouju/zouzu* (Mn, N), *Zu* (A), *Zutho* (N), *Zupui* (Mz), *Zufang* (Mz)
Fermented tea				*Pu-erh tea* (Tibet in China)

A, Assam; AP, Arunachal Pradesh; B, Bhutan, CN, Central Nepal; Dj, Darjeeling; EN, Eastern Nepal; HP, Himachal Pradesh; JK, Jammu and Kashmir; L, Ladakh; Mg, Meghalaya; Mn, Manipur, Mz, Mizoram; N, Nagaland; S, Sikkim; T, Tripura; TAR, Tibetan Autonomous Regions in China; U, Uttarakhand; WH, Western Himalayas; WN, Western Nepal

Himalayan people during preparation of fermented foods and alcoholic fermented beverages.

Methods

Documentation on oral history and legendries of some Himalayan fermented foods and alcoholic fermented beverages has been collected and obtained through questionnaire (Table 2) and interview with local communities of different villages and regions of the Himalayan countries of India, Nepal and Bhutan during 35 years of my research. During the time of survey, many criteria were questioned and sought, which included oral history on origin and antiquity of fermented foods, methods of preparation, taboo and festivals associated with the preparation, culinary practices, socio-economy, etc. I could not visit Tibetan Autonomous Regions in China; however, I could interact and tasted the Tibetan cuisine including fermented foods of the ethnic Tibetans settled in India.

Soya food culture and origin of soybeans

Antiquity and origin of soybean cultivation in South East Asia are still debatable, mostly based on legends and myths. Soybean, which is spelled as 'भटमास' (*bhaṭamāsa*) in the Nepali language, was probably introduced to the Eastern Himalayan regions of North-east India, South Bhutan and East Nepal from China from Yunnan Province of China [7], or by traders who brought soybeans via Myanmar to India [8]. Despite India being the fifth largest soybean producer country in the world, soybean was not historically and organoleptically accepted traditional food item in the Indian gastronomy except by the ethnic people of North-east India mostly belonging to the Mongolian races [9]. Local varieties of soybean seeds, viz. 'yellow cultivar' and 'dark brown/black cultivar', with a diameter of 5–7 mm are traditionally cultivated in the upland terraces as sole crop or a mixed crop with rice and maize up in edges of the hilly terraces in the sub-Himalayan regions of east Nepal, North-east India and south Bhutan. Local varieties of Chinese soybean seeds are slightly larger than that of the Eastern Himalayas. Soybeans, mostly black varieties, are also cultivated in the Central and Western Himalayan regions of Nepal and India in the lower altitudes as major or mixed crops. Recently, several high-yielding hybrid varieties of soybeans are grown in the sub-Himalayan regions.

Historically and anthropologically, the hypothetical claim of introduction of Chinese soybeans to the Himalayan regions through north-east states of India is quite remote to establish the claim. Based on the information collected from questionnaire during the survey, mythological and legendary of origin of soybeans in the Himalayas have been mentioned in the *mundhum*, the collections of ancient religious scripture and mythology of the Limboo communities [10], who are aboriginal Kirat race of Nepal [11], believed that the soybean was gifted by their God 'Yuma' to overcome famine [4]. As per the myth, cultivation of soybeans, termed as *chembi* in Limboo language, was included in the traditional agriculture system of the Limbu. The first harvested soybean seeds were offered to the God showing their gratitude and respect for saving their life from famine as mentioned in *mundhums* might have written in between 2500 and 100 BCE [12]. The actual history of Limbhuwan which was the ancient Kingdom of Limboo in eastern Nepal, currently the districts of eastern Nepal, recorded in 580 BCE till 1774 CE, after the Gorkha King Prithivi Narayan Shah merged Limbhuwan to new unified Nepal [13, 14].

During the survey, we could document few legends and myths on origin of soybeans in North-east India. Ethnic

Table 2 Questionnaire on oral history and legendries of the Himalayan fermented foods and fermented alcoholic beverages

I. General information	
1. Identification number:	2. Name of interviewee:
3. Ethnic group:	4. Name of village, district, state and country:
II. Information on oral history of fermented foods	
1. How did you learn the traditional methods of preparation of fermented foods? From your mothers/grandmothers/fathers/grandfathers/village elders?	
2. In your knowledge how long this traditional method of fermented foods at your home being practised in your family history? Tentative year?	
3. Have you heard of any oral history about the present fermented foods? If so, what and from whom?	
4. Do you have any written historical documents/monographs/monuments/relics, etc., if so in which language?	
5. Do you have any taboo to prepare/consume the fermented products you mentioned? Yes/No	
6. If yes, provide details:	
7. Do you perform any ritual or worship any particular god(s) or goddess(es) with fermented products, you consume? Yes/No	
8. If yes, provide details:	
9. Do you think that fermented products, which you have mentioned, have medicinal value(s) or play a role in promoting health?	

inhabitants in foot hills of the sub-Himalayans regions in North-east India, viz. Meitei, Naga Mizo, Khasi, Apatani, etc., also believe having their own indigenous varieties of soybeans locally cultivated in the regions; however, the authenticity of their claim is yet to be established. Who and how domesticated soybeans were brought to the Eastern Himalayan regions remains a hypothesis. It might have brought by migrated people from regions to regions during medieval days, or by traders, or by medieval soldiers fighting in the Himalayan borders of South China, Myanmar, and Manipur, Nagaland and Arunachal Pradesh in India and then shared their indigenous practice of soybeans cultivation. However, it also depends on the cultivation of soybeans as the tradition, which is not common among the highland Tibetans in TAR, due to extreme cold climate. Hence, consumption of soybeans both fermented and non-fermented is uncommon among the Tibetans.

Antiquity of fermented soybean foods

Though cultivation of soybeans has been a traditional practice in the Western and Central Himalayas, natural fermentation of locally grown soybeans into delicious foods has been observed only among Mongolian races of the Eastern Himalayan regions of east Nepal North-east India, and south Bhutan [9]. The naturally fermented soybeans with sticky texture, slight ammoniacal flavour, or *umami* flavour [15], (*umami* flavour is the flavour generated during breaking down of protein to amino acids in soybean fermentation), are commonly known as *kinema* in eastern regions of Nepal, Nepali/Gorkha-dominated Darjeeling hills and Sikkim in India, and south Bhutan, along with several other similar types of naturally fermented soybean foods with different vernacular names in North-east Indian states of Arunachal Pradesh, Assam, Meghalaya, Manipur, Mizoram, Nagaland and Tripura (Fig. 2; Table 1). The word *kinema* (Fig. 2a) is believed to have derived from the word *kinamba* of the Limboo language (*ki* means fermented and *namba* means flavour) [16], probably they were the first Himalayan ethnic people who innovated the indigenous knowledge of utilization of soybeans into naturally fermented delicious foods. However, such claim has not been substantiated by the historical documents or archaeological findings. Antiquity of *kinema*, however, is yet to be fully studied. Whether *kinema* originated first and diversified to other similar products or vice versa is a big challenge to food anthropologists and historians. *Kinema* might have originated in east Nepal around 600 BCE to 100 CE during the Kirat dynasty [4].

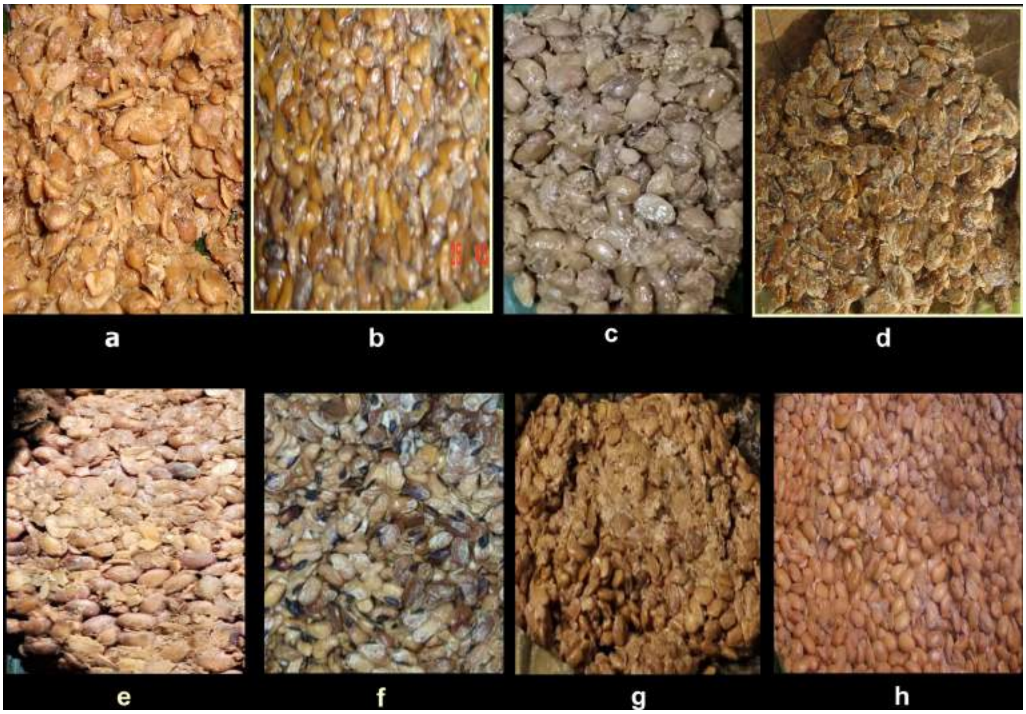

Fig. 2 Himalayan fermented soybean foods: **a** *Kinema* of Darjeeling hills and Sikkim in India; eastern Nepal and southern Bhutan; **b** *Hawaijar* of Manipur; **c** *Tungrymbai* of Meghalaya; **d** *Bekang* of Mizoram; **e** *Aakhonii* or *axone* of Nagaland; **f** *Peruyaan* of Arunachal Pradesh; **g** *Peron namsing* of Arunachal Pradesh; **h** *Bemerthu* of Assam

Preparation of sticky fermented soybean food in the Himalayas is more or less identical with slight deviation in the production steps depending on locally available leaves/paddy straw as wrapping materials for fermenting soybean seeds, fermentation duration, culinary practice and mode of consumption. In general, first local varieties of harvested soybean seeds are selected, cleaned and soaked in water for overnight, and then soaked soybeans are transferred in a big metallic container or pot and boil till seeds become soft in an open fire in an earthen oven. Boiled seeds are cooled in bamboo-made mats for few minutes, and cooked beans are wrapped loosely in fresh fern fronds or banana leave or other locally available fresh leaves with large surface area or paddy straw which are kept inside the bamboo-made baskets and are placed above earthen kitchen for natural fermentation for 1–3 days. After the fermentation, whitish viscous sticky materials appeared on the surface of soybean grits with slight ammoniacal flavour or umami taste and is commonly eaten as side dish curry or soup or condiments accordingly to the preference of the consumers, with steamed rice in main meal.

Himalayan sticky fermented soybean foods are similar to other Asian fermented soybean foods such as *pe poke* of Myanmar, *thua nao* of northern Thailand, *sieng* of Laos, *douchi* of Yunnan province of China, *cheonggukjang* of Korea and *natto* of Japan [16]. Hypothetically, an imaginary triangle, if drawn, starts from Japan (*natto)*, touching Korea (*cheonggukjang*), Yunnan Province of China (*douchi*), Laos (*sieng*), northern Thailand *(thua-nao*), North-east India (*axone/aakhonii, bekang, bemerthu, bekanthu, bezeithu, grep chhurpi, hawaijar, kinema, peruñyaan, peron, paeha/peha, tungrymbai*), south Bhutan (*kinema/bari*), and ending at eastern regions of Nepal (*kinema*). This imaginary triangle was named as '*natto* triangle' [17] and renamed as '*kinema-thua-nao-natto* triangle' [4]. Historically, the preparation and consumption of bacterial-fermented non-salted soybean foods with more to less stickiness, umami flavoured, are exclusively prepared and eaten within these imaginary triangle countries, which has not been reported from any part of the world. Unlike other Asian fermented soybean foods, which are either eaten directly without frying or cooking, e.g. Japanese *natto*, or made into soup or condiments, *kinema*, and other Himalayan fermented soybean foods are made into curry with onion, tomatoes and chillies. Probably, culinary of *kinema* is a blend of Aryan and Mongolian food culture. Though the Eastern Himalayan people have culturally adopted eating soybeans as non-fermented and fermented products, traditionally they never prepare and consume fungal-fermented soybean foods such as *miso, soya sauce, tofu, sufu and tempeh*, whereas fermented non-soybean legume products are limited and are confined only to the Central and Western Himalayas. Black gram is naturally fermented into an artisan spongy ball-like dry product and is called *maseura* in Nepal and *wari* in Himachal Pradesh and Uttarakhand in India. Non-soybean legumes, locally called *dal*, are very popular thick stew eaten with boiled rice and baked bread.

Evidence-based traditional practice for soybean fermentation

Extensive research has been conducted to understand the microbial communities, their roles in natural fermentation, nutritional values, health-promoting benefits and the fermentation mechanisms during the traditional methods of preparation of fermented soybean foods of the Eastern Himalayan regions [18–24]. The evidence-based science explains the mechanisms of fermentation during traditional preparation of fermented soybean foods and also validates the commendable indigenous knowledge of 'ethno-microbiology' of the ethnic Himalayan people (Table 3) [18, 22, 25–27]. Himalayan fermented soybean foods are rich in microbiome mostly represented by the dominant beneficial and functional bacterium *Bacillus*, which is a rod-shaped, non-pathogenic, showing several bio-functional properties including health benefits such as immunity booster (immunomodulators), antioxidants (anti-cancer), antithrombotic (to cure heart disease), and produces vitamins, biopeptides, poly-glutamic acids, etc. [28].

Himalayan fermented milk products

Animal husbandry has been a part of traditional agricultural system, depending on the altitudinal-based vegetation for grazing the domesticated animals since the ancient and the medieval times in the Himalayas. Though there is no record of antiquity of origin of the fermented milk products in the Himalayas, preparation and consumption of ethnic Himalayan fermented milk products are observed only among the ethnic communities of the Eastern Himalayas, mostly Darjeeling hills, Arunachal Pradesh and some regions of Assam in India, Nepal, Bhutan and TAR in China, the Central and Western Himalayas (Table 1). Despite the possession of domesticated animals and practising the livestock, historically there is no documentation or record of traditional fermented dairy milk products of some ethnic communities in North-east India mainly Naga, Mizo, Meitei, Apatani, Adi, Mao, Kasi, Garo, etc., of North-east India. This may be due to the Aryan-Hindu pastoral system which might have influenced consumption of milk and milk products in the early settlement in the Himalayas mostly the Central and Western regions. Local varieties and breeds of cows are reared mostly in

Table 3 Traditional processing steps during the Himalayan fermented soybean food production with scientific explanation for noteworthy ethno-microbiological knowledge of the Himalayan people

Flow sheet	Details of production steps	Scientific explanation
Soybean ↓ Cleaned and washed ↓ Soaked in water 8–10 h; drained off excess water ↓ ← Added clean water ↓ Cooked in an open cooker ↓ Drained off ↓ Cracked in a wooden mortar lightly by a wooden pestle ↓ Kept in a bamboo basket lined with jute bag and wrapped by fern leaves ↓ Fermented (25–40 °C, 1–3 d) ↓ Sticky fermented soybeans	Soaking of soybeans in water for overnight	Soaking of soybeans will make the seeds soften and also helps to develop some flavour properties [25]
	Soaked soybean seeds are boiled till it becomes soft	Boiling of soybean reduces the opportunistic and pathogenic microorganisms except heat-tolerant bacteria, and also allergenicity of soybeans [26]
	Wrapped in fresh leaves (fern fronds, banana, paddy straw)	Since the dominant fermentative bacterium is *Bacillus* which is aerobic to semi-anaerobic, loosely pack leaves or ferns will create the semi-anaerobic condition to facilitate the growth of *Bacillus* on the surface of fermenting soybeans. [18, 27]
	Naturally fermented at warm place	*Bacillus* is heat resistant and can grow up to 45 °C [27]
	Fermented beans are covered with whitish mass, mucilaginous materials if touch appears sticky with umami favour	Whitish mass is the spores of *Bacillus* with PGA production which appears as mucilaginous and sticky materials on the surface of soybeans with umami flavour. Flavour is generated during proteolysis (breaking down of protein to amino acids by proteolytic enzymes synthesized by *Bacillus* spp. during fermentation) [22]

the sub-Himalayan regions, whereas buffalos are reared in the low land hills and *tarai* areas of the Central and Western Himalayas. Yaks (*Bos grannies*) are domesticated in the high lands in Sikkim, Arunachal Pradesh in the Eastern Himalayas, north-west districts of Nepal in the Central and Western Himalayas and Ladakh and TAR in the Western Himalayas. Milk of domesticated animals is traditionally preserved and made into varieties of artisan fermented dairy products by natural fermentation for future consumption [29]. Among the fermented milk products, a soft variety of cottage-cheese-like product known as *chhurpi* (Fig. 3a) is popular among the Nepali/Gorkha, which is consumed as delicious curry or thick soup with cooked rice. Soft variety of *chhurpi* with a short duration of fermentation (24–40 h) is slightly acid in nature, rubbery texture and mild-flavoured, which is mostly preferred by the Nepali/Gorkha consumers, whereas the same product, if kept for a longer fermentation period of more than 7 days, becomes strong-flavoured and highly acidic in nature. Such product is called *chhu* in Sikkim (Fig. 3b) and *datshi* in Bhutan (Fig. 3c) and is mostly preferred by the Bhutia, Tibetans, Dukpa and Ladakhi [30–32]. *Ema datshi* is the popular delicacy in the Bhutanese traditional cuisine prepared from *datchi* and green chillies. Another popular variety of *chhurpi* is a hard-textured, artisan product accordingly designed by the different ethnic communities as per their preferences. Such hard-textured milk product is called *chhurpi* or *dudh chhurpi* (Fig. 3d) by the Nepali/Gorkha and the Bhutia, *churkam* (Fig. 3e), by the Dukpa of Bhutan and Mongpa of Arunachal Pradesh, *chhura*, by the Ladakhi and *chura loenpa*. Hard-textured artisan *chhurpi* is eaten as a natural chewing gum without any addition of sugar supplements to provide an extra energy to body by continuous movement of jaws and gum of the highlanders Himalayan people. It is a popular milk product in the sub-Himalayan regions too as a masticator. This hard-textured chew-gum-like yak milk product is unique and is innovatively made only in the Himalayan regions; there is no historically correlation of such product with other global milk products.

The yogurt-like curd product commonly called *dahi* (Fig. 3f) also plays a vital role in the Himalayan dietary culture and is religiously considered as a sacred food item by the majority populace of the Hindu and the Buddhists. Highlanders in Sikkim, Arunachal Pradesh, Ladakh in India, Bhutan, northern parts of Nepal and TAR traditionally produce *dahi* from yak milk using their indigenous knowledge. However, in the sub-Himalayan and lowlands, *dahi* is prepared from cow and buffalo milk. Unlike the Balkans and Europeans *yogurt*, *dahi* is also used as non-food items such as to make adhesive coloured spot called 'tika' mixed with rice grains and coloured powder to be applied on the foreheads of the family members by the elders during the festivals and marriage ceremonies of the Nepali/Gorkha community as well as hill tribes in Himachal Pradesh and Uttarakhand in India. Such unique application of *dahi* as non-food adhesive for other sociocultural

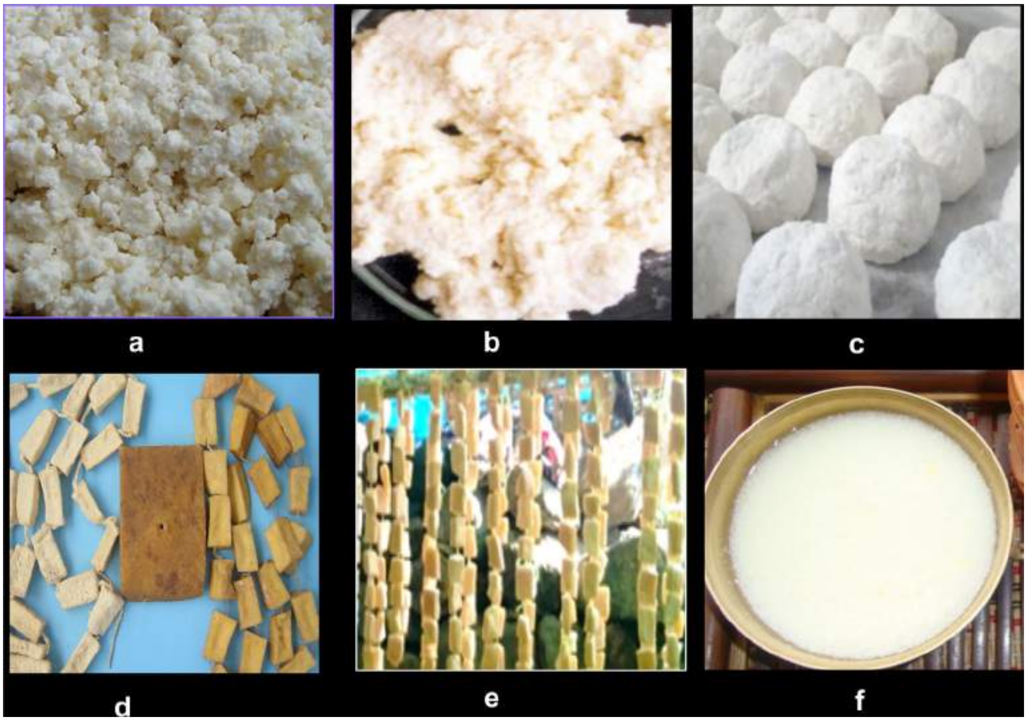

Fig. 3 Himalayan fermented milk products: **a** Soft variety of *Chhurpi* of Darjeeling hills, Sikkim and Nepal; **b** *Chhu* of Sikkim; **c** *Datshi* of Bhutan; **d** Hard-variety of Chhurpi of Darjeeling hills, Sikkim, Ladakh, Arunachal Pradesh, Nepal, Bhutan and TAR; **e** *Churkam* of Bhutan and Arunachal Pradesh; and **f** *Dahi* of the Himalayas

aspects by the Himalayan people has not been practised by other communities in the Indian subcontinent. *Dahi* is also offered to solemnize the marriage of Hindu and Buddhist.

Validation of traditional practices for milk fermentation

Unlike the European yogurt and many fermented milk products, which are produced by controlled fermentation using the standard probiotic starter culture, the Himalayan fermented milk products are still prepared in the traditional way without using any standard starter culture. Ethno-microbiological knowledge of the Himalayan people has been scientifically validated by evidence-based findings. A huge diversity of lactic acid bacteria with probiotic and other biological properties have been reported from various naturally fermented milk products of the Himalayas. These include: *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuc. mesenteroides* subsp. *jonggajibkimchii*, *Lactobacillus delbrueckii, Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *hordniae*, *Lc. lactis* subsp. *tructae*, *Lacticaseibacillus paracasei* subsp. *tolerans*, *Levilactobacillus brevis*, *Loigolactobacillus coryniformis* subsp. *torquens*, *Lentilactobacillus parabuchneri*, *Enterococcus faecalis*, *E. italicus*, *E. pseudoavium*, *Steptococcus salivarius, S. thermophiles* [30–36]. In vitro and genetic screening of lactic acid bacteria isolated from the Himalayan fermented milk products proved to have probiotic properties such as antimicrobial activities that ensure the safety of the product, reduce serum cholesterol level, making lactose-free fermented milk products for lactose-intolerant consumers, adherence abilities of bacteria to epithelial cells thus contributing the bacterial pathogenesis in the human gut [33, 37]. Though the antiquity and origin of the Himalayan fermented milk products are unknown, the scientific analysis of the traditional fermented milk products has validated the innovative knowledge of the Himalayan people on production of probiotic and functional dietary milk products for nutritional security and health benefits.

Himalayan fermented vegetable products

Intake of seasonal vegetables, mostly leafy vegetables and also wild edible herbs, in the daily diets is more common in the subtropical zones rather than temperate zones of the Himalayas. Depending on the altitudinal zones, varieties of leafy and other vegetables are grown for consumption in the sub-Himalayan regions mostly during the winter seasons. Many seasonal fresh leafy vegetables are scanty during the long monsoon seasons in the Himalayas, hence, the people have to depend on either wild edible plants as vegetable supplements or traditionally fermented vegetable products, which are stored as dry products. Some common fermented vegetable products of the Himalayas are *gundruk* (Fig. 4a); *sinki* (Fig. 4b), *khalpi, inziangsang*, etc., which are spontaneously fermented during winter seasons, when the fresh leafy vegetables such as '*rayo-sag*' (*Brassica rapa*), mustard (*Brassica juncea*) leaves and radish (*Raphanus sativus*) leaves are plenty. Unlike other Asian fermented vegetable products such as *kimchi* of Korea and *pao cai* or *suan cai* of China, which are mostly salted and spiced, and consumed immediately after the fermentation without drying or dehydrating, the Himalayan fermented vegetable products such as *gundruk* and *sinki* are non-salted and sun-dried after the fermentation, and the dried fermented vegetables are consumed as soup or curry (Fig. 4c).

Perishable vegetables are fermented naturally by the pit fermentation method which is unique and unapparelled in the Himalayas for biological preservation of perishable vegetables for future consumption. However, the genesis of such a traditional method of pit fermentation in the Himalayas has not been historically documented. Myth and oral history, documented from questionnaire during the survey narrated by elders of villages, have been recorded on the accidental origin of *gundruk* and *sinki* in Nepal [4]. Ancient and medieval histories of Nepal have seen several wars between small kingdoms prior to unification of modern Nepal [38], which compelled the local villagers to flee their homes. Before fleeing the villages, the wise villagers might have buried all available agricultural produce including freshly grown vegetables in the pits and were covered by mud and hay to hide from the enemies. After the war, the villagers returned back to their homes and started digging out the pits where they have dumped the agricultural produce. Actually, they were looking for food grains, mostly the paddy or maize, and when heaped grains were brought out from pits they pulled some dumped vegetables too. After taking out from the pits, the heaped food grains and vegetables were kept lying on the field in the sun to dry the moist foods grains and also to get rid of unpleasant odour, actually discarding the dumped vegetables. After some time, the villagers went to the pit sites and collected dried food grains and they accidentally noticed the sun-dried leftover vegetables with appealing flavour, and they tasted and found slightly sour with appealing flavour, and they liked the products which were different from the fresh

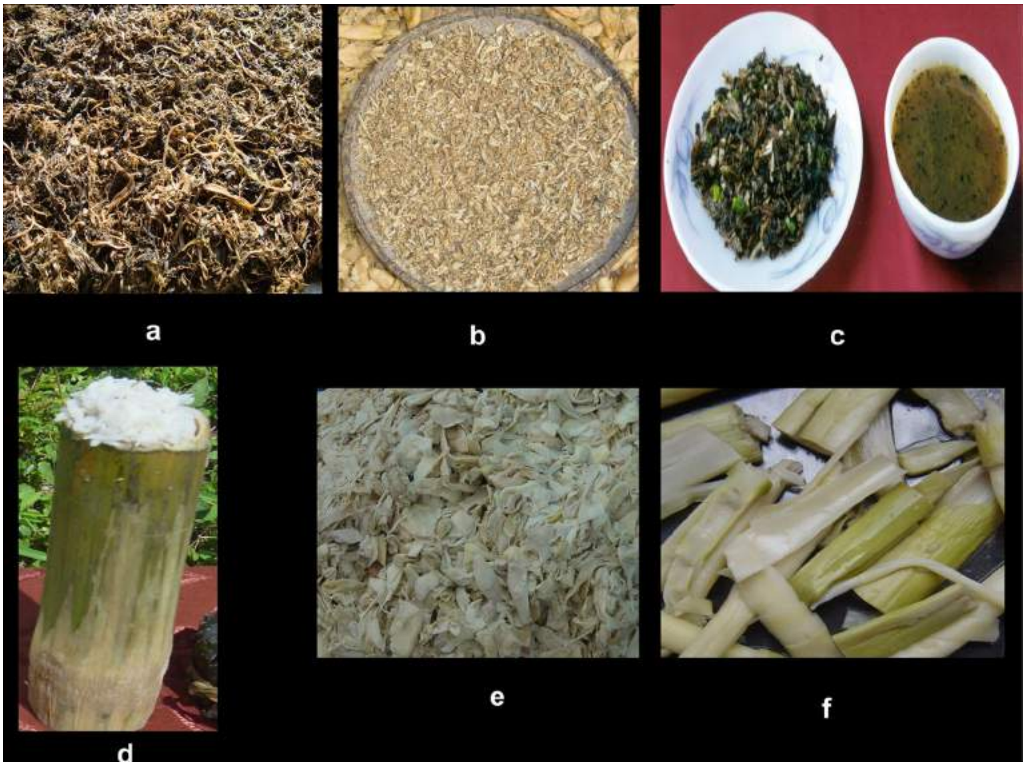

Fig. 4 Himalayan fermented vegetable products: **a** *Gundruk* of Darjeeling hills, Sikkim, Assam, Nepal and Bhutan; **b** *Sinki* of Darjeeling hills, Sikkim, Assam, Nepal and Bhutan; **c** *Gundruk* pickle and soup; Himalayan fermented bamboo shoot products: **d** *Mesu* of Darjeeling hills, Sikkim and eastern Nepal; **e** *Soibum* of Manipur; and **f** *Soidon* of Manipur

vegetables and radish. This is how *gundruk* and *sinki* have been accidentally invented in Nepal. Ancient villagers made the sun-dried fermented vegetables into different recipes as per their preferences and started standardizing the traditional methods of mass production of *gundruk* and *sinki*. Accidental invention of *gundruk* might have happened during the rule of Newar, one of the oldest inhabitants of Kathmandu valley in Nepal [39], probably the word *gundruk* might have derived from the Newari word *gunnu* meaning dried taro stalk [4]. The unique type of pit fermentation of vegetables, innovated by the Himalayan people, has not been reported in other parts of the world except in the South Pacific islands and China. Varieties of fermented bamboo shoots are also traditionally prepared and consumed as preserved pickles in the bamboo-growing regions in the Eastern Himalayas such as *mesu* (Fig. 4d) of Sikkim, Darjeeling hills and eastern Nepal, *soibum* (Fig. 4e) and *soidon* (Fig. 4f) of Manipur. Historically consumption and preparation of fermented vegetable and bamboo shoot products is not recorded in the Western Himalayas except in western Nepal (Table 1).

Validation of bio-preservation of perishable vegetables

The traditional method of bio-preservation of perishable vegetables by pit fermentation in the Himalayas is actually the lactic acid bacterial fermentation initiated by a consortium of acid-tolerant and carbon dioxide-tolerant (anaerobic condition) bacteria. During preparation, wilted and shredded vegetables or radish are pressed tightly inside the pit dug in the fields. The pressed vegetables are made into heaps in the pit lined with dry bamboo sheaths and rice straw and are covered from top and filled up with mud or cow dung on the surface and also weighted by heavy planks and stones to make it airtight and left for natural fermentation. With their 'ethno-microbiological' knowledge, the ethnic people were 'scientifically' making the anaerobic conditions by making the whole process airtight to facilitate the growth of functional fermenting low acid-tolerant (low pH) and CO_2-tolerant (anaerobic environment) lactic acid bacteria. These lactic acid bacteria present in *gundruk* and *sinki* fermentation are *Levilactobacillus brevis, Lactiplantibacillus plantarum, Lacticaseibacillus casei* ssp. *casei, Lacticaseibacillus casei* ssp. *pseudoplantarum, Limosilactobacillus fermentum, Pediococcus pentosaceus* and *P. acidilactici* [40, 41]. During the vegetable fermentation, the carbohydrate present in the vegetables is broken down to glucose by enzymes secreted by the fermenting bacteria for their growth and also lowers the pH to make the product acidic and sour in taste [42]. This will also inhibit the growth of other pathogenic and opportunistic microorganisms and thus making the fermented vegetable foods safe for consumption. Drying after the fermentation of *gundruk* and *sinki*, is to dehydrate the moisture for prolonging the shelf life of the freshly fermented moist product which can be stored at room temperature for future consumption up to 1 year or more. This is a good example of food preservation in the Himalayas where the majority of rural people cannot afford the cold storage or refrigerator. Himalayan people might have innovated such bio-preservation techniques also to carry the dried products, which are comparatively lighter than the weight of fresh substrates, while travelling for long distances in the difficult terrains of the mountains in ancient times. This ancient habit of carrying sun-dried *gundruk* and *sinki* by the Nepali/Gorkha, while travelling for long distances has become a cultural practice even today. Himalayan fermented vegetables have functional properties and antimicrobial and probiotic properties [43].

Fermented cereal products

Cooked rice is a staple cereal diet eaten with varieties of other fermented and non-fermented food items in the sub-Himalayan and low land regions of the Central and Eastern Himalayas. Maize is also a staple diet in the Eastern and Central Himalayas, whereas wheat and barley are the major staple cereal diets in the Western Himalayas. Millets, mostly finger millets and minor millets, are also high mountain cereal crops which are consumed as staple diets in the highlands of the Himalayas including TAR. Traditional fermentation of cereals as food items is rarely seen in the

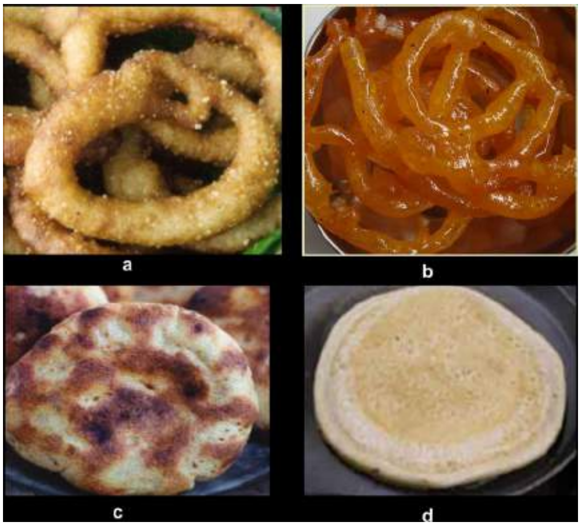

Fig. 5 Himalayan fermented cereal products: **a** *Selroti* of Darjeeling hills, Sikkim, Assam, Himachal Pradesh, Uttarakhand, Nepal and Bhutan; **b** *Jalebi* of Central and Western Himalayas; **c** *Khambir* of Ladakh; and **d** *Chira* of Himachal Pradesh

Eastern Himalayan regions except *selroti* (Fig. 5a), a ring-shaped deep-fried donut-like confectionary product in Nepal, Darjeeling hills, Sikkim and Bhutan. Rice is commonly fermented into mild alcoholic beverages in North-east India, Nepal and Bhutan in the Eastern Himalayas. Finger millet locally called 'kodo' is fermented into mild alcoholic beverages in Central and Eastern Nepal, Darjeeling hills, Sikkim and Arunachal Pradesh in India, and Bhutan. Whereas, a variety of non-alcoholic traditional fermented cereal products are prepared and consumed in the Western Himalayas as staple diets such as *khambir* (Fig. 5b) of Ladakh, *chira* (Fig. 5c) of Himachal Pradesh, etc. Besides edible, some of these ethnic fermented cereal products have ethnic values and are used during festivals and marriages in Himachal Pradesh, Uttarakhand, Kashmir and Ladakh [44]. *Jalebi* (Fig. 5d) is a popular fermented wheat flour mixed with sugar syrup and is deep-fried, circled, and crispy confectionary, in the entire Himalayan region, mostly in the Western Himalayas, except in Ladakh and TAR. Though *jalebi* is of Arabic or Persian origin, it has been known in North India since 1450 CE [45]. *Selroti* is one of the oldest heritage foods with socio-ethical values in the customary life of the Nepali/Gorkha in the Himalayas [46].

Fermented animal products

Consumption of beef is a taboo to the Hindu populace in the Himalayas, so as the pork-eating taboo to Muslims. However, traditional preservation mostly by smoking and drying and fermentation of perishable animal flesh is also performed by some ethnic Himalayan communities since immemorable time. The typical Himalayan sausage-like product such as *kargyong* (Fig. 6a) is prepared and consumed in Sikkim, Bhutan, Arunachal Pradesh in the Eastern Himalayas, *gemma* (Fig. 6b) is consumed in Uttarakhand, Ladakh, Himachal Pradesh and TAR in the Western Himalayas (Table 1). Smoked and dried meats are also common in the Himalayas.

Fish-eating culture is traditionally seen in the low lands in the sub-Himalayan regions where ponds, lakes, streams and rivers are located. Salting, drying, smoking and fermentation of locally available freshwater fish are common traditional practices among the different communities in North-east India, east Nepal, and Bhutan in the Eastern Himalayas residing nearby by water bodies and streams/rivers such as *sukuti* (Fig. 6c), *tungtap* (Fig. 6d), *ngari* (Fig. 6e, Table 1). However, such preservation practice of freshwater fish is uncommon in the Central and Western Himalayas. Antiquity of both meat and fish products in the Himalayas are still unknown. The food anthropologist claim that the Asian fermented

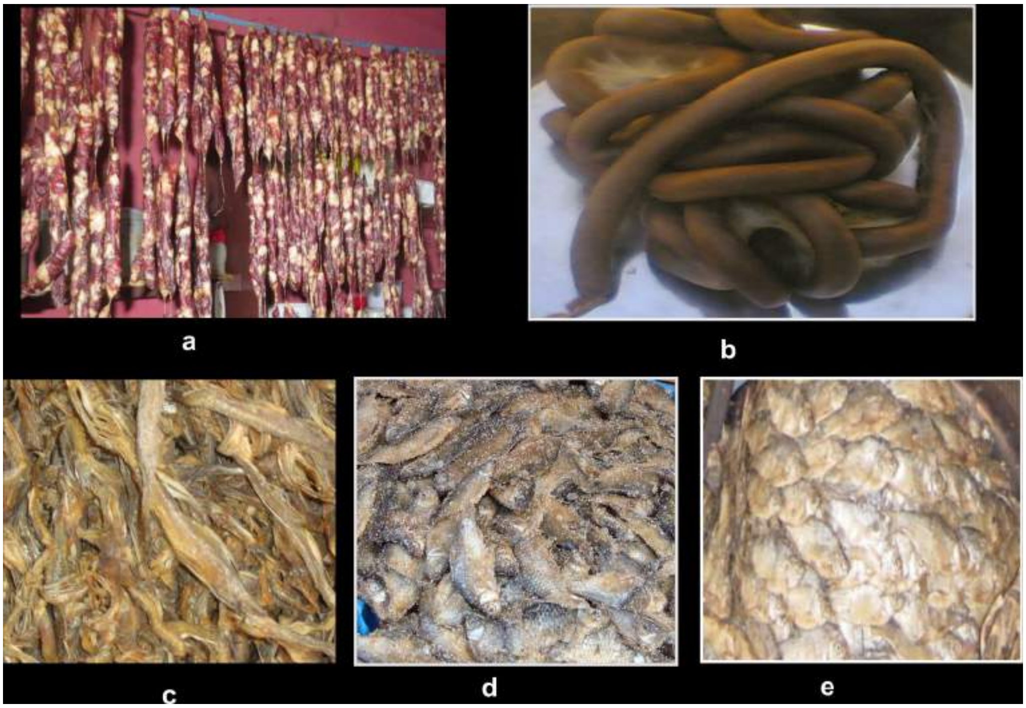

Fig. 6 Himalayan fermented sausages: **a** *Kargyong* of Eastern Himalayas and **b** *Gemma* of the Western Himalayas. Himalayan fermented sausages: **c** *Sukuti* of the Eastern and Central Himalayas; **d** *Tungtap* of Meghalaya; and **e** *Ngari* of Manipur

fish culture has originated from Makong river basin [47]; however, the traditionally preserved and fermented fish products of South East Asia are different from the Himalayan fish products in terms of traditional processing and mode of consumption. In the Himalayas, preserved and fermented fish are eaten as curry and pickle, however in South East Asia, fermented fish products are mainly made into fish sauce and used as condiments and tastemakers. Recently, a huge microbial community diversity was explored in some traditionally prepared fish and meat products and some of these bacteria have biological functional properties [48, 49]. These traditionally processed fish and meat products are also found safe to eat due to non-production of toxins and absence of some deadly pathogenic bacteria [50, 51].

Alcoholic fermented beverages

Alcoholic fermented beverages, mostly prepared from rice, millets and other cereals, and their distilled liquor are culturally and socially used for drinking as well as for worshipping the ancestors and Gods in the Himalayas by the majority of ethnic communities, except the Brahmin Hindu. Wine-making and also brewing and malting for beer production are historically unknown in the Himalayas, since grapes are not the traditional fruits, except in Himachal Pradesh where grapes have been cultivated since the eighteenth century after the Britishers introduced wine culture in India [52]. Origin and antiquity of the Himalayan alcoholic beverages and use of starter cultures for alcohol production are unknown. Like in many South East Asian countries, alcoholic beverages are traditionally prepared from cereals mostly rice, and finger millets, by mixing with traditionally made non-eatable artisanal starter culture in the form of dry, oval, round, flattened shaped with variable shapes and sizes. Such traditionally prepared starters are known by local names in each locality of the Himalayas (Table 1). *Marcha* (Fig. 7a) is one of the most common starters of the Nepali/Gorkha community which is traditionally used to ferment cereals and millets into alcoholic beverages in Central and Eastern Himalayas regions. Among the Nepali, *marcha*-making hereditary trade is historically confined to the Limboo and Rai castes, both belonging to the Kirat race. *Marcha*-like other community-specific

Fig. 7 **a** *Marcha*, an amylolytic starter culture for alcohol production; **b** fermented finger millets; **c** *Kodo ko jaanr/chyang*, fermented finger millets, mild alcoholic beverage inside the bamboo-made vessel called 'toongbaa' in the Himalayas

starter cultures with different names are also traditionally prepared by other non-Nepali ethnic communities of the Himalayas ranging from Western to the Eastern Himalayas. Conventionally, rice grains are soaked in water for 6–8 h, dewatered and soaked rice grains are crushed in pounded in a wooden mortar by a pestle, then rice flour is mixed with roots and leaves of some locally available wild plants and some spices such as ginger, dry red chilli, and 2–3% of previously made powered starter, as a mother culture (back-slopping) is added to the mixture. The mixture is then made into thick doughs by adding water and kneaded into different artisanal and variable shapes and sizes, and then they are kept in the fresh fronds of ferns frond/paddy leaves/other leaves on the bamboo stripes, covered by dry ferns and jute bags. Covered doughs are kept at a warm place for fermentation for 2–4 days depending on the seasonal weather of the regions. Swollen appearance of starter culture with mild alcoholic and ester aroma indicates the completion of fermentation. Freshly fermented doughs are taken out from wrapping leaves and are immediately placed in bamboo-made trays for sun-drying for 2–3 days to obtain dry starter culture, which can be stored in a dry condition for a year or more. The general steps followed for the preparation of crude starter cultures among the multiethnic people are more or less same, except some variation in wrapping materials, addition of locally available wild herds and incubation period from region to region in the Himalayas. The artisan starters, innovated by the Himalayan people, are not prepared by every family or community, the production skill is protected among few caste and sub-caste mostly by mountain women who also sell the artisan starters in the local markets for the livelihood and income generation.

The Himalayan alcohol-producing starters, prepared from rice/millets and few wild herbs, are actually the consortia of mixed cultures of saccharifying filamentous moulds [53], amylase and alcohol-producing yeasts [54] and acid-flavour-producing bacteria [55] for the production of alcoholic beverages. The ancient Himalayan people have been cultivating the desirable microorganisms in the form of dry artisan starter where rice serves as the starch-containing base for cultivation of the essential microorganisms. Scientifically, addition of few pinches of powdered starter is to sub-culture or transfer the essential inocula (necessary and functional moulds, yeasts and bacteria) to new starters. Hence, the ethno-microbiological knowledge of the ethnic Himalayan people has been maintaining the functional microbes for long centuries. Several bio-functional properties including probiotics [56] and enzymatic [57] have been reported in the age-old Himalayan artisan starters. Addition of wild herbs during the preparation actually gives more sweetness (glucose or carbon source) for fermenting microorganisms, and probably the addition is to inhibit the growth of the pathogenic or other unwanted microorganisms during the fermentation.

The traditional preparation methods of the Himalayan dry starter cultures are quite identical to that of dry starter cultures of South East Asia such as *daqu* and *chu* of China and Taiwan, *loogpang* of Thailand, *benh/men* of Vietnam and *dombea* or *medombae* of Cambodia *nuruk* of Korea, *ragi* of Indonesia, *bubod* of Philippines [58, 59]. Some Chinese historians claimed that the first barley-based starter called *chu* was originated in North China in 530–550 CE [4] and rice-based chu was originated in South China [60]. Baijii, one of the oldest Chinese alcoholic drinks, is prepared from rice using *daqu,* Chinese artisan starter culture, which was recorded in the second century BCE [61]. Whether *marcha*-making process has independently originated in the Himalayas, or has a historical correlation among the other Asian starters is difficult to establish.

Himalayan artisan starters are used to ferment cereals into different types of thick, mild alcoholic and sweet–sour beverages. Fermented beverages are traditionally distilled to obtain high alcoholic liquor for drinking. Sporadic history on *kodo ko jaanr*, a fermented finger millet mild alcoholic beverage was mentioned during the Kirat dynasty in 625 BCE to 100 CE [62] and also during Malla dynasty in 880 CE [63]. Drinking culture of traditional fermented millet beverages of ethnic people of Darjeeling hills and Sikkim was mentioned in some historical literature works [64–66]. Unlike in other Asian countries, the Nepali, Bhutia, Lepcha, Tibetan, Ladakhi, Mongpa and Dukpa communities of the Himalayas sip the extract of the fermented finger millet beverage locally known as *kodo ko jaanr* or *chyang* (Fig. 7b), which are poured inside an artistically designed vessel, made of up bamboo shoot, called 'toongbaa' (Fig. 7c), by a narrow bamboo-made straw. Himalayan beverage is considered as a high-calorie food beverage with a low alcohol content of 4% [67], with bio-availability of minerals [68, 69]. Though origin and history are unknown, it is assured that the Himalayan low alcoholic beverages prepared by using artisan starters contribute high-calorie food beverages and some essential bioactive compounds in the diets of highlander people in adaption to harsh climatic conditions and deficient of regular food supply. Besides drinking, alcoholic beverages have deep-rooted social values, used to solemnize the marriage ceremony, worship Gods and ancestors, other social functions and festivals and perform spirit possession by some communities.

How important the Himalayan fermented foods to the diets of the twenty-first century?

The people outside the Himalayan regions may have the curiosity to know how important the Himalayan fermented foods in the context of human health. The Himalayan fermented foods are always considered healthy and nutritive foods. For example, Himalayan fermented soybean foods, such as *kinema and hawaijar*, are inexpensive high plant protein foods with several health-promoting benefits such as immunity booster antioxidants (anti-cancer), anti-thrombotic (to cure heart disease), anti-thrombotic properties, anti-diabetic and are rich in vitamins, bioactive peptides, isoflavones, immunomodulators, etc. [28, 70–73]. Himalayan fermented milk products such as *dahi and chhurpi* have several probiotic properties [32, 33], biopeptides [74], etc. The consumers of the twenty-first century prefer foods which cure ailments, combat diseases, reduce gut dysbiosis, boost immunity and impart health-promoting benefits. Probably, the Himalayan fermented foods can supplement such demands with high functionalities and health tags to consumers. However, due to popularity and high preference of fast foods and commercially available ready-to-eat foods as well as modernization and urbanization of many regions in the Himalayan countries, the traditional food habits and dietary cultures are affected mostly among the young generations. These changeovers in food habits and more choices of fast foods over ethnic foods may affect the production and consumption of the Himalayan fermented foods and alcoholic fermented beverages. The other reasons may be the climate change which affect the yield and productivity of the agricultural produce and finally may impact the production of traditional fermented foods. Though limited information is available on the impact of climate change on production of Himalayan fermented, Tamang et al. [75] reported the cultural adaption of Himalayan fermented foods to mitigate the harsh climate change in the high mountains.

Conclusion

Diverse types of exotic, artisan, acidic, alkaline, slow, functional, nutraceutical and fermented foods and alcoholic fermented beverages are traditionally produced by the multiethnic communities of the Himalayan people by their ethno-microbiological knowledge of food fermentation. Some of these fermented foods have been scientifically studied and proved to have several health-promoting benefits to consumers.

Besides delicacy, ethnical and cultural values, the Himalayan fermented foods are also the hubs of biological resources of beneficial and functional microorganisms which have been contributing health-promoting benefits to thousands of Himalayan people with a dearth of modern food products. Since the Himalayan ethnic fermented foods are region-specific and are mostly based on the traditional knowledge of the ethnic people, the Himalayan people have culturally and historically adopted the consumption of various ethnic fermented foods and alcoholic fermented beverages even in the dearth of modern food supply, hence their traditional knowledge and dietary culture may be preserved with more awareness on the scientific findings on health tags or safety measures of their cultural foods. If so, the importance of regional, traditional, cultural fermented foods and beverages of the Himalayas towards the Sustainable Development Goals of the 2030 Agenda of the United Nations, adopted in 2015 may suffice and create an example of sustainable cultural food and nutritional security. The novelty of this study is the documentation on validation of the traditional practices of some common Himalayan fermented foods by scientific evidence, which have been extensively studied earlier. However, the limitation of this study is tracing back the origin and history of the Himalayan fermented foods and alcoholic fermented beverages due to a dearth of archaeological findings, historical monuments and chronological records of the ethnicity in the Himalayas and the unavailability of food anthropological studies. When and how the Himalayan fermented foods and alcoholic beverages originated are still unknown.

Acknowledgements
Nil.

Author contributions
The paper was written by me. The author read and approved the final manuscript.

Funding
Not required.

Availability of data and material
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The author declares that there is no competing interests.

Received: 26 May 2022 Accepted: 19 July 2022

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