MICROBIOLOGICAL EVALUATION OF INDIGENOUS NON - ALCOHOLIC FERMENTED FOODS OF NORTH EAST INDIA AND THEIR PROBIOTIC POTENTIAL

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PURBAJYOTI DEKA

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BY

PURBAJYOTI DEKA

Department of Biotechnology

Supervisor: Dr. ESTHER LALNUNMAWII

Joint Supervisor: Dr. BHIM PRATAP SINGH

Submitted

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DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF LIFE SCIENCES
MIZORAM UNIVERSITY
(A central University)
P.B. No.190
TANHRIL, AIZAWL-796004, MIZORAM, INDIA

Dr. Esther Lalnumawii
Assistant Professor Email: essie.zadeng@gmail.com

CERTIFICATE

This is to certify that the thesis entitled "**Microbiological Evaluation of Indigenous Non-alcoholic Fermented Foods of North East India and their Probiotic Potential"** submitted by **Purbajyoti Deka,** PhD Research Scholar for the award of the Degree of Doctor of Philosophy in Biotechnology is carried out under my supervision and incorporates the student bonafide research and this has not been submitted for the award of any degree in this or any other university or institute of learning.

Place: Aizawl, Mizoram **(Dr. Esther Lalnunmawii)**
Date: **(Supervisor)**

(Dr. Bhim Pratap Singh)
(Joint Supervisor)

DECLARATION

MIZORAM UNIVERSITY

JULY, 2025

I **PURBAJYOTI DEKA,** hereby declare that the subject matter of this thesis entitled is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the **Degree of Doctor of Philosophy in Biotechnology.**

(PURBAJYOTI DEKA)

Research Scholar

(Prof. N. SENTHIL KUMAR)

Head of the Department

(Dr. ESTHER LALNUNMAWII)

Supervisor

(Dr. BHIM PRATAP SINGH)

Joint Supervisor

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Place: Aizawl **(PURBAJYOTI DEKA)**

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LIST OF ABBREVIATIONS AND SYMBOLS

%	:	Percentage
+ve	:	Positive
-ve	:	Negative
≤	:	Less than or equal to
℃	:	Degree of celcius
µM	:	Micromolar
16S rRNA	:	16S- Ribosomal Ribonuceleic Acid
AOAC	:	Association of Official Analytical Chemists
BLAST	:	Basic Local Alignment Search Tool
BV	:	Bacterial vaginosis
Bp	:	Base pair
CCA	:	Correspondence analysis
DNA	:	Deoxyribonucleic Acid
EFSA	:	The European Food Safety Authority
e.g.	:	Exempli gratia: For example
et al.	:	et alii: and other
etc.	:	et cetera: and other things
GI	:	Gastrointestinal tract
g/L	:	Gram/Litre
GRAS	:	Generally recognized as safe
HCL	:	Hydro chloric acid
IBS	:	Irritable Bowl Syndrome
LC-MS	:	Liquid Chromatography-Mass Spectrometry
H	:	Hour
LAB	:	Lactic Acid Bacteria
LB	:	Luria Bertani
M	:	Molar
MEOR	:	Microbially enhanced oil recovery
Mg ml^{-1}	:	Miligram per Millilittre

mM	:	Milli Molar
mm	:	Millimeter
MEOR	:	Microbially Enhanced Oil Recovery
MTCC	:	Microbial Type Culture Collection
No	:	Numbers
NCBI	:	National Centre of Biotechnology Information
ML	:	Maximum Likelihood
NaOH	: `	Sodium hydroxide
OUT	:	Operational Insights into Microbial Ecology
PCA	:	Principal component analysis
PCR	:	Polymerase Chain Reaction
PCoA	:	Principal coordinate analysis
PBS	:	Phosphate buffer saline
P H	:	Negative Ion of Hydrogen Ion Concentration
QIIME	:	Quantitative Insights Into Microbial Ecology
SAC	:	Saccharolytic activity
SAC s	:	Surfaceactive compounds
Sp.	:	Species
TAE	:	Tris Base, Acetic Acid and EDTA
U/µl	:	Unit Per Micro Liter
UHPLC	:	Ultra-high performance liquid chromatography
UPGMA	:	Unweighted Pair-Group Method with Arithmetic means
UV	:	ULTRA-Violet
WHO	:	World Health Organization

Fermented foods are nutritionally and functionally enhanced due to the action of microorganisms, such as bacteria, fungi (mycelia and yeast) and their enzymes (Aslam et al., 2020). The International scientific association of Probiotics and Prebiotics (ISAPP) defines fermented food as food prepared by the wanted development of microorganisms and by the enzymatical change of food substances. Fermented foods, although microbially fermented, does not always contain live/viable microorganisms at the time of consumption. But the term probiotic is more specifically applied to products containing well-characterised living strains of microorganisms that have been well tested and shown by research studies to have a beneficial effect on the host health when consumed in sufficient quantities. This definition will help to distinguish between probiotics and fermented foods due to strain specificity and appropriate choice of dose and clinical studies (Hill et al., 2023). And fermented foods may be good carriers of probiotics, which can be transported to the host by the modified food matrix and therefore provide the health benefits of the food matrix and the microorganisms (Ikango and Antony, 2021). Historically, dairy, vegetables, fruits, grains, legumes (e.g., soybeans), meat and aquatic products have been fermented to make various products. This biochemical reaction can be controlled by many factors, including the microbial consortia, physicochemical characteristics of the substrate and environmental conditions. This combination has resulted in the remarkable diversity of fermented foods. Over the years, fermentation has been a vital means of food preservation and this process has been helped by the production of inhibitory compounds by microorganisms such as organic acids, alcohol and bacteriocins which prevent the growth of spoilage and pathogenic microorganisms. The process of fermentation also results in the improvement of microbiological safety and the extension of shelf-life but it also contributes to the sensory properties of foods, how they taste, smell and feelIn others, it makes foods quite tasty or harmless to eat, an example is the fermentation of olives to lower the levels of bitter phenolic compounds which otherwise would make them inedible (Marco et al., 2021).

Food fermentation generally occurs via two principal pathways. The first involves spontaneous fermentation, often referred to as "wild fermentation". This process

relies on the indigenous microflora naturally present on the raw materials or within the processing environment. Such autochthonous microbial populations initiate and sustain the fermentation without the intentional addition of starter cultures. Notable examples of spontaneously fermented foods include sauerkraut, kimchi, and various traditional fermented soy-based products. Secondly foods can undergo fermentation by adding starter cultures; these are referred to as "culture-dependent ferments" and include *natto*, *kefir*, and *kombucha* (Rezac et al., 2018). "Backslopping" is a culture-dependent fermentation technique where a tiny quantity of a previously fermented batch is combined with the raw food, such as sourdough bread (Marco et al., 2017). Alternatively, in industrial scale fermentation, the use of defined starter cultures comprising specifically selected microbial strains is increasingly preferred. These cultures are employed to ensure process consistency, enhance product safety, and achieve predictable sensory and physicochemical attributes in the final product.These commercial starters are chosen for their ability to consistently deliver desirable organoleptic qualities, such as specific flavors, aromas, and textures, thereby ensuring product uniformity and safety (Liu et al., 2022).

According to Rezac et al. (2018), the majority of fermented products have been found to have at least 10^6 microbial cells per gram, with concentrations varied based on a number of factors including the product's age, area, and time of analysis or consumption. The food matrix seems to play an important role in the survival of probiotic strains because of its buffer capacity and protection against the gastrointestinal environment such as low pH and bile acids (Bove et al., 2013). Recent studies have provided growing evidence that live microorganisms present in fermented foods are able to survive the gastrointestinal passage and are temporarily present in the intestine. But their survival and transient colonization are influenced by several factors, including the taxonomic identity and functional properties of the microorganisms, the physico-chemical characteristics and composition of the food matrix, and the host-related gastrointestinal conditions (pH and bile acids).

These factors not only affect microbial viability but also modulate their interactions with the host microbiota and potential health outcomes (Sanders et al., 2023). However, these microbes may still exert beneficial physiological effects in the gut by outcompeting pathogenic bacteria and generating fermentation by-products that

modulate immune function and support neurogenesis (Zhou et al., 2022). In addition, bioactive metabolites generated during the fermentation process have been associated with a range of potential health-promoting effects. These compounds may contribute to improved gut health, modulation of the immune system, and enhanced bioavailability of nutrients. For example, according to Pessione and Cirrincione (2016), lactic acid bacteria are important for fermented foods to both dairy and non-dairy products. Microorganisms involved in fermentation are capable of synthesizing bioactive compounds such as peptides and polyamines, which have been implicated in modulating immune function, metabolic processes, and cardiovascular health. Furthermore, fermentation can facilitate the biotransformation of certain precursor compounds into physiologically active metabolites, thereby enhancing their bioavailability and biological efficacy. For example, flavonoids and other phenolic substances can be transformed into physiologically active metabolites by lactic acid bacteria (Filannino et al., 2015).

Fourthly, nutrients like vitamins and prebiotics that are present in fermented foods may also have health benefits (Marco et al., 2017; Salazar et al., 2016). Finally, fermentation can lower the concentrations of toxins and anti-nutrients. For example, fermentation of soybeans can lower the levels of phytic acid (Abu-Salem et al., 2014) and the amount of fermentable carbohydrates (such as fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) can be decreased by sourdough fermentation which could make these products more tolerable for people with functional bowel disorders as irritable bowel syndrome (IBS) (Laatikainen et al., 2016). Thus, scientists have suggested that foods that have undergone fermentation be incorporated into dietary recommendations.

Chilton and colleagues argue that fermented food should be taken into consideration for regular intake and included in food consumption guidelines because of their long history of use in the human diet and their proven and developing nutritional and therapeutic benefits, which are partially attributed to their own microbiome (Chilton et al., 2015). Although fermented foods may act as delivery vehicles for various "biotic" components such as probiotics, prebiotics, synbiotics, and postbiotics they must fulfill rigorous criteria to be formally classified as biotic products. These

include complete description of the product (including identification of the microbial constituents at strain level, for probiotics, standardised testing and scientific substantiation of the health benefits. Importantly, similar to other functional ingredients, the health promoting effects cannot be solely attributed to the nutritional composition or structural features of the food matrix; instead, the bioactivity of the incorporated biotic component must be distinctly validated. Consequently, it is not appropriate to use the phrases "probiotic" and "fermented food" interchangeably (Vinderola et al., 2023). Since ancient times, humans have included fermented foods in their diet. Foods undergo a transformation during fermentation, which helps to maintain their nutritional content, safety, and unique, palatable organoleptic qualities (Sanlier et al., 2019).

Among other sources, randomized controlled trials with specific interventions have shown evidence that fermented foods can have health benefits (Kato-Kataoka et al., 2016), or correlation studies measuring the consumption of fermented foods with frequently unclear microbiological composition (Dimidi et al., 2019). *Yogurt* and other fermented milk products are the most researched fermented foods (Gómez-Gallego et al., 2018; Savaiano and Hutkins, 2021), They have been shown in numerous studies to have immunomodulatory effects, support gut transit and lactose tolerance, aid in the management of minor gastrointestinal symptoms, and help prevent diabetes, cardiovascular disease, and osteoporosis (Hadjimbei et al., 2022), reductions in serum cholesterol levels and gastrointestinal illnesses (Shiby and Mishra, 2013) and to potentially positively affect the gut microbiota (González et al., 2019).

Due to their capacity to alter the gut microbiota and/or interact with the host's immune system, some of these ingredients may improve health (De Marco et al., 2021; Rousseaux et al., 2023). In fact, there is growing interest in the potential of fermented foods to modulate gut microbiota due to the growing understanding of the involvement of gut microbiota in the etiopathology of several chronic diseases (Duvallet et al., 2017). Such a moderating ability may account for some of the health benefits of fermented foods (Kok and Hutkins, 2018).

As an illustration, Studies indicate that diets that are higher in consumption of particular fermented foods can change the gut microbiota and decreases inflammatory biomarkers (Wastyk et al., 2021). Fermentation is one of the oldest and most common forms of traditional food processing, traditionally used to increase shelf life and enhance microbiological food safety. The importance of this bioconversion process in food preservation is significant even before the development of modern refrigeration and thermal processing methods. The use of mixed cultures of microorganisms generates products that are highly nutritious, have improved flavors, smells, and textures (Ilango and Antony, 2021).

Martínez-González et al. (2019) note that addition of fermented foods to the diet has been linked to better overall health status, quality of life, and longevity in addition to the traditional functions of preserving foods, ensuring their safety and improving their sensory characteristics. In order to truly appreciate the functional capability of fermented foods, it is important to identify and describe the various microbial communities that participate in fermentation because it is this microbial diversity that mediates the biochemical changes as well as the health related effects that are ascribed to fermentation. Marco et al. (2021) add that the most common microorganisms involved are filamentous fungus, yeasts, and lactic acid bacteria. It is worth mentioning that not every microorganism that is involved in the fermentation process can be termed as a probiotic, regardless of their enormous contribution to the functionality and health-promoting characteristics of fermented foods. There are strict criteria of probiotic status such as strain level identification and proven health benefits in the host.

Additionally, India's geographical diversity and population diversity results in substantial food production and use variation. The country's diverse climatic zones, along with its rich ethnic, cultural, and religious heterogeneity, have given rise to a wide array of region-specific fermented food traditions and practices.

Individuals all around the country have quite distinct eating habits, but in the hilly regions in particular, individuals have developed unique ways of making fermented dishes and drinks using readily available local ingredients (Prajapati, 2003).

Northeastern India, with its rich mosaic of ethnic communities, offers a valuable context for ethnobotanical and ethnonutritional research. The dietary practices of

these indigenous populations are notably marked by a high consumption of traditionally fermented foods and regionally sourced indigenous food resources, reflecting deep-rooted cultural knowledge and ecological adaptation.

The aging advances used by the ethnic people reveal a strong connection between these people and nature as well as an appreciation of the benefits of microbes (Pohsnem et al., 2023). These ethnic communities created fermented cuisine using locally accessible raw resources that includes green vegetables, different types of bamboo, milk, soybean, pork, fish, cereal, and consumed it as a part of their regular diet (Das et al., 2024).

Northeast India has a long history of traditional fermented foods, a part of their culinary and cultural heritage. An example is in the state of Mizoram where fermented bamboo shoot (*Tuaithur*), fermented soybean (*Bekang*) and fermented pork fat (*Saum*) are widely eaten and form a significant part of everyday food. Such products are conventionally made by spontaneous fermentation techniques based on local knowledge systems over generations, and may not be inoculated with a specific microbe or subjected to standard processing parameters.

These fermented foods are relatively cheap to produce and some types naturally include bioactive compounds that have preservative effects, so they can be stored under different conditions and periods (several days-months). Some of these items are made throughout the whole year, but some are related to particular events of season or cultural celebrations. A significant percentage of fermented foods as well as the traditional alcohol beverages are also produced at household levels. Normally, the products are eaten fresh or stored without much cooking or chilling indicating how the community has adapted to the local environmental and resource conditions. Nevertheless, a significant number of such foods can be contaminated throughout the preparation process because of the absence of hygiene measures and the ignorance of food safety in the communities (Das et al., 2024).

Many of the traditional fermented foods have complex and mostly uncharacterized microbial communities, with some strains that are not identified or well studied taxonomically. Such undocumented microorganisms could potentially be a danger to food safety, such as foodborne infections or outbreaks, especially in the absence of standardized hygienic practices and microbial quality control during preparation and

storage. This is why it is extremely important to determine the safety of traditional foods, which are local specialties and consumed on a regular basis and are highly demanded in different parts of Northeast India (Pandey et al., 2023; Skowron et al., 2022).

The study of the bacterial diversity of these foods can be used to yield valuable information about the flavor and texture of the end products of fermentation. Although traditional fermented foods are commonly consumed and have been culturally relevant, they have received little scientific research and especially studies into their microbial composition and possible health-promoting effects. In the literature, there is a paucity of comprehensive studies addressing the diversity, functionality and probiotic potential of the related microbiota. Thus, the current research will focus on emphasizing this bacterial diversity, as well as the proximate composition of various traditional fermented foods and probiotic attribution (*Tuaithur*, *Bekang* and *Saum*) that are mostly eaten in the Mizoram state of North East India.

2.1 What is fermentation and why is it essential?

The derivation of the words fermentation, dates back to *fervere* in Latin, which translates to *boil*. It can be defined as any process that involves mass culture of microorganisms in order to make a product (Stanburry 1999). The fermented foods have attracted international interest as the functional foods, which improve nutrient availability and offer extra health benefits to the consumers (Durazzo et al., 2022). The list of possible benefits of fermented foods is growing, especially in relation to their ability to enhance immunity, improve digestion, and lower cholesterol (Nazhand et al., 2020). Fermented foods are very common in the Eastern Himalayas, and the consumption of such foods is an important part of the food culture of the Himalayan people living in Northeast India, Eastern Nepal, and Southern Bhutan (Tamang 2022).

In addition to preservation, fermentation increases the nutritional value of foods by fermenting valuable metabolites like organic acids, augmenting the digestibility of proteins by breaking them down into enzymes, and minimizing the presence of antinutritional factors and endogenous toxins. Moreover, fermentation may shorten cooking duration and enhance the nutrient bioavailability of foods, making them easier to digest and health-promoting (Tamang et al., 2023). It refers to a slow decomposition process of living substances that is speeded up by microorganisms or enzymes, and is essentially a process that breaks down complex substances into simpler carbohydrate alcohols or organic acids (FAO 1998). During the fermentation process, food is also preserved and, B vitamins, beneficial enzymes various strains of probiotics and Omega-3 fatty acids are produced (Sathe and Mandal, 2016). Fermentation is one of the most cheapest and traditional way of food preservation and safety. In addition, fermented foods offer additional benefits as they provide bionutrients and minerals and improve taste and aroma (Jeyaram et al., 2009).

2.2 Microorganisms in fermented foods

Microorganisms play a crucial role in altering the chemical makeup of raw materials during the fermentation process, enhancing the nutritional profile of certain fermented foods and providing health advantages to consumers. Lactic acid bacteria

(LAB) are commonly found in a variety of fermented foods and beverages. Prominent genera of LAB, including *Alkalibacterium*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*, have been identified in numerous fermented products worldwide (Tamang et al., 2021).

2.3 Safety of fermented foods

Fermented foods are also known to have a high level of safety even under low resource conditions where they are commonly prepared in informal conditions by people who lack formal microbiological and food safety training. Although in some cases these foods are cooked in places of poor hygiene, these are eaten daily by hundreds of millions of people in both developed and developing nations with limited health problems. Nevertheless, although fermentation has been shown to help in the preservation of food, it does not reduce the risk of contaminated water, poor hygiene, disease vectors or improper handling of raw or unfermented food. It is worth mentioning that even cooked foods may become sources of foodborne illness in case they are improperly stored or processed (Hutkins et al., 2023).

2.4 Nutritional aspects of fermented foods

Nutrition is the overall supply of energy (calories) and nutrients (proteins, indispensable amino acids, essential fatty acids, vitamins, and minerals) required to maintain physiological functions and homeostasis in metabolic processes (Gropper and Smith, 2021). The milk and other substrates undergo fermentation to produce bioactive compounds that have immunomodulatory and anticarcinogenic effects. The health promoting effects of fermented foods can be attributed to both the direct and indirect mechanism. Direct effects are improved protein bioavailability and essential amino acid profiles, whereas indirect benefits are improved concentrations and bioavailability of micronutrients including thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), and folate (vitamin B9) (Marco et al., 2021). The evidence shows an up-and-coming role of fermented milk products fortified with probiotics or prebiotics in reducing the risk and progression of chronic non-communicable

diseases such as cardiovascular disease, osteoporosis, hypertension, and some types of cancer (Hill et al., 2014; Meshram et al., 2019).

2.5 Diversity of Fermented Foods of North East India:

The North-Eastern Region of India, home to numerous indigenous tribes, exhibits distinctive socio-cultural practices and dietary traditions, particularly in the use of traditional technologies for preparing fermented foods and beverages (Deori et al., 2022). A diverse range of region-specific fermented foods and beverages are traditionally prepared and consumed across Northeast India, with many of these products also being commercially available in local markets (Figure 2.1). Different substrates and fermentation organisms are used to make these ethnic products, and the process used varies from place to place (Das et al., 2012). Until now, authentic fermented food such as bamboo shoots, soy products, vegetables, meat, fish, leaves, etc. form a large part the typical diet of individuals living in India's northeastern states. The ethnic population of Northeast India has been consuming fermented foods and alcoholic drinks for over 2,500 years. Every fermented food is specific to a certain area and has its own processing techniques and substrates. Locally accessible resources such as bamboo, vegetable, milk soybeans, fish, meat, and grain are often used (Tamang et al., 2012).

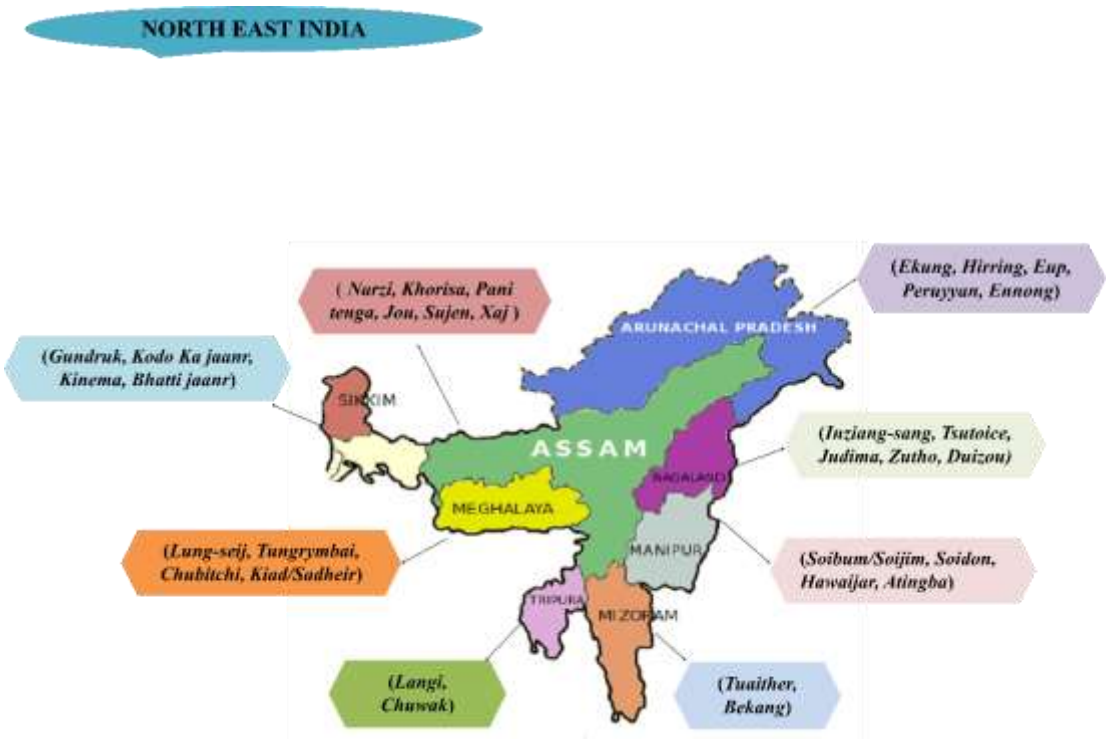


Figure 2.1: Map showing the diversity of fermented foods of North East India

2. 5.1 Traditional fermented fruits and Vegetable of North East India

The Eastern Himalayan region of India harbors a remarkable diversity of traditional fermented vegetable products, which have historically evolved as a practical means for preserving seasonally perishable vegetables for off-season consumption (Tamang 2010). Notable examples include *gundruk*, *sinki*, and *khalpi* traditional lactic acid-fermented vegetables that are culturally embedded in the dietary practices of Nepal, Sikkim, and Bhutan. These fermented foods are predominantly processed through the metabolic activity of lactic acid bacteria (LAB), including *Lactobacillus brevis*, *L. plantarum*, *Pediococcus pentosaceus*, *P. acidilactici*, and *Leuconostoc fallax* (Tamang et al., 2005; Marco et al., 2021). Similarly, ethnic bamboo shoot-based fermentations such as *mesu*, *soidon*, *soibum*, and *soijim* harbor complex LAB communities, comprising *L. brevis*, *L. plantarum*, *L. curvatus*, *P. pentosaceus*, *L. mesenteroides* subsp. *mesenteroides*, *L. fallax*, *L. lactis*, *L. citreum*, and *Enterococcus durans* (Tamang et al., 2005; Tamang and Watanabe, 2019).

Some of the tribal groups in the state of Tripura have maintained unique fermentation practices, making products of fruits and vegetables, including *Amlai Ntoi*, *Bikang*, *Bochumba*, and *Kosoi*, out of local substrates such as Indian gooseberry (*Phyllanthus emblica*), *Bombax ceiba* flower, and leguminous beans (Uchoi et al., 2015). Likewise, fermented products made using the leaf basis, like *Ziang Sang* and *Tapyo* in Manipur and Arunachal Pradesh, are produced through spontaneous fermentation (Satish Kumar et al., 2013). One of the most distinctive processes in the Himalayan belt is the pit fermentation that helps in anaerobic fermentation of microbes that helps in preservation and biochemical conversion of vegetables (Tamang, 2010). This fermentation process reduces the pH and gives it a sour organoleptic profile, by the breakdown of plant carbohydrates into lactic acid (Tamang and Samuel, 2010). They are also good sources of nutrition when fresh vegetable supplies are low in season, and contain bioavailable minerals and B-complex vitamins (Tamang, 2009; Marco et al., 2021).

Gundruk is a widely eaten, sun-dried, unsalted fermented leafy vegetable, made of Brassica juncea, *Brassica rapa*, *Raphanus sativus* and other winter vegetables, common among Gorkha people in North-East India. *L. plantarum*, *L. cellobiosus*, and *Pediococcus* species are the major fermenters of it (Mir et al., 2018). On the other hand, sinki is a pickle that is fermented in pits and is made of radish roots and is traditionally eaten during winter. It has microbial contents such as *L. brevis*, *L. plantarum*, *L. fallax*, and *L. casei*, and the product is generally attributed with digestive health effects (Ray et al., 2014).

Ziang Sang, also known as *Ziang dui* is a fermented mustard leaf product that is common in Nagaland and Manipur and is usually served in soups. LABs that are used in its fermentation production include *L. plantarum*, *L. brevis*, and *P. acidilactici* (Tamang and Tamang, 2009 a). Prepared with *Cardamine macrophylla* (magane-saag), another prominent preparation is *Goyang* by Sherpa people in Sikkim and Darjeeling. This fermented vegetable is commonly combined with thick noodle soups (thukpa) and the dominant LABs are *L. brevis*, *L. plantarum*, *L. lactis*, *P. pentosaceus*, and *E. faecium* (Tamang et al., 2008). In *Khalpi*, a fermented cucumber product that is common in Darjeeling and Sikkim, the predominant microbes are *L.*

plantarum, *L. brevis* and *Leuconostoc fallax* (Mao and Odyuo, 2007). The increasing scientific attention to these traditional fermented vegetable products is driven by their functional attributes, notably their probiotic efficacy, antimicrobial potential, and their contribution to dietary health in indigenous Himalayan populations (Marco et al., 2021; Tamang et al., 2022).

2.5.2 Traditional Fermented plant based food

The products made of fermented bamboo shoots are an important part of the traditional diet of numerous ethnic groups in Northeast India. These products are ingrained in the cuisine of local people and are mostly used in the states of Manipur, Arunachal Pradesh, Mizoram, Sikkim, Tripura, Meghalaya, Assam and Nagaland. Bamboo shoots naturally have low amounts of fat and cholesterol and high amounts of dietary fiber, potassium, and complex carbohydrates. Moreover, they are a natural source of bioactive compounds such as essential amino acids, vitamins, flavonoids, phenolics, phytosterols, and possess considerable antioxidant and therapeutic effects (Sonar et al., 2015; Choudhury et al., 2010; Satya et al., 2010; Nirmala et al., 2

Various products of fermented bamboo shoot are traditionally made in different parts of the region: *Soibum*, *Soidon* and *Soijin* in Manipur; *Ekung* and *Bamboo Tenga* in Arunachal Pradesh; *Moiya Koshak*, *Melye Amiley*, *Midukeye* and *Moiya Pangsung* in Tripura. All these products are differentiated by their mode of fermentation, the species of bamboo used and the prevalent microbial consortia.

For instance, *Mesu* a fermented bamboo shoot pickle consumed in Sikkim and Darjeelingis predominantly inhabited by lactic acid bacteria (LAB), notably *L. plantarum*, *L. brevis*, *L. curvatus*, *L. citreum*, and *P. pentosaceus* (Choudhury et al., 2012). In Manipur, *Soibum* and *Soidon* are key fermented products prepared by the Meitei community. *Soibum* is produced via spontaneous fermentation of shoots from *Dendrocalamus hamiltonii*, *D. giganteus*, *D. sikkimensis*, and *Bambusa tulda*, with microbial communities dominated by *L. brevis*, *L. plantarum*, *L. coryniformis*, *L. fallax*, *L. delbrueckii*, *L. lactis*, *L. mesenteroides*, and yeast genera such as *Saccharomyces* and *Candida* (Tamang and Tamang, 2009). The immature product is termed *Sojim*, while the mature variant is known as *Soibum*. *Soidon*, derived from the

apical parts of mature bamboo culms, is fermented anaerobically and can be stored for extended periods. It harbors LAB such as *L. lactis*, *L. brevis*, and *L. fallax* (Tamang et al., 2012).

Lung-Siej is a traditional dish made by the Khasi women of Meghalaya out of D. hamiltonii and is usually served alongside meat or fish. Its microbial ecology is characterized by LAB dominance, especially of *Lactobacillus* spp. (Mir et al., 2018). In Arunachal Pradesh, tribes like the Adi and Apatani make Ekung (also called iku or hikku) using bamboo species like *D. giganteus* and *Bambusa balcooa* where microbial communities include *L. brevis*, *L. casei*, *L. plantarum* and *Tetragenococcus halophilus* (Tamang et al., 2012). Equally, *Eup*, another fermented bamboo shoot product eaten by the Nyishi, Di and Apatani tribes is made using multiple species such as *Phyllostachys assamica*. *L. fermentum* and *L. plantarum* predominate it.

Other ethnic products are *Hirring*, made by the Apatani and Nishi tribes, containing LAB like *L. plantarum* and *L. lactis*. Interestingly, non-bamboo substrates are used by the ethnic communities in Nagaland in fermentation. *Anishi*, prepared by the Ao tribe using Colocasia leaves; Rhujuk or Bastanga, a condiment made of bamboo shoots; *Hungarung*, fermented Brassica leaves eaten in winter; and T*sutoice*, a fermented product made of cucumber, are just a few examples of the diversity of fermented foods of the region (Nongdam 2015).

In Assam, a bamboo variety is used to prepare *Miya Mecheng*, including *B. balcooa, B. tulda* and *B. vulgaris*, which has a long shelf life and can be used to cook with meats and vegetables (Narzary et al., 2016). *Khorisa* and its sour form *Poka Khorisa* (or *Khorisa Tenga*) are common dishes of Assamese. They are pickled in chili and oil, and kept as long as two years. LAB (mostly *Lactobacillus* spp.) predominates their microbial profile. (Senapati et al., 2016).

Other locally important fermented foods are *Kharoli*, a condiment made of mustard seeds that is said to have gastrointestinal curative effects (Bhuyan and Baishya, 2013), and *Narzi*, a bitter, semi-fermented jute leaf dish that is the centre of Bodo

socio-cultural activities. *Narzi* is a culinary and ritualistic food that is sun-dried, then boiled with a local alkali (*khardwi*) to alleviate bitter flavor (Narzary et al., 2016).

2.5.3 Traditional Fermented soybean based products of North east India:

In Eastern Himalayan region that includes eastern Nepal, Darjeeling Hills, Sikkim, northeastern India and southern Bhutan, traditional fermented soybean products are an important aspect of culinary culture and socio-cultural identity of the different ethnic communities. In these areas, which are mostly inhabited by the Mongoloid ethnic groups, spontaneous, salt-free fermentation of the locally grown soybeans has long been practiced, producing foods with a uniquely sticky, aromatic and rich protein content (Tamang 2015). Common representative products are aakhone, *bekang*, *hawaijar*, *kinema*, *tungrymbai*, and *peruyaan*, which are all foods and food items that have a dietary and ethnocultural value in their respective societies.

The microbial fermentation of these foods made of soybean is mainly due to *Bacillus subtilis*, a predominant organism in the naturally existing microbiota. This microbe promotes alkaline fermentation, which helps to form typical textures, aromas, and flavors (Tamang 2015). They are also of great nutritional and biomedical significance, with a variety of biofunctional properties, such as immunomodulatory, antioxidative, antithrombotic, and anticancer. The existence of bioactive metabolites that include poly-3-glutamic acid, biopeptides, essential vitamins, and phenolic compounds, among other things, is largely attributed to these health benefits (Kharnaior and Tamang 2022).

Kinema, a typical fermented soybean product of the Nepalese inhabitants of the Eastern Himalayas, is spontaneously fermented and wrapped in fig leaves (Ficus hookeriana, locally known as nevara). It is slightly alkaline, mucilaginous and has a faint aroma of ammonia.

The microbial consortium includes *B. subtilis*, *B. licheniformis*, *B. thuringiensis*, *B. cereus*, *B. circulans*, *B. sphaericus*, *Geotrichum candidum*, *Candida parapsilosis*, and *E. faecium* (Tamang, 2024). *Kinema*, a functional food, is known for its high digestibility, complete amino acid composition, B-vitamin content, antioxidative

property and low cholesterol (Tamang, 2010; Moktan et al., 2008; Sarkar et al., 1997; Omizu et al., 2011).

Likewise, *Hawaijar,* a traditional fermented soybean food of Manipur, is known for mucilage formation and a strong ammonia smell which indicates proper fermentation. It uses fig leaves (Ficus hispida, local name assee heibong) or banana leaves for packaging.

Dominant bacterial species include *B. cereus*, *B. licheniformis*, *B. subtilis*, *Staphylococcus sciuri*, *S. aureus*, *Acinetobacter* spp., and *Providencia rettgeri* (Singh et al., 2023).

Tungrymbai, consumed predominantly by the Khasi and Garo tribes in Meghalaya, involves fermentation using leaves of *Clinogyne dichotoma* (*lamet*), followed by mixing with local spices and aromatics. Key microbial isolates associated with tungrymbai include *B. subtilis* and *Enterococcus faecium* (Mishra et al., 2019).

Aakhone (also spelled *axone*), a staple of the Sema Naga tribe in Nagaland, is traditionally fermented using banana leaves (*Musa* spp.), *Phrynium pubinerve*, or *Macaranga indica*. After fermentation, tungrymbai is either dried or mixed with chili or meat (usually pork) and is served with rice (Tamang 2015).

Peruyaan, a product of the Apatani tribe in Arunachal Pradesh, is named after the local Apatani word for beans (perum) and leaf-wrapped (yannii). It is eaten without further cooking, typically by mixing the fermented soybeans with chili, salt, and hot water. The predominant microbial taxa include *B. amyloliquefaciens*, *B. subtilis*, *Vagococcus lutrae*, *Pediococcus lactic acid,* and *Enterococcus faecalis* (Singh et al., 2014).

Bekang, a fermented soybean product native to Mizoram, is prepared by wrapping soybeans in leaves of *Callicarpa arborea* or *P. pubinerve*. Its microbial diversity comprises *B. brevis*, *B. coagulans*, *B. circulans*, *B. licheniformis*, *B. pumilus*, *B. sphaericus*, *B. subtilis*, and *Lysinibacillus fusiformis* (Pohsen 2023).

Collectively, these fermented soybean foods represent a confluence of indigenous microbial ecosystems, traditional ecological knowledge, and functional nutrition.

Their sustained production, consumption, and scientific investigation are vital not only for community health and food security but also for the preservation of biocultural diversity in the Eastern Himalayas.

Figure 2.2: Different types of fermented soybean foods of North East India

2.5.4 Fermented Rice beverages of North-East India

Fermented rice based alcoholic beverages play a crucial place in the nutritional, medicinal and cultural practices of the indigenous people of the Himalayan foothills and Northeastern regions of India. These are not just drinks consumed for social recreation; they are also integral to ritual practices, religious rituals and traditional medicine (Das et al, 2023). Traditionally, in the Indian subcontinent, production of fermented foods and beverages has used plant-based substrates and microbial consortia native to the geographical area and this biocultural legacy has been maintained over generations (Roy et al., 2004). Alcoholic fermentation, an ancient method of food preservation and transformation, is still a predominant feature of ethnogastronomy among tribal people (Das and Deka 2012).

Traditional beverages play an important role in life-cycle ritualsbirth, marriage, harvest and death. For example, traditional cultures attribute medicinal properties to herbs present in the fermentation starter cultures. Ethnopharmacological and biomedical studies support the use of such rice-based ferments in alleviating conditions such as hypertension, type 2 diabetes mellitus, migraines, fatigue, and gastrointestinal disturbances (Mishra et al., 2019 a; Das et al., 2023).

Research exploring the bioactive properties of fermented rice beverages has demonstrated therapeutic effects. For instance, fermented rice bran exhibits cytotoxic activity against colorectal, gastric and bladder cancer cells (Phutthapthadoong et al., 2009). Moreover, recent studies have shown that traditionally fermented rice beers have therapeutic effects against insomnia, inflammation, parasitic diseases, urinary problems, and cholera symptoms (Das et al., 2023).

The traditional fermentation starters from these regions have a diverse microbial ecology, which typically includes filamentous molds (e.g., *Rhizopus chinensis*, Mucor circinelloides) (Anupma and Tamang 2020), ethanol-producing yeasts (e.g., *Saccharomyces cerevisiae*, *Pichia anomala*, *Saccharomycopsis fibuligera*) (Sha et al., 2019) and lactic acid bacteria (e.g., *Lactobacillus bifermentans*, *Pediococcus pentosaceus*) (Pradhan and Tamang 2019; Tamang et al., 2022). These microbial communities impart biofunctional properties such as probiotic traits (Pradhan and Tamang 2021) and enzyme activity (Anupma and Tamang, 2022) through their association, and are potential candidates for functional food production

Region-specific traditional beverages further exemplify this diversity. In Meghalaya, *Chubitchi* (Garo tribe) is produced using glutinous rice and medicinal herbs such as *Wanti*, and is consumed daily as well as during the Wangala festival (Mishra et al., 2015). The *Sadhiar* or *Kiad* (Pnar tribe) undergoes secondary distillation in a device called *Shet-kiad* (Mishra et al., 2015). Among the Rabha community in Assam, *Jonga-mod* or *Choko* is prepared using *bakhor*, and is also applied in ethnoveterinary medicine (Basumatary and Gogoi, 2014).

In the Bodo tribe, *Jou* is prepared with a medicinal starter known as *amao*, with fermentation durations modulated by ambient temperature (Narzary et al., 2016).

Judima, produced by the Dimasa Kachari tribe, incorporates a bark-based starter (*Umhu*) derived from *Acacia pennata*. Similarly, the Angami tribe in Nagaland ferments *Zutho* using a starter known as *piazu*, which contributes both saccharification and therapeutic attributes (Jamir and Rynjah, 2022 a).

Other ethnic communities contribute further to this ethnobiotechnological landscape: the Adi-Galo tribe of Arunachal Pradesh brews *Opo* using *siiyeh*; the Meitei community of Manipur produces *Atingba*; and the Deoris of Assam prepare *Sujen* with *perok kushi* (Gogoi et al., 2013; Das and Deka, 2012). The Ahom community's Xaj Pani, produced with the polyherbal starter vekur pithaa, and the Dimasa community's Jewish rice beer, also exemplify the ethnic diversity of these alcoholic beverages (Thappa and Tamang, 2006; Chakrabarty et al., 2009).

In Sikkim, the Gorkha ethnic group prepares high-calorie Bhaati Jaanr, a fermented rice beverage starters with Marcha, which is dominated by LAB (*L. bifermentans* and *P. pentosaceus*) (Tamang et al., 2022; Rai et al., 2021). Likewise, *Kodo Ko Jaanr*, made from finger millet and consumed by the Bhutia, Lepcha, Gorkha and Monpa people, has a rich microbial diversity with functional microorganisms (Thappa and Tamang, 2006).

These fermented rice-based alcoholic drinks thus represent a complex intersection of microbiology, ethnomedicine and culture. Their ongoing research not only helps us understand traditional knowledge systems but also has implications for present-day issues like food microbiology, biotechnology and public health nutrition.

The Ahom community's *Xaj Pani*, crafted with the polyherbal starter *vekur pithaa*, and the Dimasa's *Jewish rice beer*, further reflect the ethnic specificity of these brews (Thappa and Tamang, 2006; Chakrabarty et al., 2009).

In Sikkim, the Gorkha community produces *Bhaati Jaanr*, a high-calorie fermented rice drink initiated with *Marcha*; it is characterized by dominant LAB strains such as *L. bifermentans* and *P. pentosaceus* (Tamang et al., 2022; Rai et al., 2021). Similarly, *Kodo Ko Jaanr*, derived from finger millet and consumed widely among the Bhutia, Lepcha, Gorkha, and Monpa groups, exhibits a diverse microbial profile with key functional microorganisms (Thappa and Tamang, 2006).

Collectively, these fermented rice-based alcoholic beverages embody an intricate nexus of microbiology, ethnomedicine, and cultural heritage. Their continued study not only offers insights into traditional knowledge systems but also contributes to contemporary fields such as food microbiology, biotechnology, and public health nutrition.

2.5.5 Fermented milk based products

Traditional fermented milk products are an important element of the diet and cultural heritage of ethnic groups living in the Eastern Himalayan area, including the Darjeeling hills, Arunachal Pradesh and some of Assam, in India, and in Nepal, Bhutan and the Tibet Autonomous Region, China. Their use is also maintained in the Central and Western Himalayas, as well as their nutritional value and high level of ethnocultural acceptance (Tamang 2022a, 2022b). Among them, the *chhurpi*, a fermented milk product, which has a soft cottage-cheese-like texture, is especially popular among the Nepali and Gorkha communities. It is commonly used as a component of local cuisines as savory gravies or thick soups, usually accompanied by rice (Shangpliang et al., 2017).

Microbial and genomic profiling of these native fermented milk products has recently discovered a wide variety of strains of lactic acid bacteria (LAB) with emphasis on their functional probiotic characteristics. The strains of LABs have strong antimicrobial effect against food pathogens, have been shown to lower serum cholesterol levels and can hydrolyze lactose, thereby providing nutritional benefits to lactose-intolerant people. Also, their capacity to attach to intestinal epithelial cells can imply that they are useful in the modulation of gut barrier function and potentially have an effect on host immune responses, which makes them potential targets of therapeutic modulation of gut microbiota (Rai and Tamang, 2022; Ding et al., 2017).

The other widely eaten fermented milk product is dahi which is a curd-like product created by the fermentation of bovine milk (cow or buffalo), or a blend of both bovine milk and lactic acid. In the Himalayan areas with high altitudes, especially near pastoral societies, yak milk is used as a rawmaterial to produce various

fermented dairy products such as *kurut*, *chhurpi*, *chchurchurpe*n, *churkham*, *chhu*, *philuk*, and *shyowand maa*. *Chhurpi*, which has a soft white texture, can vary in intensity of flavour, and can be mild, medium, or strong, is most commonly used in making traditional curries among them (Tamang et al., 2008c). Chhu-based fermented products are mostly consumed in Sikkim, the Darjeeling hills, Arunachal Pradesh and Ladakh as a regional preference and climate and ecological adaptation (Satish Kumar et al., 2013).

2.5.6 Fermented meat based products

The conventional preservation methods used by Indigenous people in the Himalayan region that is, smoking, drying, salting and fermentation are time-tested biotechnological methods of curbing the perishability of animal-based food products. These practices are most evident in some ethnocultural communities in Northeast India where freshwater fish that are locally available are habitually processed using multi-modal processing methods. These conventional methods beyond extending shelf life will confer microbiological safety and improve the organoleptic properties of the end products by inhibiting the proliferation of pathogenic and spoilage species (Bhutia et al., 2021a; Yankowski et al., 2015).

One such traditional fermented meat product is *Kargyong*, a culturally significant sausage predominantly consumed in Sikkim and Arunachal Pradesh, formulated from yak, beef, or pork, typically blended with animal fat. Comprehensive microbial profiling has revealed the presence of a diverse microbiota, including lactic acid bacteria (LAB) such as *Lactobacillus sakei*, *L. divergens*, *L. carnis*, *L. sanfranciscensis*, and *L. curvatus*; heterofermentative species like *Leuconostoc mesenteroides*; enterococci (*Enterococcus faecium*); and various *Bacillus* spp. (*B. subtilis*, *B. mycoides*, *B. thuringiensis*). Additionally, coagulase-positive *Staphylococcus aureus*, *Micrococcus* spp., and yeasts such as *Debaryomyces hansenii* and *Pichia anomala* have been identified, all of which contribute to the product's fermentation dynamics and sensory profile (Rai and Tamang, 2022).

A parallel example is *Honoheingrain*, a traditional fermented pork or wild boar preparation integral to the Dimasa tribe of North Cachar Hills in Assam.

Microbiological assessments indicate the presence of LAB including *Lactobacillus brevis*, *L. plantarum*, and *Leuconostoc mesenteroides*; *Enterococcus faecium*; and several *Bacillus* species (*B. cereus*, *B. pumilus*, *B. firmus*, *B. circulans*, *B. stearothermophilus*). Other genera detected include *Micrococcus* and *Staphylococcus*, along with fermentative yeasts such as *Debaryomyces hansenii* and *Saccharomyces cerevisiae* (Chakrabarty et al., 2014). These microbial consortia are not only pivotal for biochemical transformations during fermentation but also contribute to the nutritional, functional, and potential probiotic qualities of the end products (Tamang et al., 2023).

2.5.7. Fermented fish based products

Fermented fish products are a part of the ethnogastronomic and microbiocultural tradition of Northeast India. These area-specific products are a result of century-old empirically developed native preservation techniques such as spontaneous fermentation at ambient conditions. Among them, *Ngari* and *Hentak* of Manipur, *Tungtap* of Meghalaya, *Karoti* and *Bardia* of Assam, *Shidal* and *Lona Ilish* of Tripura, or *Gnuchi*, *Sidra*, and *Sukuti* of Sikkim, can be distinguished (Uchoi et al., 2015). A complex interaction between autochthonous microbial consortia and endogenous fish proteases is the driving force behind these fermentative processes, which drive the hydrolysis of muscle proteins into bioavailable peptides and free amino acids. This type of proteolytic conversion increases nutritional value, as well as, provides functional bioactivities, such as antioxidant, antihypertensive, and antimicrobial properties (Tamang et al., 2023; Steinkraus, 2002).

Shidal is one of these customary products; it is a salt-free anaerobically fermented fish product that is consumed in Tripura and neighboring states. It is commonly made with Puntius sophore (yielding them *Puntishidal*) or the estuarine fish *Setipinna phaza* (yielding *Phasa Shidal*). Its strong umami profile and pungent aroma are greatly valued because they are the result of the microbial breakdown of proteins and lipids into various bioactive and flavor-generating metabolites, such as amino acids, short-chain fatty acids, indole, and skatole (Roy et al., 2014). *Shidal* has

a variety of regional names, including: *seedal*, *sepaa*, *hidal*, *verma* and *shidal*, indicating its extensive cultural assimilation.

Ngari, another emblematic fermented fish product from Manipur, is traditionally produced using sun-dried freshwater fish subjected to prolonged anaerobic fermentation in sealed containers. Microbial analyses have identified a rich and diverse community, predominantly composed of lactic acid bacteria (LAB) such as *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus plantarum*, *Enterococcus faecium*, *L. fructosus*, *L. amylophilus*, and *L. coryniformis* subsp. *torquens*, along with spore-forming *Bacillus subtilis* and *B. pumilus*, *Micrococcus* spp., and yeasts of the genera *Candida* and *Saccharomycopsis* (Jeyaram et al., 2009).

Similarly, *Tungtap* a semi-solid fermented fish condiment integral to the culinary practices of the Khasi community in Meghalaya exhibits a comparable microbial composition. Dominant strains include *L. lactis* subsp. *cremoris*, *L. plantarum*, *E. faecium*, *L. fructosus*, *L. amylophilus*, *L. coryniformis* subsp. *torquens*, and *L. puhozihii*, accompanied by *B. subtilis*, *B. pumilus*, *Micrococcus* spp., and yeasts from the genera *Candida* and *Saccharomycopsis* (Thapa 2016). These microbial consortia play pivotal roles in biochemical transformations during fermentation, leading to improved preservation, enhanced sensory attributes, and the potential development of probiotic functionalities. Dominant microbes associated with different fermented foods of North East India are described below (Table 2.1).

Table 2.1: Dominant microbes associated with different fermented foods of North East India:

Location	Fermented food	Microbes associated	References
Assam	*Khorisa*	*Lactobacillus, L. plantarum, L brevis, L. paracasei, L. pentosus, L. collinoides*	Sharma and Barooah, 2017
	Pani Tenga /Kahudi	*Enterococcus durans, L. plantarum, L. fermentum, L. casei*	Goswami et al., 2017
	Jou	*Roseburia, Faecalibacterium sp., Saccharomyces cerevisiae, Pichia burtonii, Wickerhamomyces anomalus*	Barooah et al., 2020
	Sujen	*Rhodotorula sp, Candida sp. S. cerevisiae Candida krusei, C. pelliculosa, C. utilis, C. sphaerica,C.magnolia, Rhodotorulaglutinis L. plantarum, L. brevis*	Handique and Deka, 2016
	Xaj	*L. plantarum, L. brevis, Leuconostoc lactis, Weissella cibaria, Lactococcus lactis, W. paramesenteroides,L.pseudomesenteroides*	Saikia et al., 2007
Arunachal Pradesh	*Ekung*	*L. plantarum, L. brevis, L.casei, L.fermentum, Tetragenococcus halophiles*	Thakur et al., 2016
	Hirring	*L.plantarum, L. brevis, L. curvatus, L. lactis, Leuc. Mesenteroides, Ped. pentosaceus, Leuc. Lactis, Leuc. fallax, Ent. durans, Leuc. citreum*	Thakur et al., 2016
	Eup	*L. brevis, L. plantarum, L. xylosus, L. casei, L. fermentum, L. mesenteroides, Ped. pentosaceus, Leuc. lactis, , Leuc. Fallax, Ent. Durans, Leuc. citreum*	Thakur et al., 2016; Tamang et al., 2012
	Peruyyan	*B. subtilis, B. amyloliquefaciens, Vagococcus lutrae, Ped. acidilactici, E. faecalis, P. acidilactici, E. faecium, E. lactis*	Kharnaoir and Tamang, 2023, Singh et al., 2014
Meghalaya	*Lung-seij*	*L. brevis, L. curvatus, L. mesenteroides, L. fallax, L. lactis, L. citreus*	Badwaik et al., 2015
	Tungrymbai	*B. subtilis, B. licheniformis, B. pumilus, B. amyloliquefaciens, L. brevis, Vagococcus carniphilus, Debaryomyces hansenii, Pic burtonii*	Chettri and Tamang, 2015
	Chubitchi	*S. cerevisiae, Rhodotorula mucilaginosa, W. anomalus, L. plantarum, L. fermentum , Rothia mucilaginosa, W. anomalus, Saccharomycopsis fibuligera,*	Mishra et al., 2019 a, Das et al., 2023. Mishra

		Paraburkholderia terricola	et al., 2018
	Kiad/sadheir	*Escherichia coli, Pseudomonas cepacia, Citrobacter species, S. cerevisiae, R. mucilaginosa, W. anomalus, S. fibuligera, P. terricola*	Mishra et al., 2019 a, Samati and Begum, 2007,Sawian et al., 2017
Nagaland	*Inziang-sang*	*L. plantarum, L. brevis, Pediococcus*	Tamang 2010
	Akhone	*E. faecium, B. amyloliquefaciens, B. subtilis, Bacillus tropicus, B. oleronius, Corynebacterium glutamicum, Ignatzschineria sp, B. Oceanobacillus indicireducens, B. thermoamylovorans, B. hisashii, B. fordii, B. fortis , Sporosarcina pasteurii, O. indicireducens*	Das et al., 2023
	Tsutoice	*B.subtilis, B. licheniformis, B. amyloliquefaciens*	Deb and Jamir, 2020
	Judima	*Ped. pentosaceus, B. circulans, B. catarosporous, B. pumilus, B. firmus, D. hansenii, S. cerevisiae, Lactobacillus sp., Micrococcus sp., Staphylococcus aureus, Rhizopus, Mucor*	Das et al., 2012; Buragohain et al., 2013; Ray et al., 2016
	Zutho	*S. cerevisiae, C. glabrata, W. anomalus, Pichia anomala, Rhizopus sp.*	Das et al., 2012; Ray et al., 2016
	Duizou	*S. cerevisiae, C. glabrata, W. anomalus, P. anomala*	Das et al., 2012
Manipur	*Soibum/Sojim*	*L. brevis, L. plantarum, Leuconostoc mesenteroides, L. fallax, L. plantarum, L. corniformis, L. delbrueckii, L. lactis, E. durans, Strep. lactis, B subtilis, B.licheniformis, B.coagulans and yeast Candida, S. Torulopsis, B. pumilus, B. cereus, P. fluorescens, Torulopsis* sp.	Thakur et al., 2016
	Soidon	*L. brevis, L. lactis, L. curvatus, Leuconostoc fallax, L. plantarum*	Thakur et al., 2016; Tamang and Tamang, 2009
	Hawaijar	*B. subtilis, B. licheniformis, B. cereus, B. amyloliquefaciens S. aureus, S. sciuri, A. sp., Providencia rettgeri, Proteus mirabilis, Alcaligenes sp., Brevibacillus borstelenis, E. faecium. megaterium,Weisella confuse, C. glutamicum, Ignatzschineria sp, B. tropicus, B. thermoamylovorans, S. lentus, W. confuse, B. ruris, B. coagulans*	Das et al., 2023

	Atingba	*P. pentosaceus, S. cerevisiae, Pichia anomala, Trichosporon spp., C. tropicalis, P. guilliermondii, C. parapsilosis, Torulaspora delbrueckii, P. fabianii, P. kudriavzevii, C. glabrata*	Jeyaram et al., 2008
Mizoram	*Tuaither*	*L. brevis, L. curvatus, L. plantarum, B. circulans, B. firmus, B. sphaericus, B. subtilis, Weissell sp. Pseudomonas sp. Chromobacterium sp. Acinetobacter sp. Corynbacterium sp. Ped. pentosaceou, Lc. lactis,*	Tamang and Tamang 2009,
	Bekang	*B. brevis, B. coagaulaus, B. circulans, B. lichniformis, B. pumilus, B. spahericus, B. subtilis L. fusiformis, Debaryomyces hansenii, Pichia burtonii, Bacillus rugosus, Microbacterium paraoxydans, Mammaliicoccus sp, Corynebacterium glutamicum, Ignatzschineria sp, B. Oceanobacillus indicireducens, B. tropicus, S. lentus, W. confuse, C. casei, B. fordii, O. indicireducens*	Das et al., 2023
Tripura	*Langi/Chuwak*	*C. glabrata , W. anomalus*	Majumdar et al., 2016
	Moiya pansung	*L. plantarum, L. lactis, Levilactobacillus brevis, Leuconostoc mesenteroides, Weissella paramesenteroides, L. kimchii, P. pentosaceus, L. gasicomitatum, Lacticaseibacillus casei.*	Das et al., 2023
Sikkim	*Gundruk*	*L. brevis, L. plantarum, L. paracasei, L. fallax, P. pentosaceus, P. acidilactici, Lb. fermentum, Lb. casei, Ped. pentosaceus*	Tamang et al., 2005
	Kodo ka jannr	*S. fibuligera, Corchorus capsularis, P. anomala, P. burtonii, S. cerevisiae, S. bayanus, C. glabrata*	Tamang 2020b
	Kinema	*B. subtilis, B. licheniformis, B. cereus, B. circulans, B. thuringiensis, B. glycinifermentans, B. thermoamylovorans, B. coagulans, B. paralicheniformis, Brevibacillus borstelensis, B. sphaericus, Cand. parapsilosis, Geotrichum candidum, Pediococcus acidilactici, E. faecium*	Kharnaoir and Tamang, 2023, Kharnaoi et al., 2022
	Bhatti jannr	*P. acidilactici, S. fibuligera, Corchorus capsularis, Pichia anomala, P. burtonii, S. cerevisiae, S. bayanus, C. glabrata, Hansenula anomala, Rhizopus* spp., *yeasts, L. bifermentous*	Anupama and Tamang, 2020

2.6. Fermented foods and Probiotics:

The FAO/WHO characterizes probiotics as microorganisms that are deemed safe for their intended applications, validated by at least one clinical trial involving humans, and capable of maintaining viability at the specified dosage throughout the product's shelf life (Binda et al., 2020; Misra et al., 2022). Probiotics are live microorganisms that can confer health benefits to humans when consumed in sufficient quantities (Mulaw et al., 2020). Globally, individuals have been incorporating various fermented foods into their diets for their health advantages; however, the specific mechanisms and the bacteria involved remain largely unexplored. Recent studies utilizing culture-dependent techniques and comparative genomics have aimed to identify the microbiota associated with the fermentation processes of these traditional foods (Jani and Sharma, 2021).

Probiotic bacteria must be capable of withstanding and thriving in the challenging environment of the host's intestinal tract. They should reach the target site in a viable physiological condition and provide protection against pathogens by generating antimicrobial substances such as bacteriocins or metabolites like organic acids. Lactic acid bacteria are the most notable group of probiotic bacteria that offer beneficial effects within the host's gastrointestinal tract (GI) (Bogale and Prapulla 2015).

Lactobacillus, recognized as the most widely utilized lactic acid bacteria (LAB) in food and fermentation processes, is also among the most varied groups of LAB employed in probiotic production. This genus, which consists of Gram-positive bacteria, generates lactic acid during fermentation (Giraffa et al., 2010). Both in vitro and in vivo studies are conducted on various strains of probiotic bacteria. The typical in vitro measurements are determination of the source of the bacteria, determination of antibiotic resistance patterns and determination of the safety of these bacteria with regards to their possibilities of pathogenicity. Additionally, probiotic strains should be analyzed concerning their adherence to the intestinal epithelial cells, intestinal mucosa permeability, and immunomodulatory properties (Chilton et al., 2015; Galdeano and Perdigón, 2004).

LAB plays a crucial role in maintaining the balance of gut microbiota and overall health. These microbes are predominantly found in genera *Lactobacillus* and *Bifidobacterium*, but are also found in *Bacillus*, *Pediococcus* and some species of yeast (Fioramonti et al., 2003). LAB produces antimicrobial substances such as acids, hydrogen peroxide, and bacteriocins that have great potential as biopreservatives in food (Mokoena et al., 2016). In addition to their preservative properties, LAB has been identified to have immunomodulatory properties and be able to increase gut health. Studies also show that consumption of probiotics with LAB could potentially improve symptoms related to gastrointestinal diseases like irritable bowel syndrome and inflammatory bowel disease (Mokoena et al., 2021; Mulaw et al., 2019).

The use of probiotic bacterial strains can benefit a variety of food products. As an example, yogurt supplemented with *Lactobacillus reuteri* and *Lactobacillus rhamnosus* can be mentioned (Hekmart and Reid, 2006). Fermented milk with inulin in *Lactobacillus acidophilus* and *Bifidobacterium* animalis strains (Shu et al., 2015), chocolate products with *L. paracasei* strains (Aragon-Alegro 2007), and condensed milk with *L. acidophilus* (Rodrigues et al., 2011) are also applicable. Moreover, these strains help to improve human health directly and indirectly by helping to lower blood cholesterol levels, strengthening mucosal defence, replenishing normal microflora, preventing infections and defending against food allergies (De Roos and Katan 2000).

They also play a role in mitigating cariogenic activity (Ishikawa 2005), regulating mucosal immune responses, improving digestion, and maintaining the balance of intestinal microbiota (Fioramonti et al., 2003).

Himalayan spontaneously fermented soybean products are characterized by a rich microbial community, predominantly consisting of various Bacillus species, including *B. subtilis*, *B. velezensis*, *B. siamensis*, *B. tequilensis*, *B. safensis* subsp. safensis, *B. inaquosorum*, *B. halotolerans*, *B. glycinifermentans*, *B. cereus*, *B. licheniformis*, *B. thermoamylovorans*, *B. coagulans*, *B. circulans*, and *B. paralicheniformis* (Tamang 2003; Chetri and Tamang, 2015; Kharnaoir and Tamang, 2021; Kharnaoir and Tamang, 2022; Pariyar et al., 2022; Tamang et al., 2021). The *Bacillus* species found in these fermented soybean foods exhibit a range of biological

activities, including the synthesis of poly-glutamic acid (Pariyar et al., 2022; Chettri et al., 2016 a), the generation of untargeted metabolites (Kharnaoir and Tamang 2022), anti-thrombolytic effects (Singh et al., 2014), the production of bio-peptides (Rai et al., 2017), and the enhancement of antioxidant properties (Chettri and Tamang, 2016; Sanjukta et al., 2015).

The spontaneous fermentation of soybeans has been associated with the presence of certain species of lactic acid bacteria (LAB) in lower quantities during the fermentation process. Notable species include *E. faecium*, *Lactiplantibacillus plantarum*, and *Limosilactobacillus fermentum*, particularly in kinema (Tamang 2003; Kharnaoir and Tamang, 2021; Sarkar et al., 1994; Kumar et al., 2019; Goel et al., 2020). LAB in fermented soybean products are recognized for their significant contributions during fermentation, attributed to their antibacterial properties (Lim 2018), the production of protease (Ma et al., 2022), enhancement of isoflavone levels, and increased antioxidant capacity (Sirilun et al., 2017), as well as their probiotic characteristics (Jang et al., 2021). Various LAB species with probiotic attributes have been identified, including *L. lactis* from doenjang, a Korean fermented soybean paste (Jeong et al., 2014); *Lactobacillus rhamnosus* and *Lactobacillus casei* from douchi, a Chinese fermented soybean paste (Fong et al., 2020); *Leuconostoc mesenteroides*, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, and *Lactobacillus perolens* from cheonggukjang, a Korean fermented soybean dish (Son et al., 2018); and *Lactiplantibacillus plantarum* from kinema, a fermented soybean food from the Himalayas (Goel et al., 2020).

2.7. Probiotics and human health

Probiotics play a crucial role in managing various health conditions, including diarrhoea (Lai et al., 2019), diabetes (Soleimani et al., 2017), allergies (Huang et al., 2016), cancer (Brady et al., 2000), hypertension (Toral et al., 2018), and genetic disorders (Kiely et al., 2017; Toral et al., 2019). Additionally, they enhance immune function (Tsai et al., 2012).The probiotic microorganism must possess several key characteristics: it should be resistant to acid, tolerant of bile, non-pathogenic and non-carcinogenic. Additionally, it should adhere to the epithelial tissue of the host, enhance the intestinal microflora, diminish the adherence of pathogenic organisms,

and have the capability to produce secondary metabolites that are antagonistic to pathogenic microorganisms (Pan et al., 2009; Prabhurajeshwar and Chandrakanth, 2017).

Beneficial microorganisms engage with raw food substrates to generate valuable fermentation products that offer medical benefits, including enzymes and vitamins. Additionally, the intake of probiotic microbes contributes to a protective effect on the gut environment. It is important to recognize that the effects of probiotics vary by strain, meaning that not all strains are effective for optimal fermentation or the treatment of every disease (Nath et al., 2020).

The most commonly used *Lactobacillus* species in probiotic settings include *Lactobacillus acidophilus*, *L. casei*, *L. paracasei*, *L. plantarum*, L. reuteri *L. johnsonii*, and *L. rhamnosus* (de Vos, 2011; Ahmed et al., 2019). Probiotic strains of *Lactobacilli* have a strong affinity for adhering to the gastrointestinal tract of host cells, a process that relies on their ability to aggregate (Goh and Klaenhammer, 2010). Additionally, they serve as a protective barrier against the attachment of pathogenic bacteria by disrupting the colonization and growth of these pathogens, thus helping to avert the onset of infections (Patrignani et al., 2019).

Lactobacilli species are known to generate antimicrobial peptides, such as bacteriocins, as well as organic acids like acetic acid and lactic acid, along with other metabolites including hydrogen peroxide. These substances have been documented to suppress the proliferation of pathogenic bacteria (Li and Gu, 2018). Probiotic strains of *L. plantarum* play a role in the absorption of water and sodium within the colon, helping to alleviate symptoms of diarrhoea (Mortensen and Clausen, 1996). Additionally, they provide comprehensive health advantages for various medical issues, including psoriasis, allergies, and disorders of the nervous system (Hessle et al., 1999; Das and Goyal, 2015; Kim et al., 2015).

2.8. Metagenomic profile of fermented foods

Metagenomic approaches, particularly those based on high-throughput next-generation sequencing (NGS), have revolutionized the understanding of microbial communities in these foods. Likewise, tungtap, a semi-solid fermented fish paste,

used by the Khasi people in the Indian state of Meghalaya, has a similar microbial profile. The predominant species are *L. lactis* subsp. cremoris, *L. plantarum*, *E. faecium*, *L. fructosus*, *L. amylophilus*, *L. coryniformis* subsp. torquens.

These include *E. faecium*, *Lactiplantibacillus plantarum*, and *Limosilactobacillus fermentum* species, in kinema (Tamang 2003; Kharnaoir and Tamang, 2021; Sarkar et al., 1994; Kumar et al., 2019; Goel et al., 2020). LAB play a vital role in soybean fermented foods because of their contribution during the fermentation process due to their antibacterial activity (Lim 2018), protease production (Ma et al., 2022), improved isoflavone content and antioxidant properties (Sirilun et al., 2017), and probiotic properties (Jang et al., 2021).

Metagenomics, as opposed to traditional culture-based approaches, facilitates the complete profiling of cultures and uncultured microorganisms, providing information on microbial diversity, metabolic capacity, and functional aspects (Janssen et al., 2018). Environmental factors such as altitude, temperature, and traditional processing practices, including the use of bamboo utensils and leaf wrappings, contribute to microbial variation and the development of region-specific microbial signatures (Tamang et al., 2020).

Several previous research studies which utilized microbial culturing and identification of a particular fermented food product have reported that a few dominant microbial taxa play a major role in the fermentation process (Mathara et al., 2004; Mokoena et al., 2016). During the fermentation process, bacterial species convert raw food constituents into products that enhance the flavor and nutraceutical value of the final fermented product (Caplice and Fitzgerald, 1999). A more comprehensive understanding of microbial composition in fermented food products, however, requires an approach not limited by culture-based methods. In the past decade, culture-independent approaches utilizing polymerase chain reaction (PCR)-based amplification and sequencing of 16S rRNA genes have proven to be useful for the microbiological investigation of a variety of fermented foods (Sakamoto et al., 2011). Recent advances in next generation sequencing (NGS) technologies have provided platforms to explore microbial diversity and functionality in a variety of

environments (Mehetre et al., 2016; Cao et al., 2017). NGS techniques have been used to characterize the microbial communities of a variety of fermented foods and beverages, such as *kimchi* (Jung et al., 2011, kefir grains (Nalbantoglu et al., 2014), Chinese rice wine (Hong et al., 2016), etc. Given the rich variety of fermented foods in North-East India, there have been very few studies, using NGS technologies, on North-East Indian fermented foods. Previous studies have been conducted on *Bekang* (Tamang et al., 2009), *Rawtuai rep* (fermented bamboo shoot) (Nongdam et al., 2015), and *Saum* (Lalthanpui et al., 2015), but these have been based on microbial culture methods. Thus, microbial diversity analysis, using next-generation sequencing (NGS) technologies, of traditional fermented foods native to this area will provide us with a better understanding of the bacterial diversity found in these fermented foods, and help in the conservation of the ethnic identity of our traditional indigenous fermented foods.

2.9. Probiotics as Biosurfactants

Microorganisms possess the capability to synthesize a variety of surface-active compounds (SACs) that feature both hydrophilic and hydrophobic components. These compounds can engage with surfaces, reduce surface and interfacial tensions, create micelles, and facilitate the emulsification of immiscible materials (Franzetti et al., 2010). Microbial biosurfactants, which are generated by fungi, yeast, and bacteria, exhibit emulsifying characteristics and are known to perform several functions, including the solubilization of microbial biofilms (Rani M et al., 2020). A wide variety of microorganisms, including *Pseudomonas, Bacillus*, *Streptomyce*s and *Stenotrophomonas* species, have been associated with oil wells (Yoshida et al., 2005; Cai et al., 2015).

The literature reports the lipopeptide biosurfactants produced by the genus Bacillus (Zhao et al., 2017). Much of the previous work on the use of biosurfactants has focused on the method of microbially enhanced oil recovery (MEOR). This technique involves the use of microbial surfactants or dead bacterial cells to remove crude oil from surfaces to which it adheres (e.g. rocks, crevices) (Perfumo et al., 2010), as well as bioremediate contaminants (Mulligan, 2005; Thavasi, 2011).

Recently, there has been renewed interest in commercial uses of microbial biosurfactants because of their many beneficial properties, including low toxicity, environmentally safe, biodegradable, and acceptable. These properties render biosurfactants suitable for applications in agriculture, food and medicine (Nitschke and Costa, 2007; Sachdev and Cameotra, 2013; Mani et al., 2016).

The majority of known probiotic biosurfactants have been synthesized by lactic acid bacteria (LAB). These biosurfactants are primarily characterized as complexes of proteins and carbohydrates, as well as lipids or fatty acids (Velraeds et al., 1996). LAB, which are classified as generally recognized as safe (GRAS) by the United States Food and Drug Administration, are well-known for their applications in both the medical and food sectors. Their nonpathogenic nature has made LAB a focal point of research in the production of biosurfactants.

Lactobacillus species that produce saccharolytic activity (SAC) have been identified as prevalent members of the human microbiota, particularly within the urogenital and gastrointestinal tracts. The SACs generated by lactic acid bacteria (LAB) play a significant role in the bacteria's capacity to inhibit microbial infections within their respective environments (Reid G 1989; Borges S, 2014). *Lactobacilli* are effective in preventing the colonization of the urogenital tract by various pathogens, including yeast species such as *Candida albicans, C. tropicalis*, and *C. krusei*, which are linked to vulvovaginal candidiasis. They also combat anaerobic bacteria associated with bacterial vaginosis (BV), including *Gardnerella vaginalis, Mycoplasma hominis, Atopobium vaginae, Prevotella spp., Veillonella spp.*, and *Mobiluncus spp.*, as well as uropathogens like *Escherichia coli, Proteus spp., Klebsiella spp., Serratia spp.*, and sexually transmitted viruses (Reid G 1989; Borges S, 2014; Pendharkar S 2015; Hoesl CE 2005; Lepargneur JP 2002; Barrons R, 2008).

Lactobacillus paracasei, which is acquired in the dairy industry, was examined in its ability to generate biosurfactants (Gudina et al., 2010). Thavasi et al. found one strain of *Lactobacillus delbrueckii* which provided a high yield of biosurfactant with the cheap peanut oil cake carbon source producing 5.35 g/L. This production level, 5 g/L

or higher is comparable to other studies, which means this strain is one of the most efficient producers of probiotic biosurfactants (Thavasi et al., 2011).

The overall evidence suggests that traditional fermented foods of Northeast India are complex microbial communities characterized by the abundance of lactic acid bacteria, *Bacillus* spp., and yeasts that provide benefits in terms of nutritional value, food safety, and possible probiotic functionality (Tamang et al., 2023; Marco et al., 2021). Yet, compared to the well-documented states like Sikkim, Manipur and Nagaland, relatively little microbiological and probiotic characterization is carried out on native fermented foods of Mizoram, especially fermented soybean (*bekang*), fermented bamboo shoot (*tuaithur*) and fermented pork fat products (*saum*). In addition, full molecular profiling and functional probiotic certification is lacking. Consequently, scientifically, systematic microbiological testing and probiotic testing of these native foods is justified.

The critical review of the current literature shows that there are a number of research gaps that justify the current study. Geographically, despite considerable microbiological research on fermented foods of Sikkim, Manipur, Nagaland, and sections of Assam, relatively little systematic scientific research has been carried out on the native non-alcoholic fermented foods of Mizoram, especially fermented soybean (*bekang*), fermented bamboo shoot (*tuaithur*), and fermented pork fat (*saum*) products. This underrepresentation underscores an imbalance in the research in the region of Northeast India (Tamang et al., 2020; Das et al., 2023). Methodologically, most of the previous research has been based on culture-dependent methods, which can underestimate the overall microbial diversity by ignoring viable, but non-culturable and fastidious microorganisms. Utilization of high-tech molecular technologies, including 16S rRNA gene sequence, next-generation sequencing (NGS), and phylogenetic analysis, is underutilized in fermented foods of Mizoram (Johnson et al., 2019; Mehetre et al., 2016). Morphologically, as microbial taxa have been identified in a range of fermented products, probiotic validation including acid and bile resistance, antibiotic susceptibility profiling, adhesion prospect, safety evaluation, and antimicrobial activity has not been rigorously conducted on isolates obtained in Mizoram

fermented foods thus limiting their scientific recognition as potential probiotic candidates (Hill et al., 2014; Binda et al., 2020). Moreover, there is a significant sensory-microbial correlation gap as limited studies have attempted to systematically associate microbial diversity with biochemical changes that lead to flavor, aroma, texture formation and overall organoleptic properties of these traditional foods. The knowledge of these relationships is necessary to clarify the role of microbial consortia in affecting sensory properties and consumer acceptability (Wolfe and Dutton, 2015; Tamang et al., 2022). All these knowledge deficits of geography, methodology, functional and sensory knowledge are what make it important to conduct a thorough microbiological and probiotic assessment of indigenous fermented foods of Mizoram, which is the basis and scientific rationale of the current study.

2.10. Problem Statement

Mizoram's indigenous non-alcoholic fermented foods like fermented soybean (*bekang*), fermented bamboo shoot (*tuaithur*) and fermented pork fat (*saum*) are popular and culturally important, but there is no extensive microbiological assessment, molecular identification and probiotic profiling of the microorganisms in these fermented foods. This hampers their scientific credibility and their use as functional foods or probiotics**.**

2.11. Hypothesis

- The microbial communities in indigenous non-alcoholic fermented foods of Mizoram are potential probiotics.
- Isolates from these fermented foods show functional probiotic characteristics including acid and bile resistance, antimicrobial activity and safety.
- These foods are influenced by its microbial diversity.

2.12. Need of the Proposed Study

- Scientific validation of traditional fermented foods.
- Identification of novel probiotic strains from indigenous sources.
- Preservation of microbial biodiversity of Northeast India.
- Development of region-specific functional food resources.

2.13. Rationale

Indigenous fermented foods are potential sources of beneficial microbes. As growing attention is being paid to discovering indigenous probiotics and functional foods, microbiological profiling of fermented foods of Mizoram is necessary for:

- Strain-level identification
- Safety profiling
- Functional validation
- Understanding microbial-sensory relationships

Such investigation aligns with the growing global scientific interest in ethnic and traditional fermented foods as sustainable and culturally embedded sources of novel probiotic microorganisms. Recent studies have highlighted fermented foods not only as a staple of diet but as microbial ecosystems, which may hold functionally active strains that have health benefits (Marco et al., 2021). In contrast to the standardization of probiotic strains produced for commercial use, fermented foods are naturally occurring microbial ecosystems that are influenced by local environmental, material and indigenous processing factors. Such microbial ecosystems could be potential sources of unique stress-adapted strains that can survive the hostile environment of the gastrointestinal tract and confer beneficial health effects. In addition, the international consensus on probiotics highlights the importance of strain-specific identification, safety evaluation and functional analysis to substantiate health and nutritional claims (Hill et al., 2014). Hence, scientific investigation of traditional fermented foods from Mizoram not only contributes to local food microbiology but also to global efforts to discover sustainable and natural probiotic resources that contribute to food security, biodiversity conservation, and functional food products.

Microbial diversity is critical to understanding the impact of fermentation on sensory properties of foods. Fermented foods serve as microbial habitats where fermentative microorganisms, such as bacteria, yeasts, and other microorganisms, transform carbohydrates, proteins, and lipids into a diverse range of organic acids, alcohols, peptides, amino acids, and volatile compounds that contribute to taste, aroma, texture and acceptability (Wolfe and Dutton, 2015). Lactic acid bacteria mainly contribute to

sourness and texture properties via acidification (lactic acid production). However, Bacillus spp. in particular, in soy-based fermentations, produce proteolytic enzymes that liberate free amino acids and ammonia, contributing to umami taste and alkaline aroma, respectively (Tamang et al., 2020).

Yeasts further contribute to sensory complexity by producing ethanol, esters, and other volatile aromatic compounds that enrich flavor profiles (Marco et al., 2021). Variations in microbial composition and metabolic activity, influenced by geographical conditions and traditional processing practices, therefore lead to distinct region-specific sensory characteristics. However, despite the cultural importance of fermented foods of Mizoram, systematic studies correlating microbial diversity with sensory attributes remain limited. Establishing this microbial–sensory relationship is scientifically necessary to justify the inclusion of microbial diversity analysis as a central objective of the present investigation.

2.14. Aims and objectives

Characterization of the bacterial diversity present in traditional fermented foods can yield valuable insights into the development of sensory attributes such as flavor and texture in the final fermented products. However, comprehensive investigations into the microbial composition and associated health-promoting properties of these foods remain limited and are largely underrepresented in the scientific literature. Therefore, the present study aims to highlight this bacterial diversity, along with the proximate composition of different traditional fermented foods and their probiotic attribution (*Tuaithur*, *Bekang* and *Saum*) primarily consumed in Mizoram state, India.

The objectives of the proposed study are:

- To investigate the microbial diversity of traditional fermented foods of Mizoram, Identify and characterize dominant microbial species using advanced molecular technique.

- Isolation and Molecular characterization of the isolates using 16S rRNA gene and their phylogenetic analysis.

- To evaluate the probiotic potential of microbial strains isolated from the foods assess acid and bile tolerance, antimicrobial activity and adhesion properties.

3.1 Collection of Food Samples

Three traditional fermented food products native to the state of Mizoram in Northeast India were selected for this study and sampled in triplicate. The fermented products included *tuaithur*, prepared from bamboo shoots, *bekang*, produced from soybean, and *saum*, made using pork fat. These foods are commonly prepared and consumed by local communities and represent important components of the region's traditional diet. The preparation of these products follows indigenous processing practices based on spontaneous fermentation, a technique that has been traditionally used for generations. (Figure 3.1).

Figure 3.1: Sampling locations in Aizawl, Mizoram, North-East India, and representative images of traditional fermented foods.

(a) *Tuaithur*

(b) *Bekang*

(c) *Saum*

These food products are prepared using traditional methods of processing involving spontaneous fermentation, as has been conducted since ancient times.

Samples were obtained from local vendors in the marketplaces of Aizawl. The vendors were chosen based on their geographical location and the traditional processing methods employed in preparing the fermented foods. For each product, samples were collected in triplicate on the 3rd, 5th, and 7th days of fermentation, ensuring that they were obtained from the same container and from the same vendor to maintain consistency. Prior to further analysis, the triplicate samples corresponding to each fermentation day were pooled together. Consequently, a total of three representative samples for each fermented product (*Tuaithur*, *Bekang*, and *Saum*) corresponding to the 3rd, 5th, and 7th fermentation days were prepared for subsequent analyses. All collected samples were stored at 4 °C and processed immediately for the assessment of bacterial diversity.

3.2 Proximate Compositional Analysis

A portion of the collected samples was utilized for the determination of the proximate composition of each fermented product at the time of sampling and was sent for analysis to CSIR-Central Food Technological Research Institute, Mysuru 570020, India. The analysis of different proximate parameters was carried out following the standard procedures recommended by the Association of Official Analytical Chemists (AOAC, 2016). The protein content of the samples was estimated by determining the nitrogen concentration using the micro-Kjeldahl method. The lipid content was measured through solvent extraction using a Soxhlet apparatus in accordance with the prescribed AOAC protocols.

For the estimation of moisture content and dry biomass, approximately 2 g of each sample was weighed and dried in a hot air oven at 110 °C for 2 hours until a constant weight was achieved, after which the samples were reweighed. All proximate parameters were expressed as percentage values following the standard reporting format recommended in AOAC guidelines (AOAC, 2000). In addition, other physicochemical parameters including pH, moisture, total ash, crude fiber, carbohydrate content, and calorific value were also determined using the standard analytical procedures outlined in the 20th edition of AOAC

methods (2016).

3.3 DNA Extraction and Amplicon Sequencing

Approximately 1 mL of each sample was suspended in phosphate-buffered saline (PBS) and centrifuged at 800 × g for 1 min to remove large debris, after which the supernatant was carefully collected. The recovered supernatant was further centrifuged at 11,000 × g for 3 min to obtain microbial cell pellets. Genomic DNA was subsequently extracted from the pellets using the QIAGEN DNeasy Kit following the manufacturer's recommended protocol provided by QIAGEN.

The quality and concentration of the extracted DNA were evaluated spectrophotometrically using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity of the DNA was further confirmed by electrophoresis on a 1% agarose gel. High-quality DNA samples were preserved at −20 °C until further processing.

For amplicon library preparation, the extracted DNA was processed using the Nextera XT Index Kit according to the 16S metagenomic sequencing library preparation protocol provided by Illumina. The V3–V4 hypervariable region of the 16S rRNA gene was amplified using the forward primer 16SrRNAF (5′-GCCTACGGGNGGCWGCAG-3′) and the reverse primer 16SrRNAR (5′-ACTACHVGGGTATCTAATCC-3′). The resulting amplicon libraries were purified with AMPure XP beads, quantified using a Qubit Fluorometer, and subsequently sequenced on the Illumina MiSeq platform to generate 2 × 300 bp paired-end reads. The sequencing process was carried out at a commercial sequencing facility, Eurofins Genomics India Pvt.Ltd. Doddanakundi Industrial Area 2, Hoodi Whitefield, Bengaluru 560048, Karnataka, India.

3.3 Bioinformatics and Statistical Analysis

Raw sequencing reads generated in FASTQ format were first demultiplexed into individual samples using their respective barcode sequences. The sequences were then subjected to quality filtering using Trimmomatic v0.38, where adapter sequences, ambiguous reads, and low-quality reads were removed. Reads containing more than 10% bases with a Phred quality score below 20 were excluded to obtain

high-quality sequences. Subsequently (Bogler et al., 2014), the filtered forward and reverse paired-end reads were assembled using FLASH (v1.2.11) to generate merged sequences for downstream analysis (Magoc and Salzberg 2011).

Further processing and analysis of the sequence data were carried out using the QIIME v1.9.1 bioinformatics pipeline (Caprasco et al., 2010). Operational Taxonomic Units (OTUs) were assigned based on sequence similarity, and a representative sequence from each cluster of reads was selected. OTU clustering was performed at a 97% sequence similarity threshold using the uclust algorithm (Edgar 2010) against the Greengenes reference database (version 13_8) through the pick_open_reference_otus.py script in QIIME. Taxonomic classification of OTU sequences was performed by BLAST comparison against the Greengenes database, employing the RDP classifier (version 2.2) algorithm with default parameters (Wang et al., 2007).

Subsequent analyses, including microbial community composition and diversity assessment, were conducted using Microbiome Analyst software (Dhariwal et al., 2017). Bacterial abundance and diversity were evaluated using alpha diversity indices, including Observed Species, Shannon index, and Chao1, based on the generated OTU matrix within the QIIME environment (Capraso et al., 2010). Multivariate principal component analysis (PCA) was carried out using STAMP software (Park et al., 2014), where the distance matrix was calculated using the unweighted UniFrac method. Statistical significance among OTUs was assessed using unweighted UniFrac distance metrics followed by UPGMA cluster analysis. Additionally, Canonical Correspondence Analysis (CCA) was performed using PAST v3.14 to explore the relationship between nutritional parameters and the normalized abundance of dominant microbial taxa (Hammer et al., 2001).

3.5 Isolation of bacteria

The isolation of bacteria from these three fermented foods was based on the method described by Poornachandra Rao et al. (2015). 10 gm of samples were homogenised with 90 ml of 0.85% (w/v) phosphate buffer saline (PBS) for 1 minute and serially diluted (10^{-1} to 10^{-5}) in the same diluent. 1 ml of these dilutions were poure plated in

the respective nutrient media such as LB (Luria Bertani) and then incubated at 37 ℃ for 24 h. The colonies growing over the incubated plates were picked up carefully and streaked on the LB agar medium for further purification. Purity of the isolates were checked by streaking again and subculturing on fresh agar plates followed by microscopic examinations and cultures were maintained at 4 ℃ in a refrigerator for further studies. Purified strains were preserved in nutrient broth using 15% (v/v) glycerol at −20 ℃ in glycerol stock for further analysis.

3.6 Morphological and Biochemical characterization of the isolates:

A total of 32 bacterial isolates were initially obtained and subjected to preliminary identification based on microscopic examination following standard microbiological procedures (Sharpe, 1979). Subsequently, all isolates were screened using Gram staining and catalase test to determine their basic physiological characteristics. Gram staining was performed to identify the Gram reaction and cellular morphology of the isolates, while the catalase test was conducted by adding 3% hydrogen peroxide to a fresh bacterial colony to detect the presence of catalase enzyme, indicated by immediate bubble formation. Based on the results of these preliminary tests, isolates that were found to be Gram-positive and catalase-positive were selected for further characterization. Out of the 32 isolates, a total of 7 isolates exhibited both Gram-positive reaction and positive catalase activity. These selected isolates were subsequently subjected to detailed biochemical tests, which include methyl red test, Voges–Proskauer test, citrate utilization test, oxidase test, urease test, phenylalanine deaminase test, gelatin liquefaction test and sugar fermentation ability test of the isolates assimilating different sugar supplements (Holt et al., 1994; Cappuccino and Sherman, 1996; Ni et al., 2015).

3.6.1 Colony Morphology and Cultural Characteristics

The preliminary identification of bacterial isolates was carried out by examining colony morphology and cultural characteristics on appropriate growth media. Pure colonies obtained after incubation were evaluated based on colony size, shape, color, surface appearance, margin, elevation, opacity, sconsistency/texture, motility.These characteristics provide important preliminary clues about the identity of bacterial

isolates. The observations were recorded after incubation at suitable temperature and time under aseptic conditions (Madigan et al., 2021).

3.6.2 Gram Staining

Gram staining was used to identify the Gram reaction and shape of the bacterial isolates. A smear of bacterial culture was fixed on a glass slide, and stained with crystal violet, Gram's iodine, alcohol, and safranin. The slides were viewed under a microscope with oil immersion. Gram-positive bacteria retain the purple stain and Gram-negative bacteria are counterstained pink (Cappuccino and Welsh 2019).

3.6.3 Catalase Test

Catalase testing was used to establish if the bacterial isolates produced the enzyme catalase, which breaks down hydrogen peroxide into water and oxygen. A loopful of culture was placed on a clean slide and a drop of 3% hydrogen peroxide solution was added. The presence of effervescence (bubbles) was observed immediately as a result of the release of oxygen gas (Leboffe and Pierce 2021).

3.6.4 Methyl Red (MR) Test

A methyl red test was conducted to test for mixed-acid fermentation of glucose. The bacterial strains were cultured in MR-VP broth and incubated at 37 °C for 24-48 h. Following incubation, the culture was tested with a few drops of methyl red indicator. A red colour indicated a positive reaction (due to stable acid production) while a yellow colour was negative (Leboffe and Pierce 2021).

3.6.5 Voges–Proskauer (VP) Test

The Voges–Proskauer test was performed to detect the production of acetoin, an intermediate compound formed during glucose fermentation via the butanediol pathway. Bacterial cultures grown in MR-VP broth were treated with α-naphthol and potassium hydroxide reagents. Development of a pink to red color after exposure to oxygen indicated a positive result, while no color change indicated a negative reaction (Cappuccino and Welsh 2019).

3.6.6 Citrate Utilization Test

The citrate utilization test was performed to determine the ability of bacteria to use citrate as the only source of carbon.The isolates were inoculated onto Simmons citrate agar slants and incubated at 37 °C for 24–48 h. Utilization of citrate results in alkaline by-products that change the medium color from green to blue, indicating a positive reaction (Leboffe and Pierce 2021).

3.6.7 Oxidase Test

The oxidase test was used to test the presence of the cytochrome c oxidase enzyme in bacterial isolates. A loopful of bacterial culture was placed on filter paper soaked in oxidase reagent. Development of a dark purple color within 30 seconds indicated a positive oxidase reaction, whereas absence of color change indicated a negative result (Madigan 2021).

3.6.8 Urease Test

The urease test was carried out to check the ability of bacteria to break down urea to ammonia and carbon dioxide using the enzyme urease. The isolates were inoculated into urea broth or urea agar and incubated at 37 °C for 24–48 h. Hydrolysis of urea leads to alkalization of the medium, resulting in a color change from yellow to pink, indicating a positive result (Cappuccino and Welsh 2019).

3.6.9 Phenylalanine Deaminase Test

The phenylalanine deaminase test was performed to detect the ability of bacteria to deaminate phenylalanine to phenylpyruvic acid. Bacterial isolates were inoculated on phenylalanine agar slants and incubated at 37 °C for 24 h. After incubation, ferric chloride reagent was added. The formation of a green color indicated the presence of phenylpyruvic acid, confirming a positive test (Leboffe and Pierce 2021).

3.6.10 Gelatin Liquefaction Test

The gelatin liquefaction test was used to determine the ability of bacterial isolates to produce gelatinase enzyme that hydrolyzes gelatin. Bacteria were inoculated into nutrient gelatin medium and incubated at 37 °C. After incubation, the tubes were

refrigerated to observe solidification. Liquefaction of gelatin after cooling indicated gelatinase production and a positive result (Cappuccino and Welsh 2019).

3.6.11 Sugar Fermentation Test

The sugar fermentation test was done to check the ability of the bacterial isolates to ferment sugars. The bacteria were inoculated into phenol red carbohydrate broth containing different sugars and a Durham tube. After incubating the cultures at 37 °C for 24-48 hours, yellow colour of the medium indicated acid production from sugar fermentation and bubbles in the Durham tube indicated gas production (Madigan et al. 2021).

3.7 Identification of the Isolates using 16S rRNA Sequencing:

3.7.1 Genomic DNA Isolation

All the 7 isolates were grown overnight and the genomic DNA was extracted from 1.5 mL cultures using a bacterial genome extraction kit (PureLink Genomic DNA Mini Kit) following the manufacture protocol.

3.7.2 Quantification of Genomic DNA by agarose gel electrophoresis

0.8g of Agarose was mixed with 100ml of 1X TAE buffer (Mixed 10ml of 50X TAE buffer and 90ml of distilled water from stock of 50X TAE buffer). 4µl of 10mg/ml of Ethidium bromide was added to the solution and mixed. Gel comb was fixed in the gel casting tray and the Agarose gel was poured into it. 4µl of DNA samples were mixed with loading buffer and were carefully loaded on the wells using micropipettes. The gel tray was fixed on the Electrophoresis chamber and the DNA was allowed to run on the gel by supplying 100 Volt of electricity. After around 30 minutes, DNA bands were then observed on Genei UVITech Cambridge gel documentation system and the genomic gel photos were taken.

3.7.3 Amplification of 16S rRNA gene, sequencing and phylogenetic analysis

All the isolates were subjected to the amplification of 16S rRNA gene universal primers –PA (5’-AGA GTT TGA TCC TCC TGG CTC AG-3’) as the forward primer and PH (5’-AAG GAG GTG ATC CAG CCG CA-3’) as the reverse primer

(Qin et al., 2009). The PCR reactions were carried out in a VERTI THERMAL CYCLER (Applied Biosystem, Singapore) in a total volume 25 µL containing 1.0 µL of genomic DNA (50ng), 0.5 µL of each primer (10 pmol), 2.0 µL of deoxynucleotide triphosphates (2.5 Mm each), 2.5 µL of 1x PCR buffer and 1.0 µL of Taq DNA polymerase(1U/ µL). PCR conditions consists of an initial denaturation at 94ºC for 1 minute followed by an extension phase at 72 °C for 1.2 minutes with a final extension at 72 °C for 10minute. the amplifies PCR products were analysed by electrophoresis through 1.5% agarose gels and documented using a Bio-Rad Gel Doc XR+ system (Hercules, CA,USA). The PCR products were purified using Purlink PCR Purification Kit (Invitrogen) and sent for sequencing. The PCR products were purified by using Purlink PCR Purification Kit (Invitrogen) and sequenced. The 16S rRNA sequences were compared to the bacterial sequences from the NCBI database using basic local alignment search tool (BLASTN). The 16S rRNA gene sequences obtained were compared with sequences in the NCBI GenBank database using the BLASTN algorithm to determine their closest relatives.

The sequences showing the highest similarity were selected for further analysis. Subsequently, both pairwise and multiple sequence alignments were carried out using the ClustalW algorithm integrated in MEGA 11 (Tamura et al., 2021). Based on the aligned sequence dataset, a phylogenetic tree was generated using the neighbor-joining method as described by Naruya Saitou and Masatoshi Nei (1987). The reliability of the inferred phylogenetic relationships was evaluated through bootstrap analysis performed in MEGA 11, employing the Kimura two-parameter (K2P) model proposed by Motoo Kimura (1980).

3.8 In vitro screening for Probiotic Properties of the isolates

3.8.1: Acid tolerance

All the 7 bacterial isolates were initially cultivated in Luria-Bertani (LB) broth at 37 °C for 24 hours, after which the cultures were harvested and resuspended in sterile saline. The acid tolerance of the isolates was evaluated following the protocol outlined by Dowarah et al. (2018). To simulate a range of pH conditions, the pH of nutrient broth was adjusted to values of 2, 3, 4, 5, 6, 7, 8, and 9 using 1 M HCl and 1

N NaOH. A 100 µL aliquot of each overnight-grown culture was inoculated into 5 ml of pH-adjusted LB medium and incubated at 37 °C for 24 hours. Growth was assessed by monitoring the turbidity of the cultures, and the optical density was measured at 600 nm (OD600) to quantify cell proliferation. Uninoculated medium served as the negative control and showed no bacterial growth, in accordance with the findings of Chen et al. (2022). All experiments were performed in triplicate for each isolate. The growth percentage was calculated using the formula:

% Growth = (OD in test medium / OD in control medium) × 100.

3.8.2 Bile salt tolerance

The survivability of all the 7 isolates in bile salt was evaluated by using 5 mL of LB broth supplemented with 0.2%, 0.4%, 0.6% 0.8% and 1%, 1.2%, 1.4% and 1.6% bile salt. The culture was incubated at 37 °C, and samples were monitored after 24h. The negative control, which was only the media, showed no growth (Chen et al., 2022). OD_{600nm} was measured using a BioSpectrometer (Eppendorf, Germany). The growth rate was calculated as follow:

% of growth = Growth in particular medium/Growth in control medium × 100

3.8.2 Auto Aggregation activity

The ability of the 7 isolates to auto aggregate was tested as per Zommiti et al., (2017). The overnight grown bacterial culture was collected via centrifugation at 8,000 rpm for 10 minutes at 4 °C. The resulting pellet was subsequently washed twice with phosphate-buffered saline (PBS) to remove residual medium components and then resuspended in fresh PBS for further use. The sample was left for some time, incubating the sample anaerobically at 37 °C, and the suspension on top was measured for absorbance at 600 nm at 0, 1, 2, 3, 4, and 5 h.

The auto aggregation (in percentage) was calculated using

Auto aggregation % = $[1 - (A_{time}/A_0) \times 100]$, where, A_{time} represents the absorbance at a particular time and A_0 represents the absorbance at time 0.

3.9 Safety evaluation of the isolates:

3.9.1 Assessment of Antimicrobial Activity

The antimicrobial activity of all the 7 isolates were determined, using agar well diffusion method (Sim et al., 2010) against *Staphylococcus aureus* ATCC BAA-44, *Enterococcus faecalis* ATCC-51575, *Klebsiellia pneumoniae* ATCC-BAA-2814, *Candida albicans* ATCC-64124, *Streptococcus pneumoniae* ATCC-10015, *Pseudomonas aeruginosa* ATCC-10145, *Micrococcus luteus* ATCC-10240, *Bacillus subtilis* ATCC-11774, *Saccharomyces cerevisiae* ATCC-2601, *Salmonella typhimurium* ATCC-51812, *Escherichia coli* ATCC-10536. Overnight cultures of the bacterial isolates were subjected to centrifugation at 8,000 rpm for 10 minutes at 4 °C to separate the cellular biomass. The resulting supernatant was subsequently passed through a 0.2 µm pore-size syringe filter to ensure the removal of any remaining cellular debris or particulate matter. For antimcirobial activity screening, Mueller Hinton Agar media was prepared in petri plates and 100 µl of freshly grown pathogens were spreaded thoroughly on the entire surface, a well of 5mm diameter was made by punching the agar media. 70 µl of the culture were added to each 5 mm diameter wells . The plates were incubated at 37 ºC and observed after 24 hours.

3.9.2 Antibiotic Susceptibility Test

The antibiotic susceptibility profiles of all the 7 isolates were determined using the disc diffusion assay on Luria-Bertani (LB) agar plates. A panel of antibiotics, selected in accordance with the recommendations provided by the European Food Safety Authority (EFSA), was employed for this analysis. The antibiotic susceptibility pattern of the isolates was assessed using ampicillin (10 µg/disc), gentamicin (50 mcg/disc), kanamycin (5 µg/disc), streptomycin (25 µg/disc), chloramphenicol (10 µg/disc), erythromycin (15 µg/disc), clindamycin (2 µg/disc), and tetracycline (30 µg/disc), Polymysin B (50 mcg/unit), Fluconazole (25 mcg/unit), Ciprofloxacin (10 mcg/unit), Azithromycin (15 mcg/unit,) Neomycin (10 mcg/unit), Tetracycline (30 mcg/unit), Miconazole (30 mcg/unit), Amphotericin B (50 mcg/unit), Norfloxacin (10 mcg/unit), Itraconazole (10 mcg/unit), Nalidixic Acid (30 mcg/unit), Methicillin (5 mcg/unit), Levofloxacin (5 mcg/unit), Nystatin (50

mcg/unit), Ketoconazole (10 mcg/unit), Trimethoprim (10 mcg/unit) (Singh et al., 2012). The overnight cultures (100 µl) were spread on LB agar plates. Sterile antibiotic-impregnated discs were carefully positioned on the surface of LB agar plates previously inoculated with the test isolates. The inoculated plates were incubated at 37 °C for a duration of 48 hours. Following incubation, the diameters of the inhibition zones were measured using a standardized antibiotic zone scale, as per Clinical and Laboratory Standards Institute (CLSI, 2016) guidelines. Based on the measured zone diameters, the isolates were categorized as susceptible, intermediately susceptible (moderately susceptible), or resistant to the respective antibiotics.

3.10 Screening of bacteria for Biosurfactant production

Both the isolates in their log phase growth were inoculated into MRS-Lac broth (glucose was replaced with lactose from the media composition) and incubated for 72 h at 30 ºC. Later, to obtain the supernatant, the culture was centrifuged at 10,000 rpm for 10 min (at 4 ºC). The supernatant was checked for the production of biosurfactant by oil displacement test (Vallejo et al., 2021).

3.10.1 Oil Displacement Assay

The oil displacement assay was conducted according to Joe et al., 2020. In a Petri dish, the distilled water (25mL) was filled and the crude oil (2 mL) was spread on the surface of the water. Then, 5-10 µl of culture supernatant was dropped on the surface of oil layer. The diameter of the oil-free zones was measured for 30 s. The distilled water was used as a negative control.

3.10.2 Production and Extraction of Biosurfactant Compounds

The most popular interesting group of biosurfactants are lipopeptide biosurfactant. Lipopeptides are characterized by structural diversity and diverse functional properties which allows their usage in various domains (Ines and Dhouha, 2015). Several microbes are responsible for the production of different types of lipopeptide surfactants. Isolates having antagonistic activity were further processed for lipopeptide production and extraction using the method Kim et al., (2004) with a minor modification.

Loop full colonies of pure cultures were inoculated into freshly prepared MRS –Lac broth and incubated at Thermo Scientific NSW-159 shaking incubator at 120 rpm for 3 days at 37 ºC. The broth suspension was then centrifuged at 6000 rpm for 20 minutes and the cell free supernatant were acidified with 3N HCl to pH 2 to precipitate the lipopeptide and left overnight at 4 ºC. After that the supernatant were discarded and the pellet were washed with water and centrifuged again at 6000 rpm for 20 minutes. The pellet were dissolved with water and centrifuged at 6000 rpm for 20 minutes. The pellet was dissolved with water and the pH was adjusted to 7. It is further transferred to a separating funnel and dissolved in ethyl acetate (2:1, v:v) and the lower layer (organic phase) were collected and evaporate the solvent at 45ºC using Buchi Heating Bath B-100 rotary vacuum evaporator and the extract were prepared until it completely evaporate. The final collected extract was sent for compound analysis to Cytogene Research & Development, Uttar Pradesh.

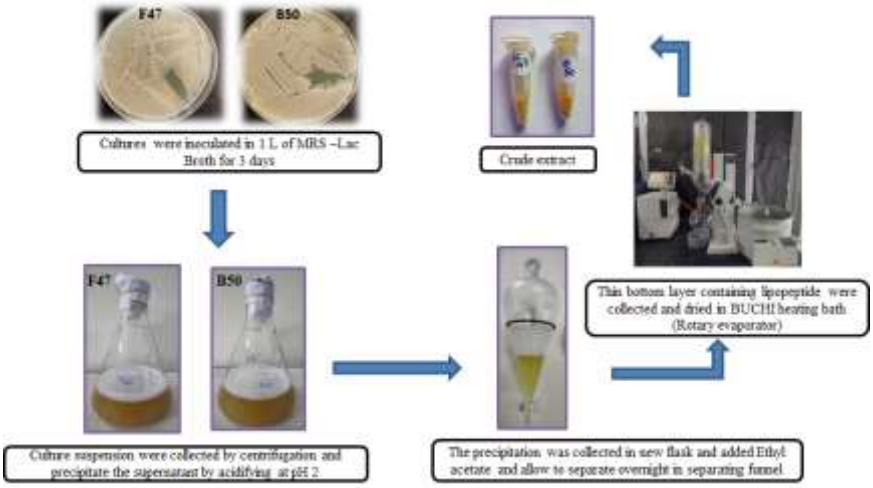


Figure 3.2: Production and extraction of crude biosurfactant of isolate F47 & B50 using Ethyl acetate

3.11. Characterization of the extracted biosurfactant

Biosurfactant product was dissolved in methanol at 1 mg/mL concentration and filtered (0.22 μm). The electrospray ionization (ESI) mass spectra of the product were analyzed using ultra-high performance liquid chromatography (UHPLC, Ultimate 3000, Dionex) system coupled with TSQ Endura™ Triple Quadrupole Mass Spectrometer (Thermo Scientific, USA).

The separation was performed using a Hypersil Gold C_{18}, 1.9 μm, 100 × 2.1 mm column. The mobile phase comprised of water, 1% formic acid and acetonitrile. The linear gradient system: 0–3.5 min, 60 to 93% acetonitrile; 3–20 min, keeping 93% acetonitrile and 7% water (1% formic acid); injection volume 5μL; flow rate 0.300 ml/min. Ultra-high-performance liquid chromatography electrospray ionization mass spectrometry (UHPLC-ESI–MS) was performed in the positive mode, with full mass spectral scans acquired over an m/z range of 200 to 2000 (Smyth et al., 2010).

4.1 Proximate Compositional Analysis

Microbial fermentation plays an important role in transforming the chemical constituents of food substrates into nutritionally enriched compounds that enhance the flavor, texture, and overall quality of fermented food products. During the fermentation process, microorganisms metabolize the available nutrients in the raw materials, leading to biochemical modifications that improve the nutritional value and sensory properties of the final product (McFeeters, 2004; Anal, 2019; Sharma et al., 2020; Hu et al., 2021). In the present study, the proximate composition of three fermented food samples was evaluated at different stages of fermentation, specifically on the 3rd, 5th, and 7th day. The results of the proximate composition analysis at these fermentation intervals are summarized in Table 4.1.

Table 4.1: Proximate composition of fermented bamboo shoots (*Tuaithur*), soybean (*Bekang*), and pork fat (*Saum*) collected after 3, 5, and 7 days of fermentation

Proximate Parameters (weight in %)	**Fermented Food samples**								
	Tuaithur			***Bekang***			***Saum***		
	3rd D	**5th D**	**7th D**	**3rd D**	**5th D**	**7thD**	**3rdD**	**5thD**	**7thD**
Moisture content	86.8	86.4	87.6	58.3	57.1	56.4	3.8	3.8	2.5
Total ash content	1.1	1.1	1.1	2.7	2.5	1.1	-	-	-
Fat	1.1	0.9	1.2	4.6	5.3	5.4	90.6	94.6	95.6
Protein	2.7	3.0	2.8	19.7	19.9	18.5	1.3	1.3	1.5
Crude fiber	3.1	2.1	2.2	10.6	2.4	2.5	-	-	-
Carbohydrate	5.2	6.5	7.9	4.1	12.8	16.2	4.2	0.2	0.3
Calorific value (K cals/ 100gm)	3.97	4.19	3.98	7.20	8.36	8.23	5.89	3.99	4.04

The pH values of the fermented food samples indicated variations depending on the type of substrate used for fermentation. The products *Tuaithur* and *Saum* showed an acidic pH value of 4.04, which is characteristic of lactic acid fermentation. The acidic nature of these products may be attributed to the production of lactic acid by lactic acid bacteria during the fermentation process (Mugula et al., 2003). In contrast, the fermented soybean product *Bekang* exhibited a slightly alkaline pH (7.93), which may be associated with the metabolic activities of microorganisms involved in soybean fermentation.

The analysis of moisture content revealed that bamboo shoot samples contained the highest moisture level (86.93%), followed by soybean samples (57.26%), whereas the lowest moisture content was observed in pork fat samples (3.36%). Similar observations of high moisture levels in fermented bamboo shoots have been reported in *Bambusa balcooa* (ChingSanei-bi), with moisture values ranging between 90.73% and 91.5% (Singh et al., 2011).

As expected, the fat content was considerably higher in the pork fat samples (93.60%) compared to the other fermented foods. The soybean samples contained a moderate fat level (5.3%), while the bamboo shoot samples showed the lowest fat content (1.06%). In contrast, the soybean samples were found to be comparatively richer in protein (19.36%) and carbohydrate (11.03%), exceeding the levels observed in bamboo shoot and pork fat samples. The lowest protein concentrations were recorded in bamboo shoot (2.83%) and pork fat (1.36%) samples. A similar trend was also observed for carbohydrate content, which was 6.53% in bamboo shoot samples and 1.56% in pork fat samples.

A relatively low amount of crude fiber was detected in bamboo shoot (2.46%) and soybean (1.56%) samples, while crude fiber was not detected in the pork fat samples. During the course of fermentation, a slight increase in protein, carbohydrate, and fat contents was observed between the 3rd and 5th day of fermentation. However, this increase was not pronounced, as a consistent trend was not observed during the later stage of fermentation, as presented in Table 4.1.

4.2 Bacterial Diversity

High-throughput sequencing of the fermented food samples generated a substantial number of sequence reads across the different fermentation stages. In the fermented bamboo shoot samples, a total of 93,857, 267,981, and 187,190 reads were obtained on the 3rd, 5th, and 7th day of fermentation, respectively. For the fermented soybean samples, the number of reads recorded were 165,177 on the 3rd day, 252,177 on the 5th day, and 259,761 on the 7th day. Similarly, the fermented pork fat samples yielded 208, 302, 152,718, and 126,281 reads on the 3rd, 5th, and 7th day of fermentation, respectively.

After quality filtering and clustering of all high-quality sequences, a total of 3,424 operational taxonomic units (OTUs) were identified collectively across all samples. The relative distribution of OTUs among the samples was found to be largely comparable, indicating that the sequencing depth was adequate for evaluating and comparing the bacterial diversity present in the different fermented food products. Furthermore, rarefaction analysis was performed using the OTU datasets for each sample at the respective fermentation time points (Figure 4.1). The rarefaction curves approached a plateau for all samples, suggesting that the sequencing effort was sufficient and that the majority of the bacterial diversity present in the samples had been successfully captured.

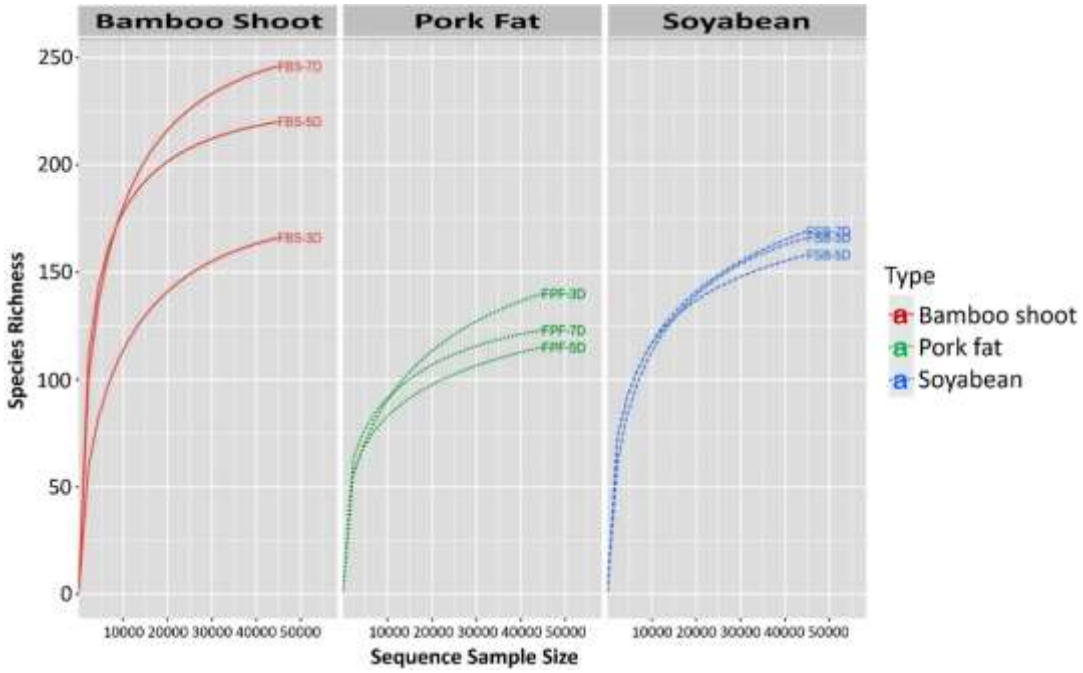


Figure 4.1: Rarefaction curves for the sequences obtained from fermented bamboo shoots (FBS), soybeans (FSB), and pork fat (FPF) collected at 3, 5, and 7 days of fermentation.

The alpha diversity of bacterial communities in the fermented food samples was evaluated using Simpson, Chao1, and Shannon diversity indices (Figure 4.2). The results indicated that the fermented bamboo shoot samples (FBS) possessed the highest bacterial diversity, followed by the fermented soybean samples (FSB), whereas the fermented pork fat samples (FPF) exhibited the lowest level of microbial diversity.

Both Chao1 and Shannon indices, which are commonly used to estimate microbial richness and diversity within a community, demonstrated similar trends across the samples. The Shannon diversity analysis of the FBS samples showed that the sample collected on the 7th day of fermentation had the greatest species richness compared to the earlier fermentation stages. In contrast, the FSB and FPF samples displayed a relatively uniform distribution of species richness throughout the fermentation period, with only minor variations observed between the 3rd and 7th

day of fermentation.

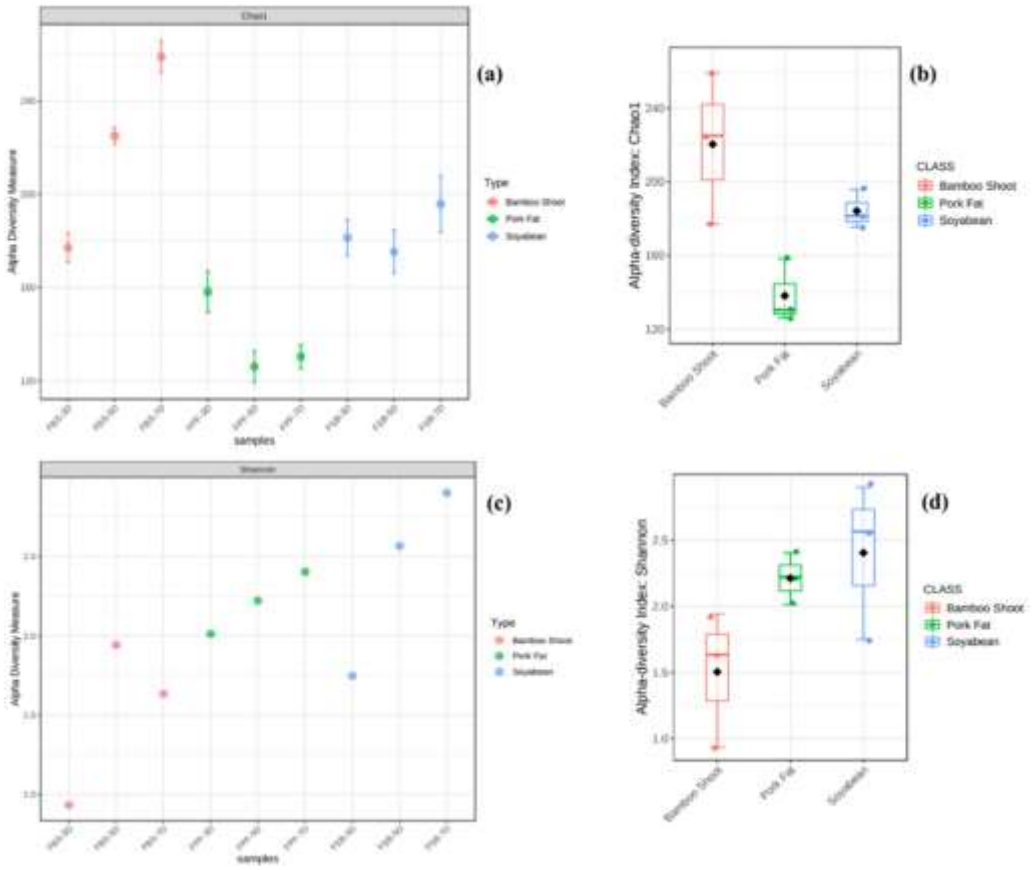


Figure 4.2: Alpha diversity of bacterial communities in fermented food samples (bamboo shoots, soybean, and pork fat).

(a) Alpha diversity across fermentation days (3rd, 5th, and 7th day).

(b) Chao-1 diversity index of fermented food samples.

(c) Alpha diversity comparison among food types across sampling days.

(d) Shannon diversity index of fermented food samples.

Sequencing data were normalized to the minimum library size (93,857 reads; sample FBS-3D).

4.3 Bacterial Community Composition

The analysis of microbial composition demonstrated that all fermented food samples contained a wide diversity of bacterial taxa. The relative abundance of the dominant bacterial groups at the phylum and genus levels is illustrated in Figures 4.3 and 4.4, respectively.

At the phylum level, the microbial communities on the 3rd day of fermentation were predominantly composed of Firmicutes, followed by Proteobacteria, Bacteroidetes, Actinobacteria, and Verrucomicrobia across the bamboo shoot (FBS), soybean (FSB), and pork fat (FPF) samples. The relative abundance of Firmicutes was recorded as 94.99%, 82.72%, and 92.91% in FBS, FSB, and FPF samples, respectively. Proteobacteria represented the second most dominant phylum, accounting for 4.67%, 15.01%, and 6.82% in the respective samples. Other phyla such as Bacteroidetes (0.13%, 0.027%, 0.038%), Actinobacteria (0.077%, 2.14%, 0.18%), and Verrucomicrobia (0.023%, 0.002%, 0.0049%) were detected in comparatively lower proportions.

Overall, Firmicutes was the most prevalent phylum across all samples, with an average relative abundance exceeding 90.22%, whereas Proteobacteria represented the second most abundant group with an average abundance above 8.83%. Comparable findings have been reported in previous studies on traditional fermented foods, including ‘‘*zha-chili*”, where Firmicutes and Proteobacteria were identified as the dominant bacterial phyla (Cai et al., 2021). Similarly, investigations on *Suancai*, a widely consumed fermented vegetable product in northern China, also reported these two phyla as the principal microbial groups present during fermentation (He et al., 2020).

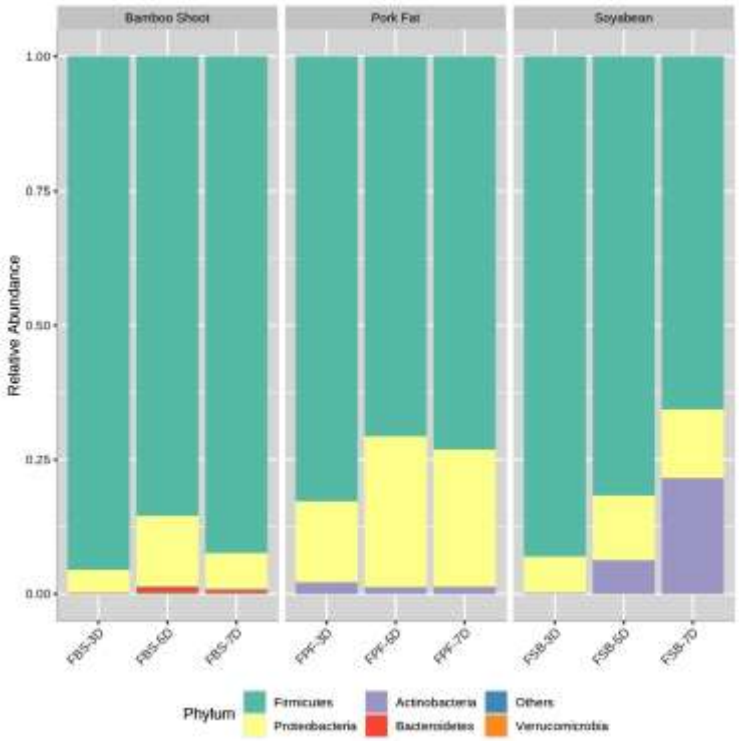


Figure 4.3: Relative abundance of top bacterial phyla in fermented bamboo shoots (FBS), pork fat (FPF), and soybeans (FSB) samples collected on the 3rd, 5th, and 7th day of fermentation.

Other bacterial phyla were also detected in the samples, but their relative abundance remained very low (less than 1%). These minor groups included Planctomycetes, Fusobacteria, Chloroflexi, Acidobacteria, Nitrospirae, Spirochaetes, Gemmatimonadetes, Synergistetes, Lentisphaerae, Elusimicrobia, and Chlorobi. On the fifth and seventh days of fermentation, the proportion of Firmicutes showed a slight decline, whereas the abundance of Proteobacteria increased notably in soybean samples, reaching approximately 70.85%. During these later stages of fermentation, several additional phyla such as Chloroflexi, Fusobacteria, Acidobacteria, Tenericutes, Fibrobacteres, and Caldiserica were also identified. The gradual rise in bacterial diversity as fermentation progressed indicates that these microbial groups may contribute to the development of the characteristic texture and flavor of the fermented food products. Furthermore,

considerable variation in genus-level composition was observed among the three fermented foods across the different fermentation days (Figure 4.4)

In fermented bamboo shoots, the genus *Lactobacillus* showed the highest dominance, accounting for 91.64% of the total bacterial population. In contrast, its relative abundance was considerably lower in fermented soybeans (4.81%) and was minimal in fermented pork fat (0.042%). In fermented soybean samples, the microbial community was mainly dominated by the genera *Staphylococcus* (52.36%), *Bacillus* (38.47%), and *Pseudomonas* (6.40%). Approximately half of the sequencing reads obtained from soybean samples on the third day of fermentation were assigned to operational taxonomic units (OTUs) affiliated with *Staphylococcus*. Interestingly, this genus was not detected in the other two fermented food products analyzed in the present study. In fermented pork fat samples, the predominant genera identified were *Clostridium* (72.48%), followed by *Sutterella* (12.54%) and *Lactobacillus* (4.81%), indicating a distinct microbial composition compared with the other fermented foods.

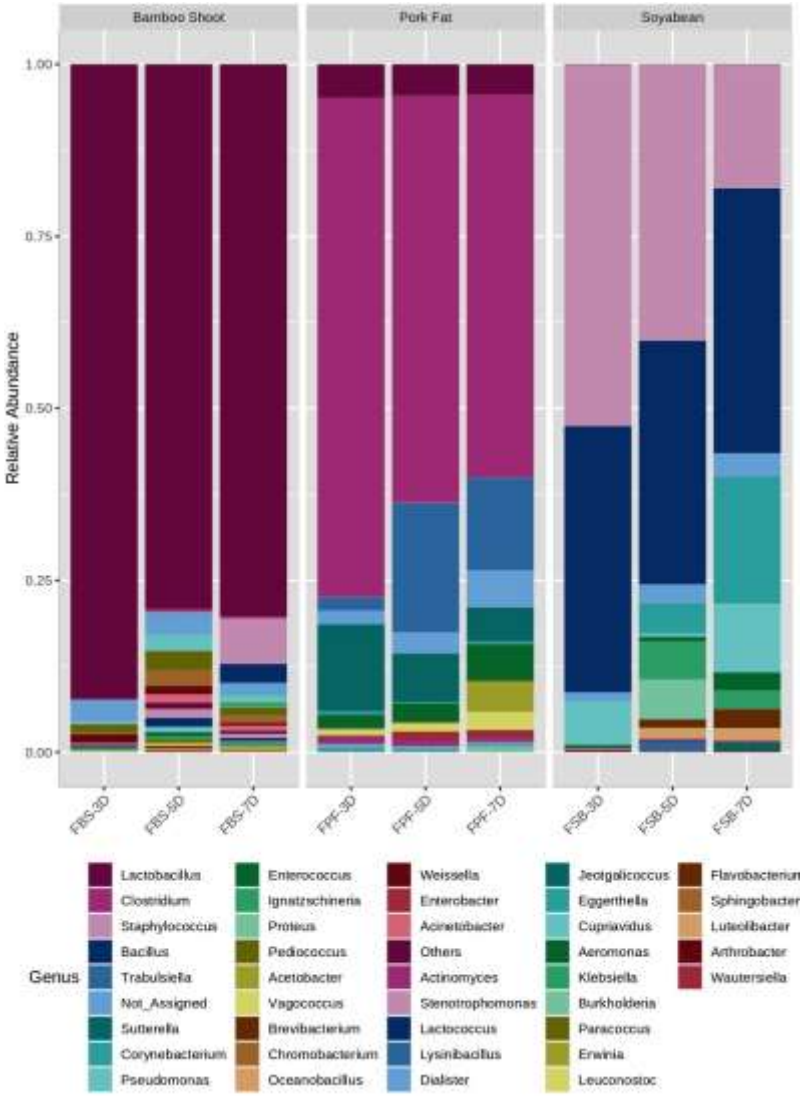


Figure 4.4: Relative abundance of bacteria at the genus level in fermented bamboo shoots (FBS), pork fat (FPF), and soybean (FSB) on the 3rd, 5th and 7th day of fermentation

The genera *Lactobacillus*, *Staphylococcus*, and *Clostridium* together represented more than 60.53% of the total bacterial sequence reads obtained from the three fermented food products analyzed in this study. Among them, *Lactobacillus* was particularly prominent in fermented bamboo shoot samples. Members of this genus are well known for their important role in bamboo shoot fermentation, and previous studies have reported lactic acid bacteria as the dominant microbial group in several fermented bamboo shoot products (Sonar and Halami 2014). For example, the

traditional fermented bamboo shoot product *Khorisa*, widely prepared and consumed by indigenous communities of Assam, has been reported to contain diverse lactobacilli with potential antimicrobial properties and considerable pharmacological significance (Badwaik et al., 2015). In addition, *Lactobacillus* species are capable of producing various volatile metabolites that contribute to the characteristic aroma and sensory quality of fermented foods (He et al., 2020).

Apart from *Lactobacillus*, other lactic acid bacteria such as *Pediococcus*, *Enterococcus, Lactococcus*, and *Streptococcus* are also known to influence the flavor profile and enhance the nutritional value of fermented food products (Stiles et al., 1997; Salminen et al., 2004). Consistent with these observations, the present study detected several lactic acid bacteria and related genera, including *Pediococcus*, *Enterococcus*, and *Lactococcus*, in fermented bamboo shoot samples with relative abundances greater than 1%. In fermented soybean samples, *Bacillus*, *Pseudomonas*, and *Enterococcus* were among the notable bacterial taxa identified. In contrast, fermented pork fat samples were characterized by relatively higher abundances of genera such as *Sutterella*, *Lactobacillus*, *Enterococcus*, and *Trabulsiella*, with their relative proportions ranging from 1.92% to 13.36%. These findings indicate distinct microbial community structures among the different fermented food products studied.

In the fermented soybean samples analyzed in this study, the genus *Staphylococcus* constituted more than 50% of the total bacterial relative abundance. The occurrence of *Staphylococcus* has previously been documented in a wide range of fermented food products (Majumdar and Gupta, 2020). This genus, along with several other bacterial groups, has been associated with the formation of synthetic esters that contribute to the characteristic flavor and aroma of fermented foods (Hasan et al., 2006). In particular, coagulase-negative *Staphylococcus* species are frequently reported as dominant microorganisms in many fermented foods across different regions of the world. Due to their functional properties, certain *Staphylococcus* species are commonly used as starter cultures in the fermentation of cheese, meat, and soybean-based products (Heo et al., 2020).

However, it is also important to note that some members of the genus *Staphylococcus* are recognized as food-borne pathogens affecting humans and animals (Fetsch and Johler, 2018). Consequently, further investigations using multiple taxonomic and molecular approaches are necessary to accurately identify the species present in fermented foods, particularly when this genus occurs in relatively high abundance. Such analyses are especially important for traditional fermented foods that are produced under open and less controlled environmental conditions (Arora and Baldi, 2017).

In contrast, the genus *Clostridium* showed the highest relative abundance in the fermented pork fat samples. The occurrence of several enteric bacterial genera, including *Clostridium*, in traditional fermented foods from North-East India has also been reported in earlier studies (Keisam et al., 2019). These observations highlight the distinct microbial composition associated with different fermented food products.

4.4 Comparison of Bacterial Communities during the Fermentation Process

Beta diversity analysis was performed to compare the microbial diversity among the different samples by evaluating the similarities and differences in community composition. This analysis reflects the degree of variation in microbial populations between samples. To assess these differences, weighted and unweighted UniFrac distance metrics were applied, followed by principal coordinate analysis (PCoA). The PCoA plot illustrates the distribution and variation of microbial species across all samples, highlighting the differences in microbial community structure (Figure 4.5). The results demonstrated clear differences in the microbial community composition among the three fermented food samples. Principal coordinate analysis (PCoA) showed a distinct separation in the microbial profiles of the different fermented foods, indicating considerable variation in their bacterial communities. Despite these differences between food types, samples collected at different fermentation stages (3rd, 5th, and 7th days) within the same food category exhibited relatively similar microbial compositions.

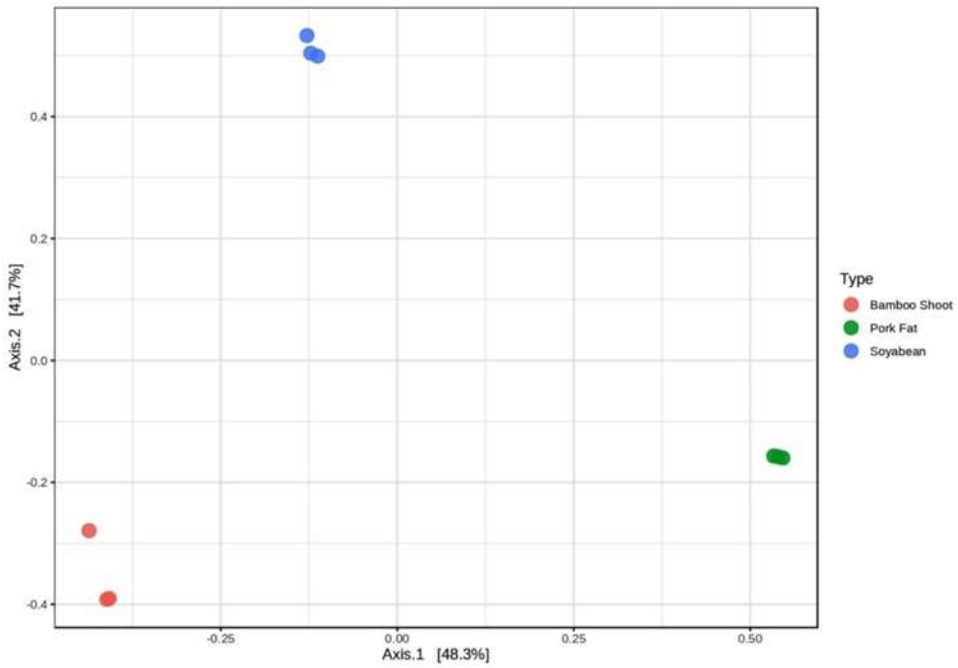


Figure 4.5: Principal component analysis of bacterial communities in the three different fermented foods (bamboo shoot, soybean, and pork fat).

In the PCoA plot, samples derived from bamboo shoots, soybean, and pork fat formed separate clusters, reflecting the distinct microbial communities associated with each substrate. However, the samples corresponding to different fermentation days within each food type were positioned close to one another, suggesting that the microbial composition remained relatively consistent throughout the fermentation period for that particular food (Figure 4.5).

The first and second principal coordinate axes explained 41.7% and 48.3% of the total variation among the samples, respectively. These values indicate a substantial degree of variation in bacterial genera between the three fermented food types, suggesting that their microbial communities are considerably different from one another. In addition, a hierarchically clustered heat map based on the bacterial community composition at the genus level was generated to further visualize the distribution and relative abundance of bacterial genera across the samples (Figure 4.6).

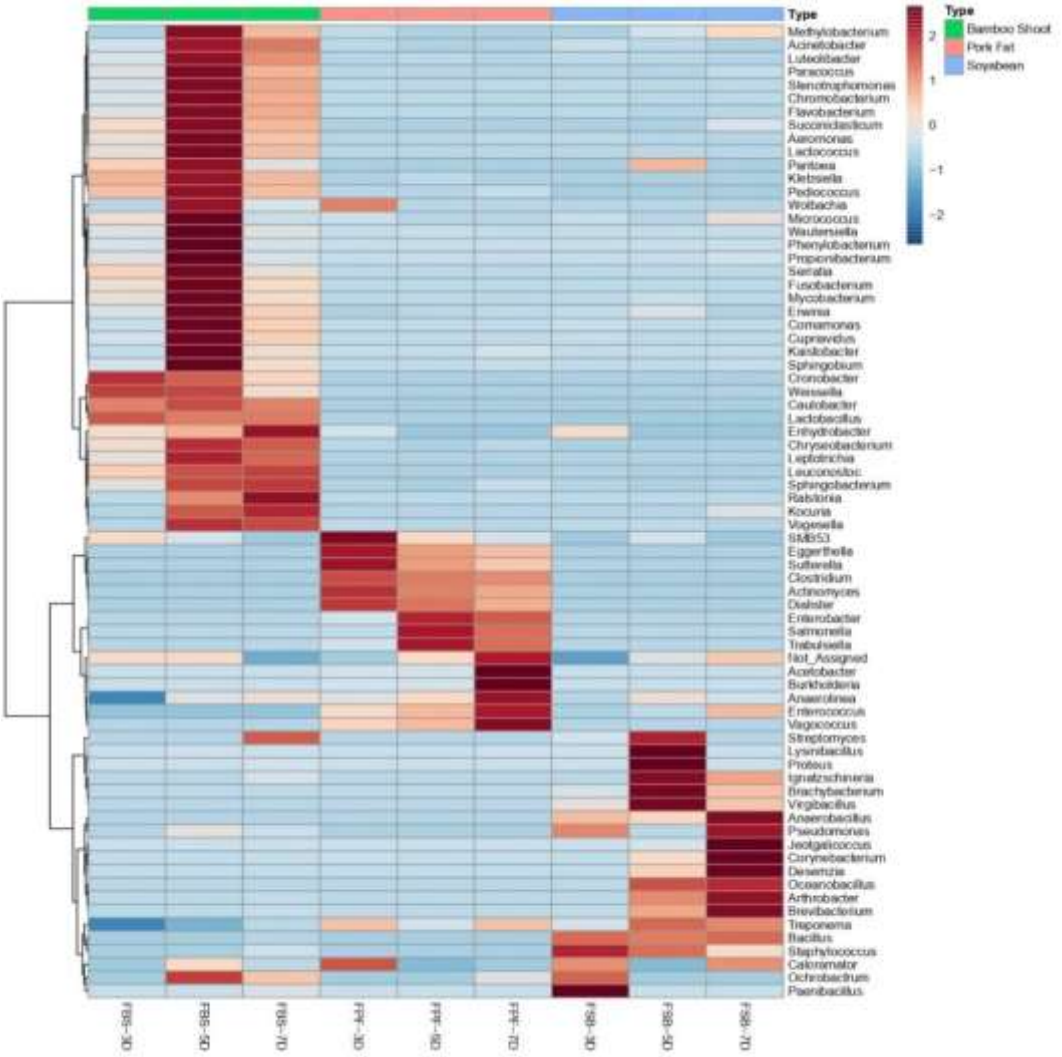


Figure 4.6 Heat map showing genus-level bacterial community composition in fermented food samples.

FBS – bamboo shoots; FPF – pork fat; FSB – soybean, collected on the 3rd, 5th, and 7th day of fermentation.

Color intensity from blue to red represents low to high relative abundance.

The hierarchical clustering analysis revealed distinct patterns in the distribution of bacterial phyla among the fermented food samples. The predominant phylum detected in bamboo shoot samples formed an independent cluster, whereas the major bacterial phyla identified in soybean and pork fat samples grouped together in

another cluster, which was further subdivided into smaller sub-clusters. At the genus level, the dominant bacterial genera associated with each food type were organized into three clearly separated clusters, indicating substantial differences in microbial composition among the fermented products. Among the detected genera, *Lactobacillus*, *Staphylococcus*, and *Clostridium* were identified as the most prevalent across the three fermented food types.

Microbial communities present in fermented foods are known to undergo dynamic changes throughout the fermentation process, with different microbial groups contributing to the development of nutritional and functional properties of the final product (Zang et al., 2018). The relative abundance of the most dominant bacterial genera detected in the fermented food samples collected on the 3rd, 5th, and 7th days of fermentation is presented in Table 4.2.

Table 4.2: Relative abundance of the top most bacterial genera in day wise samples of the fermented foods

Fermented Bamboo Shoots (FBS)			
Genus	**FBS-3D**	**FBS-5D**	**FBS-7D**
Weissella sp.	1.27	0.0	0.0
Pediococcus sp.	1.17	2.80	0.0
Pseudomonas sp.	0.36	2.24	1.25
Chromobacterium sp.	0.27	2.29	0.0
Acinetobacter sp.	0.0	1.35	1.14
Corynebacterium sp.	0.0	0.0	6.58
Sphingobacterium sp.	0.0	0.0	2.74
Unclassified	3.13	2.28	2.27
Others	1.86	11.58	7.11
Fermented Pork Fats (FPF)			
Genus	**FPF-3D**	**FPF-5D**	**FPF-7D**
Clostridium sp.	72.48	59.48	55.40
Sutterella sp.	12.54	7.01	4.85
Lactobacillus sp.	4.81	4.44	4.41
Enterococcus sp.	1.92	2.67	5.28
Trabulsiella sp.	1.81	18.45	13.36
Unclassified sp.	2.07	3.26	5.24
Others	3.17	4.67	11.43
Fermented Soybean (FSB)			

Genus	**FSB-3D**	**FSB-5D**	**FSB 7D**
Staphylococcus sp.	52.36	39.48	17.90
Bacillus sp.	38.47	35.56	37.87
Pseudomonas sp.	6.40	0.0	9.67
Enterococcus sp.	0.47	0.0	0.0
Paenibacillus sp.	0.19	0.0	0.0
Proteus sp.	0.0	5.89	0.0
Ignatzschineria sp.	0.0	5.49	0.0
Corynebacterium sp.	0.0	4.35	18.13
Brevibacterium sp.	0.0	0.0	2.77
Unclassified	1.12	2.98	3.61
Others	0.95	6.23	10.02

The bacterial community composition at the genus level in fermented bamboo shoots during different stages of fermentation (3rd, 5th, and 7th day) is presented in Table 4.2. On the 3rd day of fermentation (FBS_3D), the microbial population was predominantly dominated by *Lactobacillus*, which accounted for 91.64% of the total relative abundance. In contrast, other genera were present only in minor proportions, including *Weissella* (1.27%) and *Pediococcus* (1.17%). As fermentation progressed, the relative abundance of Lactobacillus showed a gradual decline, decreasing to 77.16% on the 5th day (FBS_5D) and 78.88% on the 7th day (FBS_7D). Conversely, the proportion of *Pediococcus* increased during the fermentation period, reaching 2.8% by the 7th day.

Accurate differentiation of lactic acid bacteria (LAB), including *Lactobacillus*, *Weissella*, and *Pediococcus*, at the species and strain level requires more precise and advanced identification techniques. Rapid and reliable identification of these microorganisms is particularly important in the field of food microbiology (Adesulu-Dahunsi et al., 2017). Certain LAB species, especially those belonging to the genus *Pediococcus*, are known for their functional properties, such as the production of exopolysaccharides, improvement of aroma characteristics in fermented foods, and enhancement of lactic acid levels within the fermented product (Adesulu-Dahunsi et al., 2021).

In addition, a noticeable increase in the relative abundance of several other bacterial genera was observed on the 7th day of fermentation. Genera such as *Chromobacterium* (2.29%), *Pseudomonas* (2.24%), *Acinetobacter* (1.35%), *Corynebacterium* (6.58%), and *Sphingobacterium* (2.74%) showed a marked rise in their population during the later stage of fermentation, indicating a shift in the microbial community structure as fermentation progressed

The bacterial community composition of fermented soybean samples collected at different stages of fermentation is presented in Table 4.2. The sample obtained on the 3rd day of fermentation (FSB_3D) showed a predominance of *Staphylococcus* (52.36%) and *Bacillus* (38.47%). In addition to these dominant genera, several other bacteria were detected at relatively lower abundances, including *Pseudomonas* (6.40%), *Enterococcus* (0.47%), and *Paenibacillus* (0.19%). As fermentation progressed, the relative abundance of *Staphylococcus* declined markedly, decreasing from 52.36% on the 3rd day to 17.90% by the 7th day of fermentation. In contrast, the proportion of *Bacillus* remained comparatively stable throughout the fermentation period, with values of 38.47%, 35.56%, and 37.87% recorded on the 3rd, 5th, and 7th days, respectively.

Fermented soybean products are commonly associated with the activity of *Bacillus* species, which contribute to the characteristic sticky texture observed in the final product. Similar microbial profiles have been reported in several traditional fermented soybean foods, including natto from Japan and kinema from Nepal and North-East India (Tamang et al., 2016). In the present study, an increase in the relative abundance of several genera other than *Bacillus* was observed after the 5th day of fermentation (FSB_5D). These included *Proteus* (5.89%), *Ignatzschineria* (5.49%), and *Clostridium* (4.35%).

A further shift in the microbial community structure was evident by the 7th day of fermentation. At this stage, *Clostridium* (18.13%), *Pseudomonas* (9.67%), and *Brevibacterium* (2.77%) exhibited a substantial rise in their relative abundance. Among these, *Clostridium* showed the most pronounced increase, rising from approximately 0.05% on the 3rd day to 4.35% on the 5th day and exceeding 18% by the 7th day of fermentation. This trend indicates a progressive change in the

microbial population during the later stages of soybean fermentation

A high abundance of *Clostridium* in fermented foods may pose potential health concerns for consumers; therefore, careful consideration of the fermentation duration is essential. In the fermented pork fat sample collected on the 3rd day of fermentation (FPF_3D), the bacterial community was predominantly composed of *Clostridium* (72.48%), followed by *Sutterella* (12.54%), *Lactobacillus* (4.81%), *Enterococcus* (1.92%), and *Trabulsiella* (1.81%). As fermentation progressed, the relative abundance of Clostridium showed a gradual decline, decreasing by approximately 13% on the 5th day of fermentation (FPF_5D) and by a further 4.08% on the 7th day (FPF_7D).

Previous investigations using MiSeq amplicon sequencing have also reported the presence of *Clostridium* and several other foodborne bacterial pathogens, as recognized by the World Health Organization (WHO), in Indian fermented foods including fermented pork fat (Keisam et al., 2019). In the present study, certain genera such as *Trabulsiella* (18.45%), *Sutterella* (7.01%), *Lactobacillus* (4.44%), and *Enterococcus* (2.67%) showed relatively stable levels of abundance and did not display major fluctuations during the fermentation process.

Overall, the bacterial composition of the traditional fermented food samples from Mizoram mainly differed in the relative abundance of a few dominant genera rather than in the presence or absence of specific taxa. These observations indicate that while the type of raw material and local fermentation conditions may influence bacterial richness, they may not substantially alter the overall microbial diversity. Comparable patterns have also been documented in other fermented food systems reported in previous studies (Liang et al., 2019; Leech et al., 2020; Fang, 2021)

4.5 Correlation Analysis of Nutritional Parameters with Bacterial Diversity

Canonical correlation analysis (CCA) was conducted to evaluate the relationship between bacterial members (at the level of phylum and genus) and the proximate composition of the food materials (Figure 4.7). Results of the CCA indicated

that Lactobacilli were positively correlated with fermented bamboo shoot samples, *Staphylococcus* was correlated with fermented soybean samples, and *Clostridium* was correlated with fermented pork fat samples. *Staphylococcus* was positively correlated in soybean samples with protein, carbohydrate and crude fiber content. Determining the correlation of microbial members with food components is important for understanding the microbial communities of related food samples and can lead to the design of synthetic microbial consortia for optimum fermentation of specific food types (Parente et al. 2018).

The Canonical Correspondence Analysis (CCA) revealed that microbial distribution across fermented bamboo shoot (FBS), fermented soybean (FSB), and fermented pork fat (FPF) was significantly influenced by substrate-specific nutritional attributes. Fermented soybean demonstrated a strong positive association with protein content and clustering of fermentative and potentially probiotic genera, including lactic acid bacteria and *Bacillus* spp., indicating that the protein-rich matrix may support metabolically active strains capable of producing bioactive compounds and contributing to functional benefits. Fermented bamboo shoot showed correlation with fiber content and comparatively higher microbial diversity, suggesting possible synbiotic interactions that may enhance gut microbial modulation. In contrast, fermented pork fat was primarily associated with lipid content and selective microbial adaptation, with relatively lower dominance of classical probiotic-associated taxa. Based on the integrated assessment of nutritional parameters, microbial diversity indices, and multivariate clustering patterns, fermented soybean appears to represent the most promising probiotic candidate among the three studied foods. Nevertheless, fermented bamboo shoot also demonstrates considerable functional relevance due to its diverse microbial profile and fiber-associated microbial enrichment. The revised discussion thus provides a clearer deduction linking compositional attributes with microbial ecology and probiotic potential.

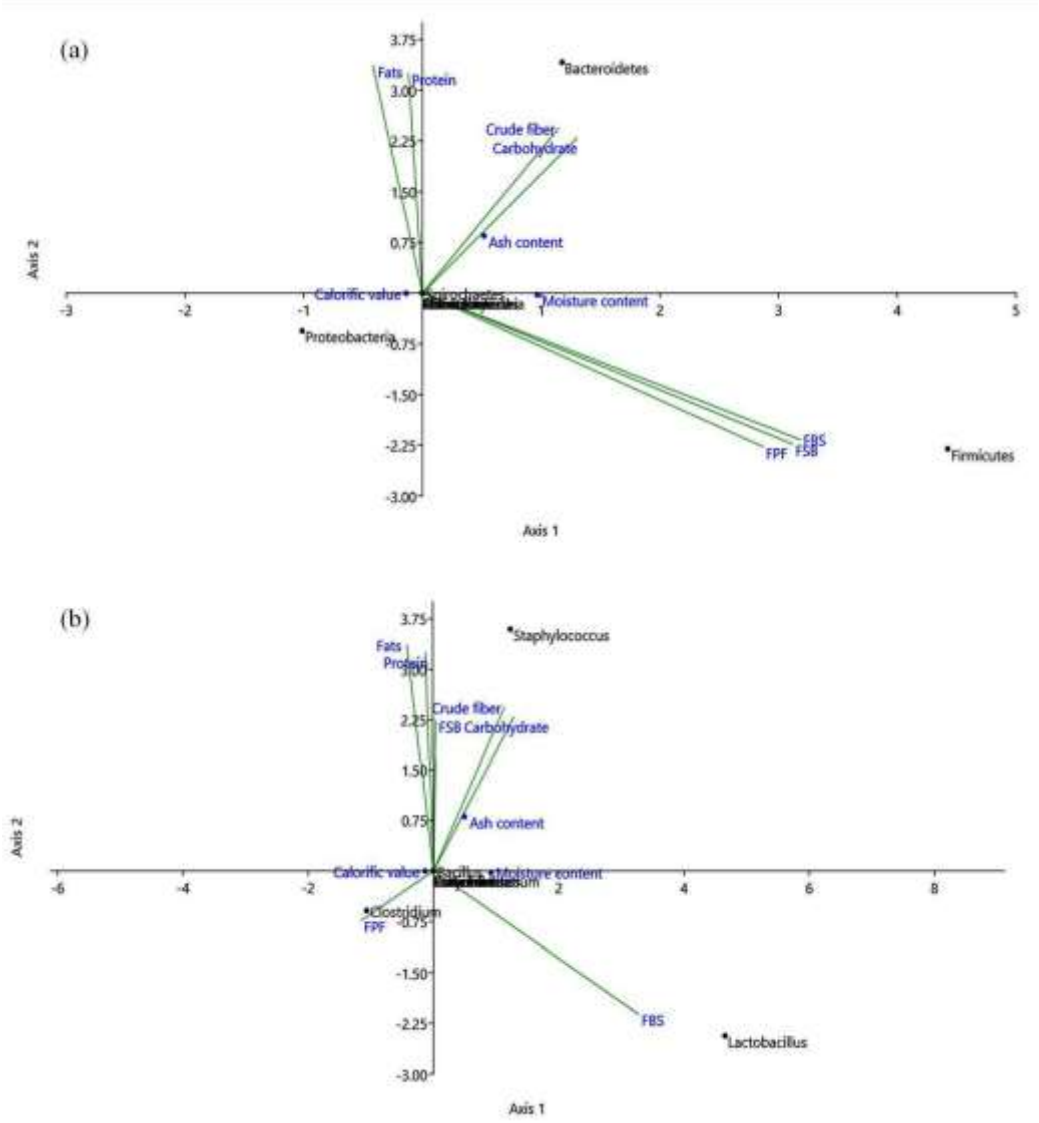


Figure 4.7: Canonical Correspondence Analysis (CCA) triplot showing the relationship between bacterial communities and fermented food samples.

(a) Bacterial phyla

(b) Bacterial genera

FBS – fermented bamboo shoots; FSB – fermented soybeans; FPF – fermented pork fat.

4.6 Preliminary Characterization of the isolates

4.6.1 Morphological and microscopic characterization

We have isolated 32 no.s of bacteria and based on microscopic and biochemical properties 7 bacteria were selected for further experiments (Figure 4.8). The seven bacterial strains (S27, S31, S45 - from fermented soybean; F34, F47 - from fermented pork fats; B50, B57 - from fermented bamboo shoots) showed considerable morphological variability in several phenotypic characters (Table 4.3). Colony colours varied from white (S27, B57), orange (S31, F34), yellow (S45) to creamy white (F47, B50) indicating differential pigment production, possibly due to environmental influences and strain-specific metabolic processes. Colony morphology was diverse, ranging from irregular (S27, S31, B57), circular (S45), spindle (F34) to filamentous (F47, B50), also suggesting diversity. All strains were Gram positive (Figure 4.9), with rods being the most common cell shape (except for S45 and B57, which were cocci), typical of genera like *Bacillus* and *Lactobacillus*. `The majority of the colonies showed convex and umbonate elevations with smooth surfaces, characters commonly observed *Bacillus* spp. isolated from foods and soils (Rajendran et al., 2022). (Rajendran et al., 2022). All isolates except S45 showed motility, which is consistent with environmental rod-shaped bacteria that use flagella for motility to colonise and acquire nutrients (Kumar et al., 2021).

4.6.2 Biochemical Characterization

Biochemical tests revealed conserved and variable features of the isolates. All isolates were positive for catalase (Figure 4.10), suggesting a common aerobic or facultatively anaerobic metabolic pathway, typical of *Bacillus* spp. and catalase-positive cocci (Nguyen et al., 2020). On the other hand, the negative oxidase (Figure 4.16) and urease (Figure 4.14) reactions in all isolates highlight a conserved inability to produce these enzymes, which is consistent with bacteria living in non-host, nutrient-rich environments. Results from the Voges-proskauer test (Figure 4.13) were strain-specific (S31, F34, F47, B50), implying the existence of acetoin fermentation (via butylene glycol pathway) found in Enterobacteriaceae and some *Bacillus* spp. (Singh et al., 2023). However, the positive methyl red test (Figure 4.12) for F47 and B50 only suggests strain-specific use of the mixed acid fermentation pathway.

Citrate utilization (Figure 4.11) was confined to B50, suggesting metabolic adaptation to use citrate as the sole carbon source. Phenylalanine deaminase activity (Figure 4.15) was negative for all isolates, suggesting a lack of conversion of phenylalanine to phenylpyruvic acid. However, the isolates were positive for gelatin liquefaction (Figure 4.17), indicating the presence of extracellular gelatinase enzymes and proteolytic enzymes.

4.6.3. Carbohydrate fermentation profile

The carbohydrate fermentation patterns of the bacterial strains (S27, S31, S45, F34, F47, B50, and B57) showed variation in their carbohydrate utilization, suggesting metabolic diversity between the bacterial strains. The majority of the isolates were positive for cellobiose, lactose, mannitol and trehalose fermentation, indicating the presence of various carbohydrate-degrading enzymes that enable energy generation from multiple carbohydrates. This metabolic diversity is often seen in many beneficial microorganisms from fermented foods and probiotics, where the capacity to ferment different carbohydrates is a trait that promotes survival and adaptation in different environments such as the gut (Hill et al., 2014; Markowiak and Śliżewska, 2017).

There were also variations in the fermentation of sugars. Fructose fermentation was restricted to isolates S31 and F34, whereas sucrose fermentation was detected only in isolate B57, suggesting variation in individual enzyme activity (such as invertase or fructokinase) between the different isolates. Moreover, the fermentation of melibiose and raffinose was found in isolates S27, S31, S45 and F34, suggesting the ability to ferment oligosaccharides through the enzymes α-galactosidase. Conversely, these sugars were not fermented by the isolates F47 and B50, which may have different carbohydrate-degrading enzymes.

Interestingly, some of the isolates fermented pentose sugars (arabinose and xylose). Isolates S27, F34, F47 and B50 were able to ferment these sugars, suggesting the ability to ferment plant polysaccharides.

This is frequently described in probiotic and fermentative bacteria as it aids in the degradation of dietary fibres and other complex carbohydrates, thus stimulating beneficial metabolic activities in the host (Marco et al., 2021).

In general, the results of carbohydrate fermentation showed significant variation between the isolates, indicating differences in metabolic capacities. In our study, isolates F47 and B50 showed relatively wider metabolic profiles, including the ability to metabolize a range of mono and disaccharides, as well as pentose sugars. This trait is desirable for potential probiotic strains as carbohydrate metabolism provides energy for bacterial growth and survival in the gut environment and contributes to the production of valuable metabolites, such as organic acids (Sanders et al., 2019; Marco et al., 2021). On the other hand, S45 and B57 had comparatively restricted carbohydrate metabolism patterns, which potentially reflect narrower metabolic niche.

Overall, the variability in carbohydrate metabolism patterns reflected the metabolic diversity of the strains and provided initial evidence for the potential probiotic qualities of F47 and B50, as their metabolic versatility may contribute to their adaptation to the gut environment and beneficial metabolic activities.

Table 4.3: Morphological and biochemical characterization of the isolates

"+": Positive reaction, "-'': Negative reaction

Test parameters	**S27**	**S31**	**S45**	**F34**	**F47**	**B50**	**B57**
Morphological Characterization							
Color	White	Orange	Yellow	Orange	Creamy white	Creamy white	White
Shape	Irregular	Irregular	Circular	Spindle	Filamentous	Filamentous	Irregular
Size	Moderate	Large	Moderate	Moderate	Large	Large	Small
Surface appearence	Smooth	Smooth	Smooth	Smooth	Rough	Rough	Smooth
Margin/Edge	Undulate	Undulate	Entire	Lobate	Lobate	Lobate	Lobate
Elevation	Convex	Umbonate	Raised	Umbonate	Umbonate	Umbonate	Raised
Opacity	Opaque	Opaque	Opaque	Opaque	Translucent	Translucent	Opaque
Consistency/ Texture	Smooth	Dry	Smooth	Smooth	Mucoid	Mucoid	Smooth
Motility	Motile	Motile	Nonmotile	Nonmotile	Motile	Motile	Motile
Gram reaction	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Shape	Rod	Rod	Cocci	Rod	Rod	Rod	Cocci
Biochemical Characterization							
Methyl red test	-ve	-ve	-ve	-ve	+ve	+ve	-ve
Voges-praskauer test	-ve	+ve	-ve	-ve	+ve	+ve	-ve
Citrate utilization test	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Catalase test	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Urease test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Phenylalanine deaminase test	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Gelatin liquefaction test	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Carbohydrate fermentataion							
Cellubiose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Lactose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Manitol	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Melibiose	+ve	+ve	+ve	+ve	-ve	-ve	+ve
Raffinose	+ve	+ve	+ve	+ve	-ve	-ve	+ve
Trehalose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Fructose	-ve	+ve	-ve	+ve	-ve	-ve	-ve
Galactose	+ve	-ve	+ve	+ve	-ve	-ve	+ve
Arabinose	+ve	-ve	+ve	+ve	+ve	+ve	+ve
Sorbitol	-ve	-ve	+ve	+ve	-ve	-ve	+ve
Sucrose	+ve	-ve	-ve	-ve	-ve	-ve	+ve
Maltose	-ve	-ve	-ve	+ve	+ve	-ve	-ve
Mannose	+ve	-ve	+ve	-ve	-ve	-ve	+ve
Xylose	+ve	-ve	-ve	+ve	+ve	+ve	+ve

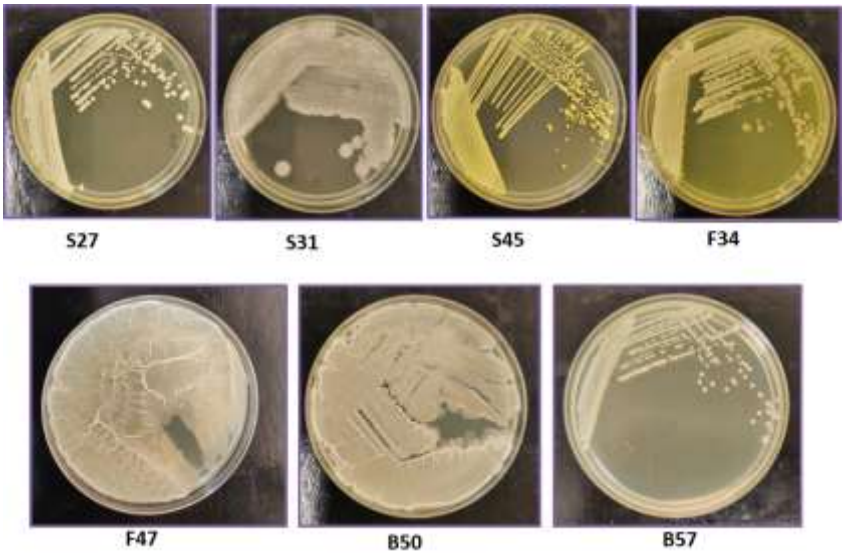


Figure 4.8: Morphological Characterization of the isolates

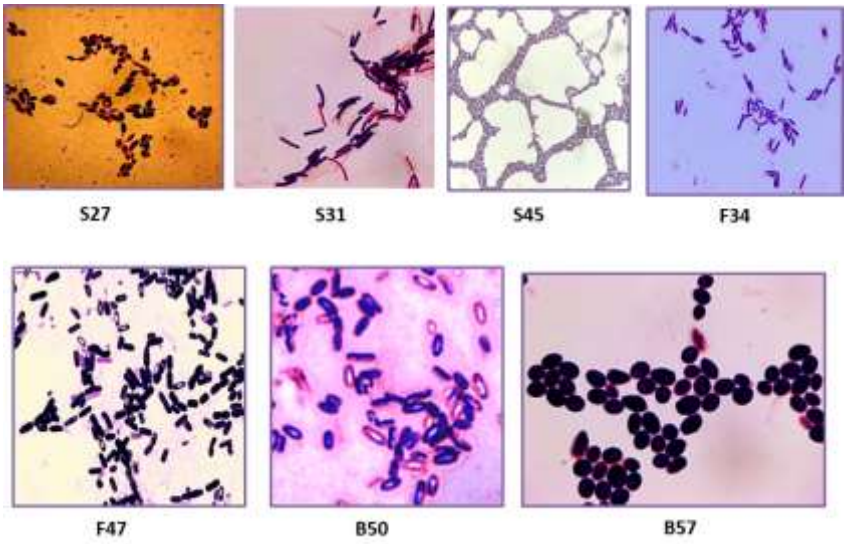


Figure 4.9: Microscopic observation of the isolates after gram staining

Figure 4.10: Catalase test of the isolates
("+ve": Production of bubbles; "-ve": No bubbles)

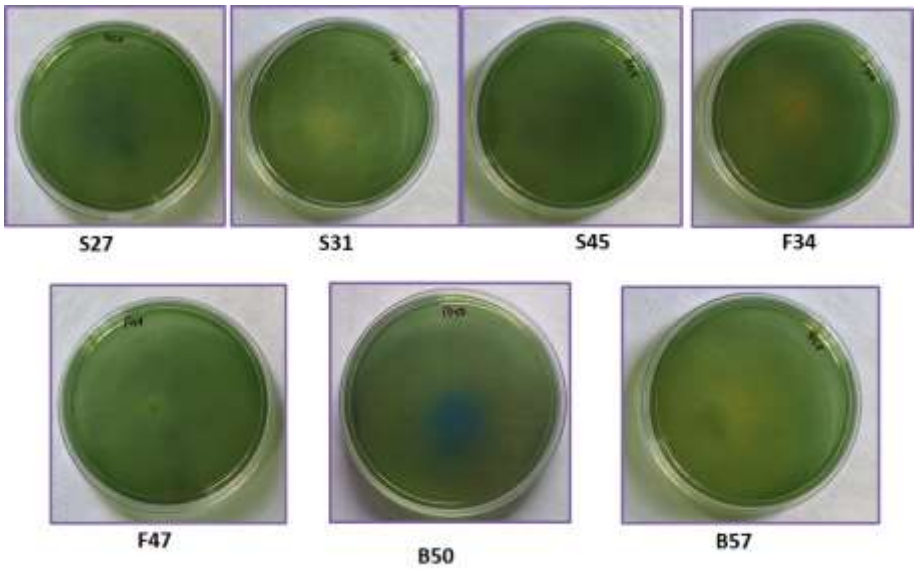


Figure 4.11: Citrate Utilization test of the isolates
("+ve": Blue colour production; "-ve": No color production)

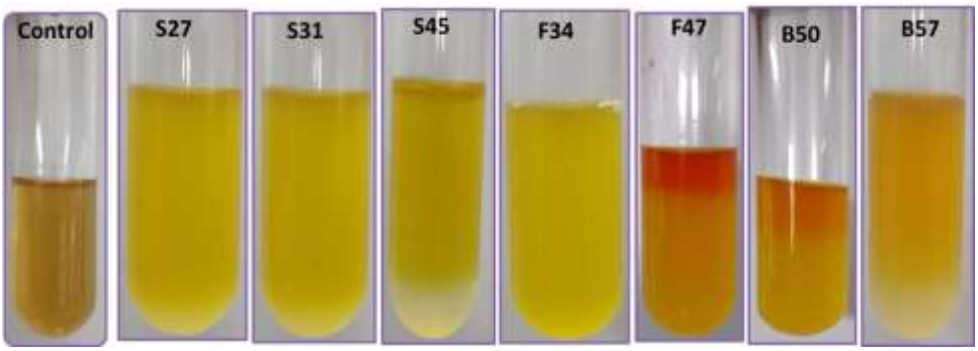


Figure 4.12: Methyl red test of the isolates
("+ve": Change of colour; "-ve": No color change)

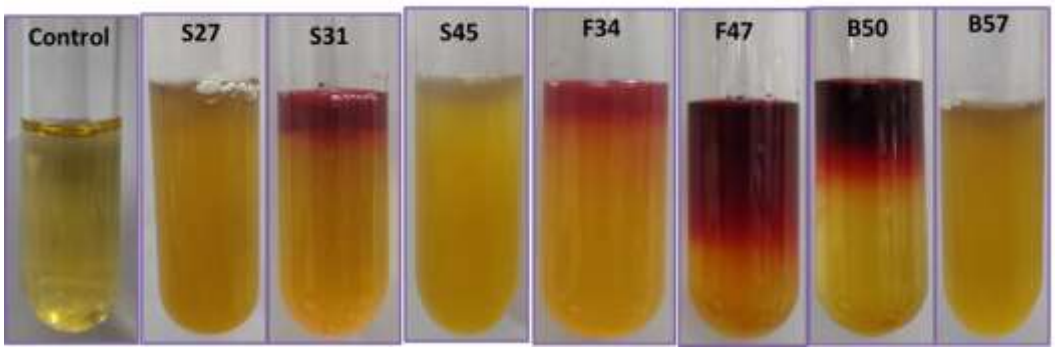


Figure 4.13: Voges-Praskauer Test of the isolates
("+ve": Change of colour; "-ve": No color change)

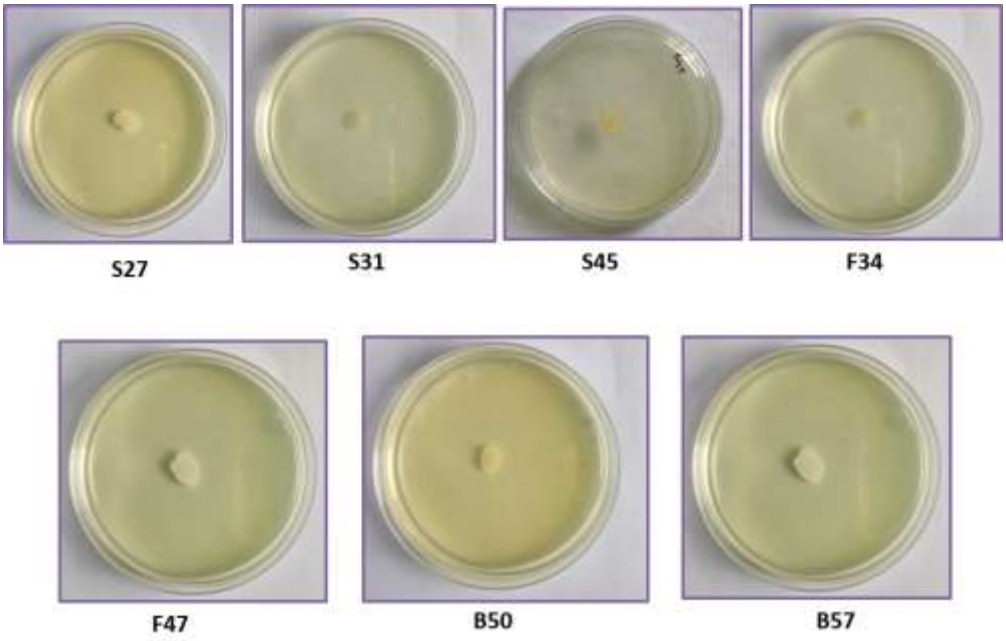


Figure 4.14: Urease test of the isolates
("+ve": Change of colour; "-ve": No color)

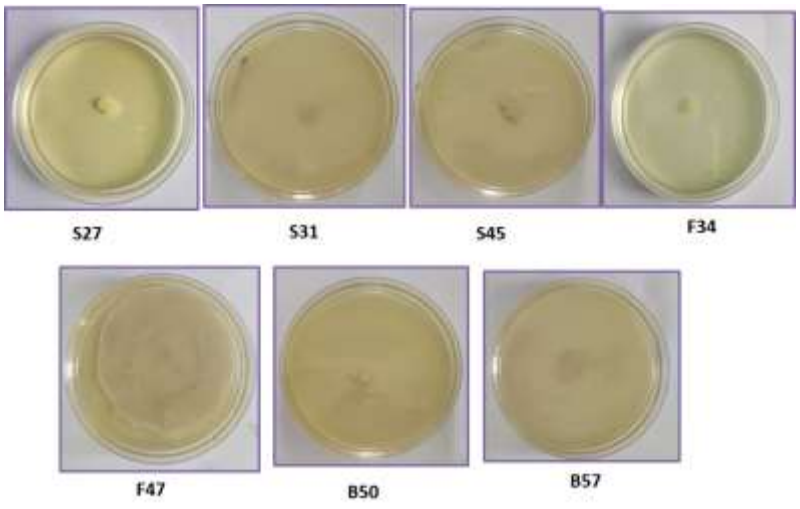


Figure 4.15: Phenylalnine deaminase test of the isolates
("+ve": Change of colour; "-ve": No color change)

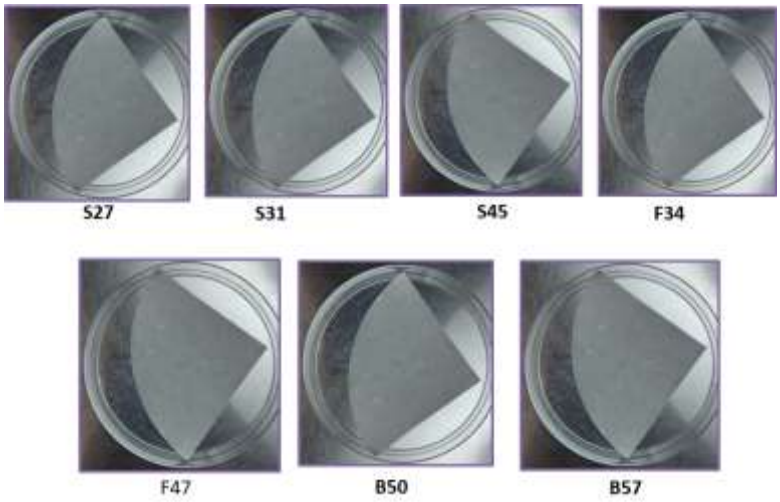


Figure 4.16: Oxidase test of the isolates
("+ve": Change of colour; "-ve": No color change)

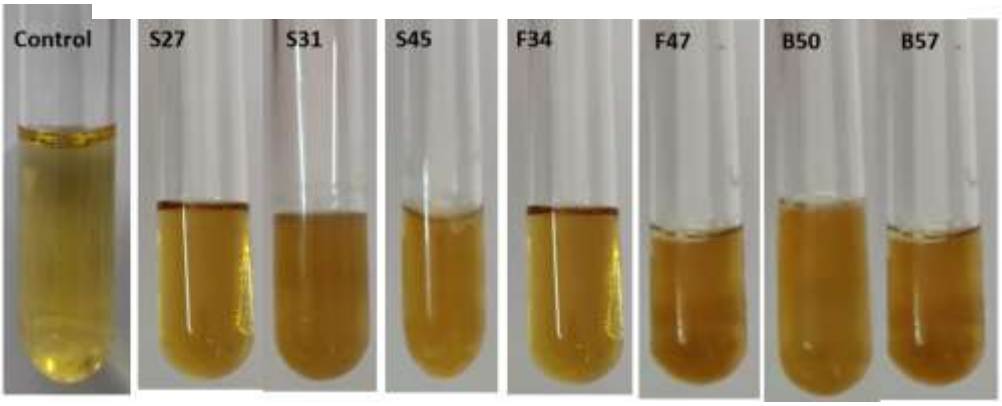


Figure 4.17: Gelatin liquefaction test
("+ve": Partial or Total Liquefaction; "-ve": Complete solidification)

4.7 Molecular Identification of 16S rRNA gene Sequencing and phylogenetic Analysis

4.7.1 DNA Isolation of the isolates

The Genomic DNA was isolated from the potential isolates and tested for their quality & quantity on 0.8% of agarose gel. A distinct genomic DNA band was observed upon ultraviolet (UV) illumination and subsequently documented using the Bio-Rad Gel Doc XR+ imaging system (Hercules, CA, USA) (Figure 41.18). DNA extracted from all bacterial isolates was found to be of high quality, showing intact

structure without any detectable fragmentation, confirming its appropriateness for further molecular applications

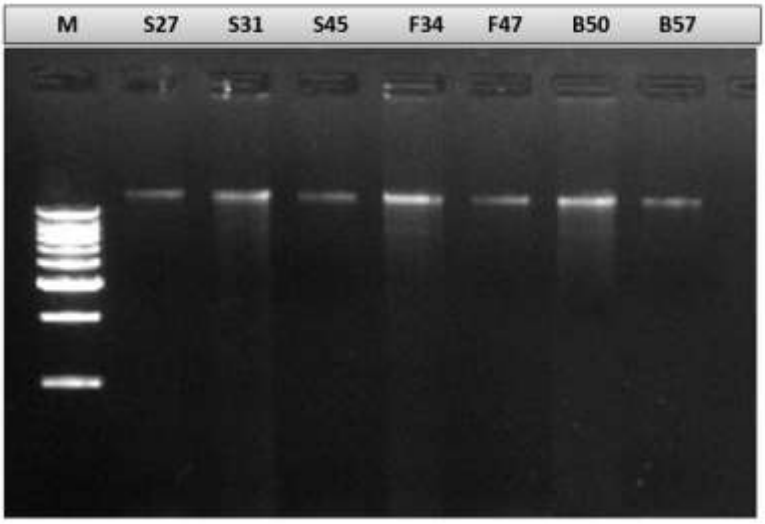


Figure 4.18: The genomic DNA bands of the bacterial isolates were visualized under ultraviolet (UV) illumination and documented using the Bio-Rad Gel Doc XR+ imaging system.

4.7.2 Amplification of 16S rRNA Gene

The 16S rRNA gene of all validated bacterial isolates was amplified using a thermal cycler (Applied Biosystems). Each polymerase chain reaction (PCR) mixture included 50 ng of genomic DNA as the template, along with universal primers specific to the 16S rRNA gene (forward and reverse). Amplified products were resolved through electrophoresis on a 1.2% agarose gel, and 3 kb DNA ladder was utilized as a molecular size standard to determine the approximate length of the amplified PCR products. Amplified PCR product was sent for sequencing to Eurofins Pvt. Ltd. Bangalore, India (Figure 4.19). The obtained sequences were compared with reference sequences available in the NCBI Information genomic database using the BLASTN search tool to determine the percentage of sequence similarity. Reference strains showing the highest similarity scores were retrieved from the NCBI database for subsequent comparative and phylogenetic analyses.

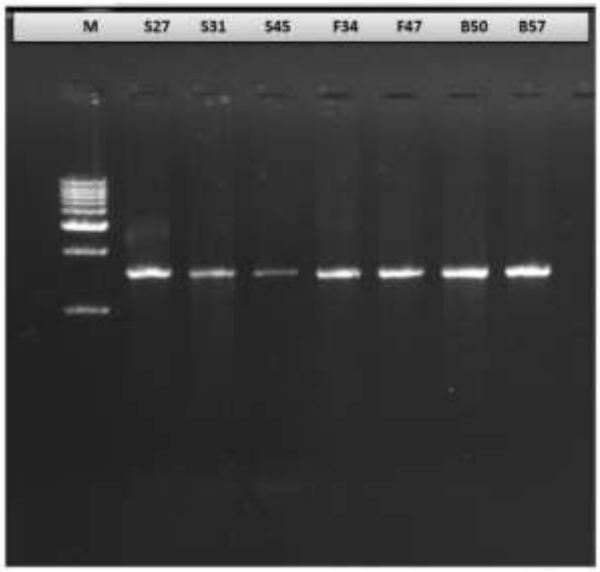


Figure 4.19: Agarose gel electrophoresis showing the PCR-amplified 16S rRNA gene fragents obtained from the bacterial isolates.

Table 4.4: Molecular Identification of 16S rRNA gene sequencing

Strain Name	Closet Homology hit	Strain	Accession	Pairwise Similarity (%)
S27	*Alkalihalobacillus hemicentroti*	JSM076093(T)	HM460885	97.76%
S31	*Bacillus sp.*	CR-502	AY603658	99.83%
S45	*Kocuria rhizophila*	TA68(T)	RCOM01000116	99.98%
F34	*Bacillus safensis subsp.*	FO-36b(T)	ASJD01000027	99.97%
F47	*Bacillus sp.*	CR-502(T)	AY603658	99.77%
B50	*Bacillus velezensis*	NCIB 3610(T	ABQL01000001	99.87%
B57	*Bacillus sp.*	CR-502(T)	AY603658	98.57%

4.8 In Vitro Testing for Probiotic Potential of the Isolates:

4.8.1 Tolerance to Low pH conditions

The acid tolerance of bacterial isolates was determined by measuring their optical densities (OD600) in acidic environments (pH 2-4) (Figure 4.20). Low pH growth is a good indicator of acid tolerance, an important characteristic for survival in challenging environments like the gut or acidified fermentations Of the seven bacterial isolates (S27, S31, S45, F34, F47, B50, B57) screened, the isolates F47 and B50 showed the best growth under acidic conditions, suggesting high acid tolerance. Isolate F47 showed consistent and improved growth at all acidic pH values. The significant OD600 at pH 2 (0.16) indicates the isolate has survival strategies and sufficient acid tolerance to start growth under strong acidic conditions. The gradual rise in optical density from pH 2 to pH 4 suggests a strong ability to adapt to an increasing acid stress, and demonstrates acid resistance and tolerance. While B50 showed limited growth at pH 2, it had the highest OD600 at pH 3 and 4 of all the isolates. This behaviour might indicate a reduced tolerance to acid shock at pH 2, but a quick adaptation and strong growth at moderately lower acidity. B50's growth at pH 3 and 4 stresses a potential use of the isolate in lower acidic conditions.

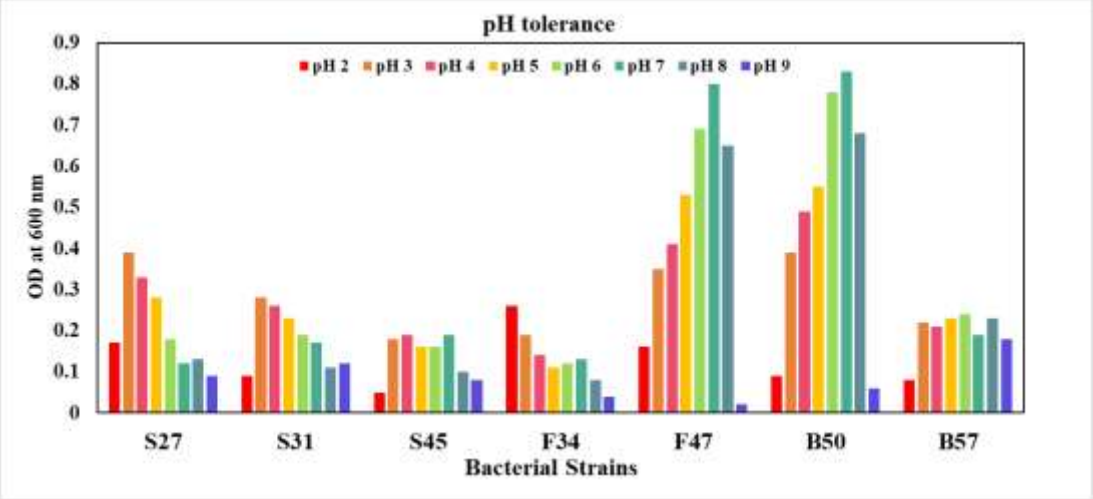


Figure 4.20: Tolerance to low pH of the isolates

These isolates should be further investigated for use in processes or environments where acid tolerance is a key functional trait, such as probiotics for the human gut or fermentations in acidic foods. Our results are in agreement with recent research on lactic acid bacteria (LAB) from traditional fermented foods. For example, Woldemariam et al. (2023) observed that LAB from Ethiopian fermented foods had greater than 75% survival at pH 2.5 but less than 50% survival at pH 2, with Afkari et al. (2020) reporting that the Indonesian isolates survived pH 2.5 stress but exhibited no growth at pH 2. Moreover, Kim et al. (2020) reported that LAB isolated from Korean kimchi and fermented fish had moderate resistance at pH 2.5 and were sensitive at pH 2. Against these standards, isolate F47 showed greater acid tolerance than many traditional LAB strains, as it was able to grow at extremely low pH, while isolate B50 showed similar acid recovery as previous studies of moderately acid-tolerant LAB. These characteristics indicate that F47 and B50 have the potential to be used in probiotic formulations or fermentations of acidified foods

4.8.2 Tolerance to bile salt

Bile salts, with their detergent-like nature, are highly toxic to microbes by disrupting their cell membranes and can present a daunting challenge in the gastrointestinal tract. As such, bile salt tolerance is a key characteristic of probiotic bacteria. Among the eight strains tested (S27, S31, S45, F34, F47, B50, B57), varied levels of tolerance were seen, with some strains showing an impressive level of resistance at high bile salt concentrations (Figure 4.21).The evaluation of bile salt tolerance in a range of bacterial strains, as shown in the figure shows distinct patterns of resistance to various bile salt concentrations (0.2% to 1.6%).

Isolate F47 showed a high level of bile salt tolerance, with growth observed up to around 1.0 % (w/v) bile salts. By contrast, isolate B50 displayed medium tolerance, with growth at lower concentrations (0.3-0.5 %) and significant decrease in growth at concentrations greater than 0.8 % bile. These results are in line with those of Hu et al. (2022), who found that lactic acid bacteria (LAB) strains isolated from kefir had a high bile tolerance, with some strains showing less than 0.7 log CFU/mL reduction at 0.3 % bile salt. Likewise, Grosu-Tudor and Zamfir (2012) reported that the majority

of LAB isolated from fermented vegetables were able to grow at 0.3 % bile concentration, but growth was significantly reduced at 0.4-0.5 % bile. Moreover, in a screening assessment of *Lactobacillus* strains, only about 13 % of the strains were found to be able to grow at more than 90 % growth at 1.2 % bile salt (Hu et al., 2022; Kodba et al., 2024). Compared to these established standards, F47 shows high bile salt tolerance, with improved physiological adaptive traits to bile exposure. In contrast, moderate tolerance of B50 is consistent with those of probiotic LAB strains derived from fermented foods. In conclusion, the findings demonstrate the potentials of both F47 and B50 as probiotic strains and their applications in fermentation processes in a bile-rich (or gastrointestinal) environment.

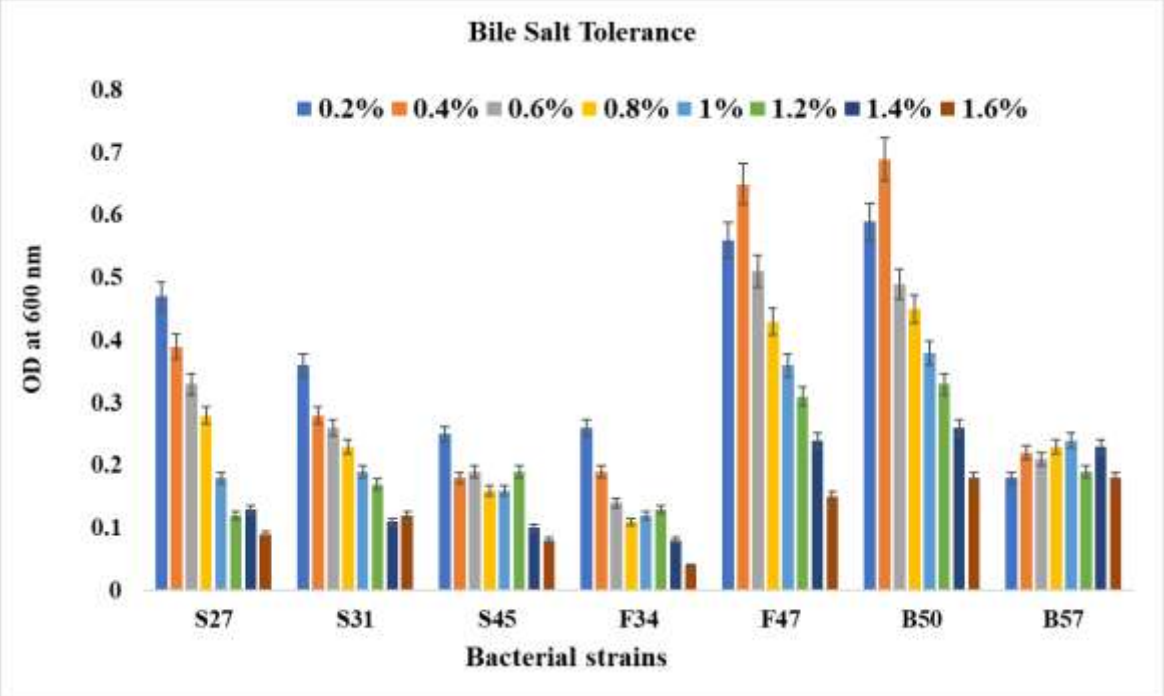


Figure 4.21: Tolerance to bile salt of the isolates

4.8.3 Autoaggregation activity of the isolates

The autoaggregation ability of bacterial strains (S27, SS31, S45, F34, F47, B50 and B57) was evaluated between 1-5h (Figure 4.22). Most strains showed a temporal rise in aggregation levels with considerable strain-specific differences. Strains F47,

B50 and S31 displayed high auto-aggregation values. F47 peaked at 40% after 5h, indicating significant self-adhesion properties, which can facilitate biofilm development and adherence to mucosal surfaces. Likewise, B50 consistently increased from 9.43% (1h) to 37.76% (5h), suggesting strong and reliable auto-aggregation. Similarly, S31 reached 19.29% at 5h, suggesting a good probiotic potential. B57 showed moderate aggregation activity, with levels increasing from 12.52% (1h) to 15.62% (4h) and then decreasing to 9.72% (5h). This indicates initial aggregation stability with some disaggregation. By contrast, other isolates (S27, S45 and F34) showed moderate and unstable aggregation.

In particular, S45 increased from 4.56% (1h) to -1.49% (5h) suggesting no aggregation and possible dispersion or death. F34 also had a minor peak at 6.28% (4h) with minimal aggregation ability. Such strains may have no structures to facilitate autoaggregation, such as fimbriae or exopolysaccharides.

Aggregation is one of the probiotic characteristics that affects mucosal attachment, biofilm formation and antagonism against pathogens. Cell surface hydrophobicity and extracellular polymeric substances (EPS) are related to auto-aggregation ability, promoting cell aggregation. The strong autoaggregation ability of F47 and B50 might be associated with high cell surface hydrophobicity or high EPS production, and can be considered for probiotic development. In addition, the persistent aggregation observed in B50 isolate is a key process for biofilm and gut colonization. The autoaggregation behaviour of different strains suggests that probiotic selection should be at strain level. Highly aggregating isolates (F47 and B50) could be used as potential candidates for studying colonization and pathogen exclusion

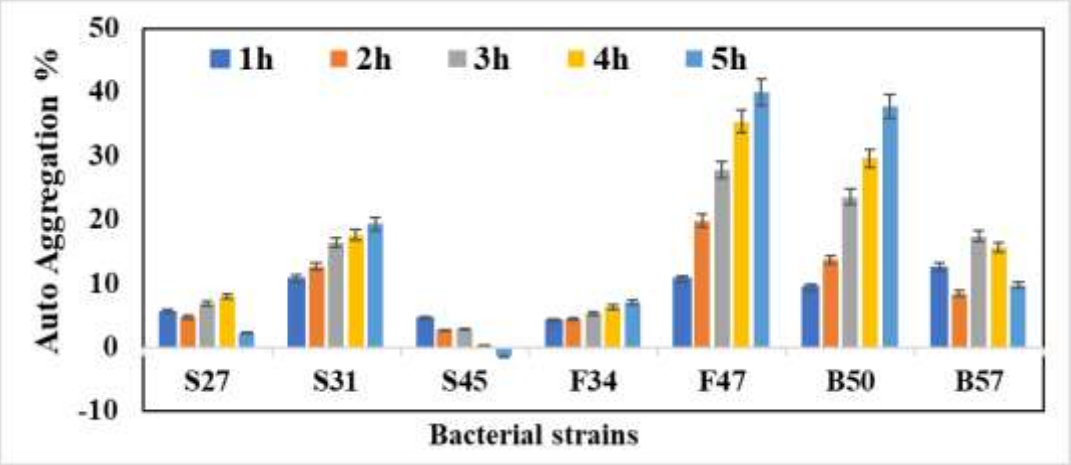


Fig 4.22: Autoaggregation activity of the isolates

This is in line with recent findings on lactic acid bacteria (LAB) from fermented foods. For instance, Zannini et al. (2023) reported that LAB from fermented cereal foods had percentages of autoaggregation ranging from 30% to 45% at 5 hours, which was in line with the isolate F47 in the present study.

Similarly, Alemayehu et al. (2021) found that probiotic LAB isolated from fermented beverages in Ethiopia showed increases in autoaggregation over time, with percentages ranging from 35% to 40% at 4 to 6 hours, confirming the low aggregation percentage observed for B50 in this study. Similarly, Deepika et al. (2020) reported that *Lactobacillus plantarum* strains derived from pickled vegetables exhibited time-dependent aggregation, with percentages of 28-43% after 5 hours, supporting the aggregation behavior of F47 and B50 in this study. These observations collectively verify that F47 and B50 possess aggregation potential in the high range of previously reported probiotic strains. The gradual rise in autoaggregation over the time period (particularly for B50) is not only indicative of adhesion but also potentially competitive and resilient nature in terms of colonization (such as intestinal epithelium). Their high aggregation capacities could also prove useful in the competition with pathogens via co-aggregation or biofilm barrier mechanisms. Hence, based on the aggregation patterns and literature knowledge, F47 and B50 strains are promising candidates for further probiotic functional trials,

especially in surface adherence, mucosal colonization, or competitive exclusion of gut pathogens related applications,

mucosal colonization, or competitive exclusion of gut pathogens.

4.8.4 Antimicrobial activity of the isolates

The antimicrobial potential of the isolates (S27, S31, S45, F34, F47, B50, B57) was assessed against Gram-positive, Gram-negative bacteria and fungal pathogens using the well diffusion method (Figure 4.23). F47 and B50 were the only isolates that showed a considerable zone of inhibition and hence exhibited antimicrobial activity. The remaining isolates (S27, S31, S45, F34 and B57) did not exhibit any zones of inhibition. F47 had a wide spectrum of activity, with zones of inhibition against *Staphylococcus aureus* (15 mm), *Klebsiella pneumoniae* (22 mm), *Bacillus subtilis* (26 mm), *Salmonella typhimurium* (14 mm) and *Escherichia coli* (23 mm). B50 also showed significant inhibition against the same organisms with slightly lower zones: *S. aureus* (6 mm), *K. pneumoniae* (18 mm), *B. subtilis* (24 mm), *S. typhimurium* (12 mm), and *E. coli* (22 mm) (Table 4.5). Such results suggest that F47 and B50 release active metabolites that are active against Gram-negative enteric pathogens (K. pneumoniae, E. coli, S. typhimurium) and Gram-positive B. subtilis. F47 was more effective than B50 against all susceptible strains, implying greater production or effectiveness of bioactive compounds. The absence of activity against fungi (*C. albicans, S. cerevisiae*) and Gram-positive bacteria (*M. luteus, E. faecalis, S. pneumoniae*) suggests either a limited spectrum of activity or resistance in the unaffected organisms.

Overall, isolates F47 and B50 show potential as sources of antimicrobial agents, with F47 being the more potent candidate.

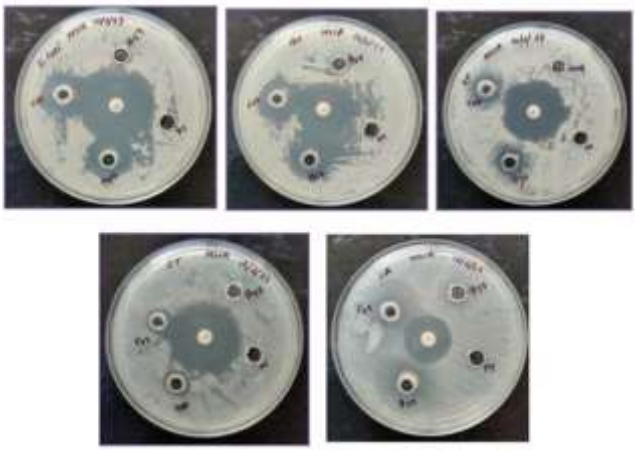


Figure: 4.23: Antimicrobial activity of the isolates against MDR pathogens

Table 4.5: Antimicrobial activity of the isolates with zone of inhibition

Pathogen	**Strain name**	**S27**	**S31**	**S45**	**F34**	**F47**	**B50**	**B57**
		Zone of Inhibition						
Staphylococcus aureus	ATCC BAA-44	-	-	-		15mm	6 mm	-
Enterococcus faecalis	ATCC-51575	-	-	-				-
Klebsiellia pneumoniae	ATCC-BAA-2814	-	-	-		22 mm	18 mm	-
Candida albicans	ATCC-64124	-	-	-				-
Streptococcus pneumoniae	ATCC-10015	-	-	-				-
Pseudomonas aeruginosa	ATCC-10145	-	-	-				-
Micrococcus luteus	ATCC-10240	-	-	-				-
Bacillus subtilis	ATCC-11774	-	-	-		26 mm	24 mm	

Saccharomyces cerevisiae	ATCC-2601	-	-	-				
Salmonella typhimurium	ATCC-51812	-	-	-		14 mm	12 mm	
Escherichia coli	ATCC-10536	-	-	-		23 mm	22 mm	

The activity against enteric pathogens is promising for possible applications in intestinal infections or food preservation. The results for strains F47 and B50 are consistent with recent findings of lactic acid bacteria (LAB) and *Bacillus* isolated from fermented foods. For instance, Ghosh et al. (2019) reported that *Bacillus subtilis* isolated from fermented soybean showed zones of inhibition of 16-24 mm for *Escherichia coli* and *Klebsiella pneumoniae*, which is similar to the inhibitory activity of F47 and B50 in this work. Similarly, Tenea (2020) described the potential of LAB derived from traditional fermented cassava in Ecuador against tested pathogens with inhibition zones of 20-24 mm against *E. coli* and 12-18 mm against *Staphylococcus aureus*, which is consistent with the results from the present study, especially those related to F47. Similarly, Abriouel et al. (2006) reported that LAB strains isolated from fermented olives produced antimicrobial peptides (bacteriocins) which were active against a range of Gram-negative bacteria including *Salmonella* and *Klebsiella*. This highlights that the isolate F47 possesses broad-spectrum antimicrobial activity, given that it showed greater antimicrobial activity than that of B50 in both Gram-negative and Gram-positive organisms. It is conceivable that the greater effectiveness of F47 may be related to increased capacity for the synthesis of antimicrobial compounds such as the production of bacteriocins, organic acids (lactic acid, acetic acid) or volatile antimicrobial compounds. Overall, the results signify that the two isolates have significant antimicrobial activity, with F47 being more potent. Such characteristics suggest their potential use for the control of enteric pathogens in food or in the gut, thus providing important insights for food safety and probiotic therapy.

4.8.5 Antibiotic Susceptibility Test

Antibiotic susceptibility testing for the seven bacterial isolates (S27, S31, S45, F34, F47, B50 and B57) shows a wide range of strain specific responses to 23 different antibiotics (Figure 4.24). This reveals considerable variation in resistance (R) and sensitivity (S), which is an important consideration for the safety of potential probiotic strains. In particular, the highest number of antibiotics (Gentamicin, Streptomycin, Ampicillin, Azithromycine, Tetracycline, Ciproflaxacin, Levoflaxacin etc.) commonly used in the clinic were found to be effective against S31, F34, F47 and B50, which are thus promising candidates for probiotic development. These strains are therefore, promising probiotic candidates as they are unlikely to carry transferable genes for resistance (EFSA, 2018) (Figure 4.25).

Fluoroquinolones, particularly ciprofloxacin and levofloxacin, were effective against most isolates, consistent with prior studies in which lactic acid bacteria from fermented dairy and non-dairy matrices exhibited similar susceptibility (Somashekaraiah et al., 2023; Sharma et al., 2022). Ciprofloxacin sensitivity was observed in 4 out of 7 isolates, reinforcing its diagnostic utility in initial probiotic screening.

In contrast, widespread resistance was noted against polymyxin B and fluconazole, suggesting potential intrinsic resistance mechanisms. Intrinsic resistance, particularly in Gram-positive bacteria like *Lactobacillus* and *Pediococcus*, is often non-transferable and therefore not a safety concern in probiotic evaluation (Salvetti et al., 2016). However, resistance to β-lactams (e.g., ampicillin and methicillin) in some isolates, including S27 and B57, may warrant molecular confirmation to determine if the genes are chromosomally encoded or mobile (Figure 4.26).

S45 emerged as the most antibiotic-sensitive isolate, showing susceptibility to nearly all tested drugs except for resistance to methicillin and fluconazole. Conversely, B57 showed the most resistance, including to multiple antibiotic classes, which may disqualify it from probiotic candidacy unless further genetic analysis proves the resistance determinants are non-transferable.

According to FAO/WHO guidelines (2002), probiotic candidates must not carry transferable resistance genes, particularly to antibiotics critical for human health. The isolates in this study especially S31 and F34 largely comply with these requirements. Their sensitivity to fluoroquinolones, macrolides, aminoglycosides, and tetracyclines is a strong indicator of safety and supports their consideration for functional probiotic assessment.

In a recent study, Ouwehand et al. (2023) found that *Lactiplantibacillus plantarum* strains isolated from kimchi and fermented cereals displayed similar profiles, with high sensitivity to ciprofloxacin and erythromycin but moderate resistance to β-lactams

Antibiotics	S27	S31	S45	F34	F47	B50	B57
Polymyxin B	R	S	S	R	R	R	S
Fluconazole	R	R	S	S	R	R	S
Streptomycin	R	S	S	R	S	S	R
Ampicilin	R	S	S	R	R	R	R
Gentamycin	R	S	S	R	S	S	R
Kanamycin	R	S	S	S	S	R	R
Sparfloxacin	R	S	S	S	S	S	R
Azithromycin	R	S	S	R	S	S	R
Chloroamphenicol	R	S	S	R	S	S	R
Neomycin	R	S	S	R	S	S	S
Tetracycline	R	S	S	S	S	S	R
Miconazole	S	R	S	S	R	R	S
Amphotericin B	S	R	R	R	R	R	S
Norfloxacin	R	S	R	S	R	S	R
Itraconazole	S	S	S	S	S	S	S
Ciprofloxacin	R	S	S	R	S	S	R
Methicilin	R	S	R	R	R	S	R
Levofloxacin	R	S	S	S	S	S	R
Trimethoprim	R	S	S	S	S	S	R
Cefixime	R	R	S	R	S	S	R
Nystatin	S	R	R	S	R	R	S
Nalidixic acid	R	S	R	S	S	S	R
Ketoconazole	S	R	S	S	R	R	S

Figure 4.24: Antibiotic susceptibility test of the isolates

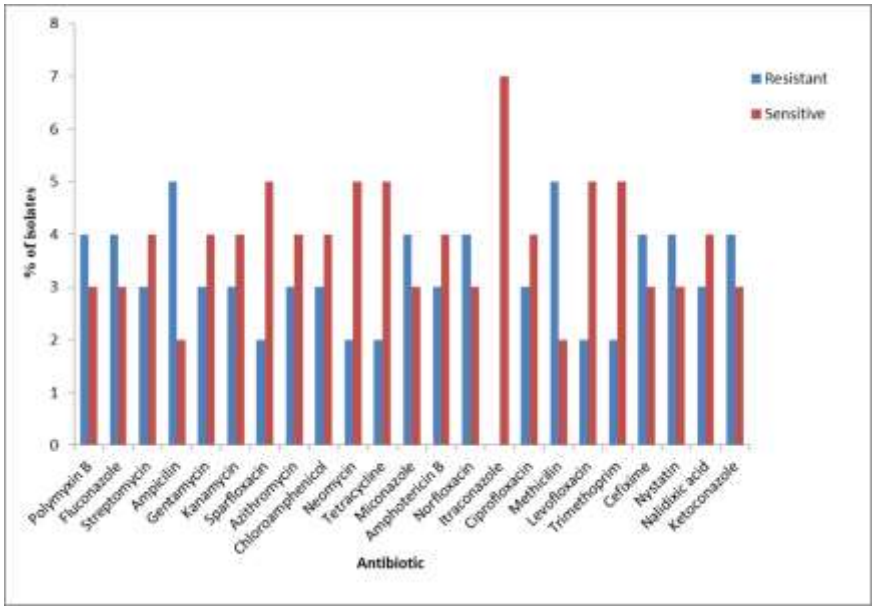


Figure 4.25: Antibiotic resistance and sensitivity patterns of bacterial isolates.
The highest resistance was observed for ampicillin (50%) and methicillin (50%), while the highest sensitivity was recorded for itraconazole (70%).

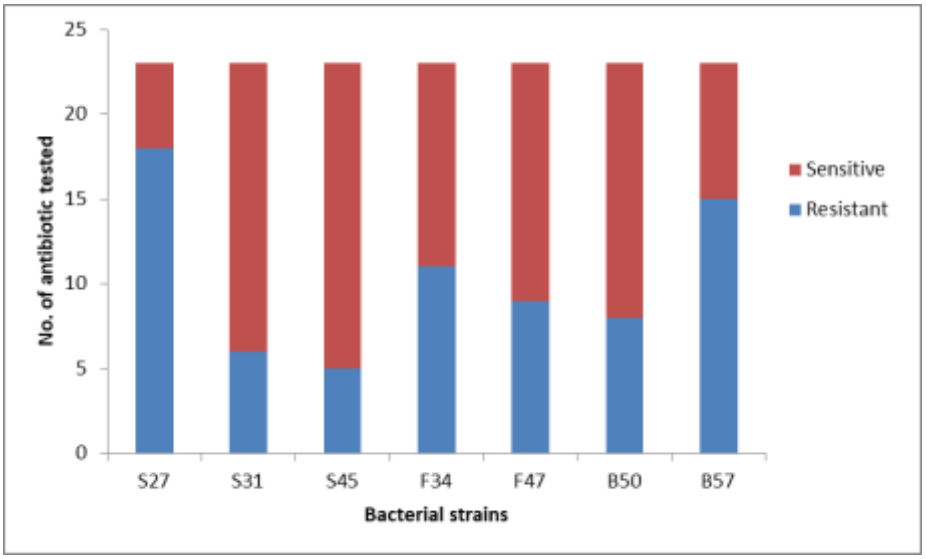


Figure 4.26: Antibiotic susceptibility profile of selected bacterial isolates.
S27, F34, and B57 showed resistance to 18, 11, and 15 antibiotics, respectively, while S31, S45, and B50 exhibited sensitivity to 17, 18, and 15 antibiotics, respectively.

4.8.6 Identification of the potent isolates

In most of the probiotic parametrs two isolates (F47 and B50) were found to be most efficient. Therefore these two strains were identified using 16S rRNA gene and phylogenetic tree were constructed. Both cultures were identified by the 16S rRNA sequencing method. The strains F47 and B50 were 99.40% and 99.53% similar to *Bacillus tequilensis*_OR899234 and *Bacillus velezensis*_OR899244, respectively.

4.8.7 Evolutionary relationships of the taxa

The evolutionary distance was measured by the Kimura-2 parameter model and the phylogenetic tree was inferred by using the Neighbor-Joining method. The tree is also drawn to scale (the length of the branches in the same units as those of the evolutionary distances used to determine the phylogenetic tree). Next to the branches are the percentages of the trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates).

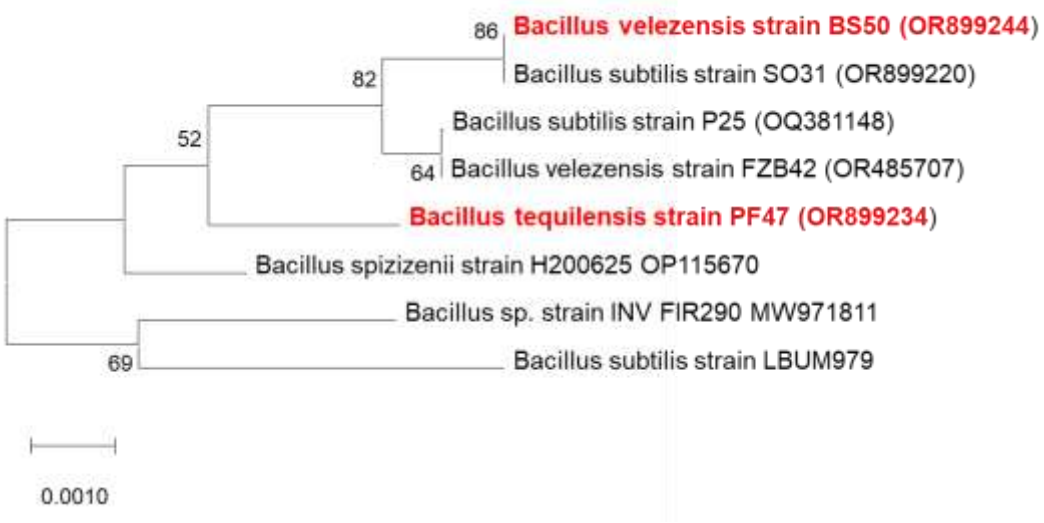


Figure 4.27: Phylogenetic tree for F47 and B50 constructed using Neighbour joining method using Kimura-2 parameter model

4.8.8 Screening for biosurfactant production

Both the isolates F47, and B50 tested positive for biosurfactant production using the oil displacement method. The formation of a clear zone of oil displacement around the point of culture supernatant application confirmed the presence of extracellular biosurfactant molecules produced by both isolates. The visible oil displacement

indicates that both isolates secrete compounds capable of disrupting the hydrophobic oil layer, a hallmark of biosurfactant activity.

This finding is supported by other studies on biosurfactant-producing fermented food bacteria. For example, Bacillus subtilis strains from traditional Korean fermented soybean paste (doenjang) showed large oil displacement zones, which were positively related to biosurfactant production (Lee et al., 2018). Likewise, strains of Lactobacillus plantarum isolated from fermented dairy products in India displayed oil displacement zones, indicating the production of biosurfactants (Joshi et al., 2016).

The presence of biosurfactant activity in isolates F47 and B50 (Figure 4.28) indicates the production of surface-active substances such as lipopeptides, glycolipids or phospholipids. Such biomolecules not only play a role in emulsification and surface tension reduction but are also linked to anti-microbial and anti-adhesive effects, and biofilm regulation which are important in probiotic functionality and industrial processes. Overall, the oil displacement results support the candidacy of both isolates as biosurfactant producers. This property enhances their potential application in food biotechnology, bioremediation, and as functional probiotics, particularly in formulations requiring emulsification or anti-pathogenic surface activity.

Figure 4.28: Oil Displacement assay of both the isolates (F47 and B50)

4.8.9 Characterization of the extracted biosurfactant

The biosurfactant product was confirmed by UHPLC-ESI/MS. In total, in both the bacterial extract of B5O And F47 different surfactin-like isoforms (C^{13}-C^{15}) of lipopeptides, at *m/z* 1036.686 with retention time (Rt 80.77), I biosurfactant-like compound N,N-Diethyldodecanamide at *m/z* 256.263 with retention time (Rt 76.63) was detected by LC/MS as shown in the chromatogram. UHPLC-ESI/MS analysis of the extract (F47 and B50) revealed several bioactive compounds with potential biosurfactant properties. Among these, key compounds were identified based on molecular characteristics and established surfactant activity in the literature.

In the extract of F47 One of the most prominent biosurfactant-related compounds identified was CDP-2, 3-bis-(O-phytanyl)-sn-glycerol, with a retention time of 82.06 min and a molecular formula of $C_{52}H_{101}N_3O_{13}P_2$. This complex phospholipid-like molecule resembles archaetidylglycerol derivatives, known for amphiphilic behavior, which is critical in emulsification and surface tension reduction. Its large molecular weight (1038.3 g/mol) and large area under the curve also verify its presence and importance in the sample (Koga et al. 2000) (Figure 4.29).

N, N-Diethyldodecanamide, with a retention time of 76.63 min, has a molecular formula of $C_{16}H_{33}NO$ and a molecular weight of 255.44 g/mol. Amides of fatty acids are known to have surfactant properties and are also used as part of commercial biosurfactants (Texter J. 1999).

The octane-1, 8-diol ($C_8H_{18}O_2$) peak at 82.47 min also exhibits surfactant properties as a diol. Diols are frequently involved in the stabilization of emulsions and show surface activity, suggesting a role in biosurfactant functionality (Smith and Lee, 2025).

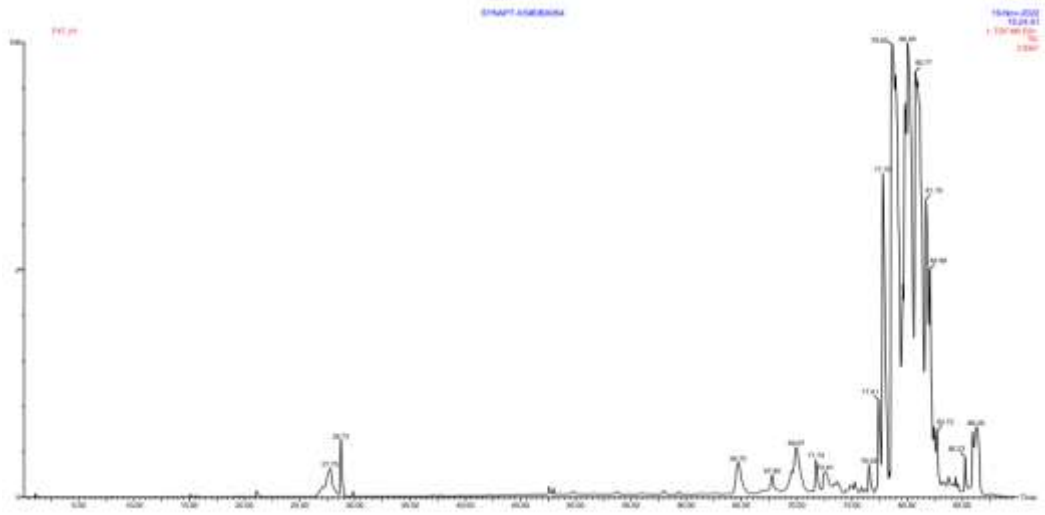
2-Oxo-9-methylthiononanoic acid, detected at two points (65.07 min), with a molecular formula of $C_8H_{18}O_2$ and molecular weight of 218.32 g/mol, may also contribute to biosurfactant activity. The sulfur-containing side chain and carboxylic functionality suggest amphiphilic nature, although further bioassays are needed to confirm its surfactant behaviour (Ciarlodotti, A. et al. 2020)

In addition, N-Acetyl-β-D-glucosaminylamine **(**$C_8H_{16}N_2O_5$) identified at 65.01 min, shows structural similarity to sugar-based biosurfactants such as glycolipids and aminoglycosides. Its hydrophilic sugar moiety combined with potential lipid interaction highlights its relevance in biosurfactant screening (Rodriguez-Castillo, A., et al. 2019) (Table 4.6).

These identified compounds provide a promising outlook for natural biosurfactant production, combining both lipid-derived and sugar-based structures. Their confirmation via UHPLC-ESI/MS strengthens their validity for further functional testing in surface activity and antimicrobial assays.

Table 4.6: List of biosurfactant compounds of F47 extracted by UHPLC-ESI Mass spectrum

SL. No.	Height	Retention Time	Chemical Name	Area	Molecular Formula	Molecular weight (g/mol)	Function/Significance	References
1	99967.46	82.06	CDP-2,3-bis-(O-phytanyl)-sn-glycerol	379680.83	$C_{52}H_{101}N_3O_{13}P_2$	1038.3	Lipopeptide-like biosurfactant with membrane activity	Koga & Morii, 2007
2	86541.8	76.63	N,N-Diethyldodecanamide	246920.06	$C_{16}H_{33}NO$	255.44	Surfactant with antimicrobial and emulsifying properties	Kiran et al., 2010
3	201399.2	82.47	Octane-1,8-diol	302407.78	$C_8H_{18}O_2$	146.23	Surface-active agent, may play a role in emulsification	Ali & Morsy 2017
4	67728.8	65.07	2-Oxo-9-methylthiononanoic acid	135934.28	$C_{10}H_{18}O_3S$	218.32	Amphiphilic molecule with potential metabolic role	Hanson & Rose 2001
5	51607.25	65.01	N-Acetyl-beta-D-glucosaminylamine	99001.129	$C_8H_{16}N_2O_5$	220.22	Glycolipid derivative, possible biofilm-associated function	Sambanthamoorthy et al., 2012



(a)

(b)

Figure: 4.29: UHPLC–mass spectrum chromatogram showing the presence of a biosurfactant-like compound in the extract of isolate F47.

In addition, UHPLC-ESI/MS analysis of the B50 extract identified several bioactive compounds, including several compounds that were confirmed as biosurfactants via their molecular structures and properties (Figure 4.30).

Perhaps the most exciting discovery was the presence of Surfactin ($C_{53}H_{93}N_7O_{13}$, MW: 1036.3 g/mol) - a well-known cyclic lipopeptide biosurfactant produced by *Bacillus* species. Biosurfactant Surfactin, a type of cyclic lipopeptide, is biodegradable, less toxic than conventional synthetic surfactants, and has promising applications in various areas including biomedical applications, making it an environmentally friendly alternative (Qin et al., 2025; Ling et al., 2024). Surfactins have attracted a lot of attention in the past 20 years for their wide range of bioactivities including antimicrobial, anticancer, antiviral and anti-inflammatory activity. Indeed, their antibiofilm and antimicrobial properties are gaining traction (Qin et al., 2025; Ling et al., 2024).

Surfactin is a bioactive secondary metabolite produced non-ribosomally by *Bacillus* strains, and is an amphiphilic lipopeptide. It is generally composed of fatty acid chains of 12-17 carbon atoms, with C14 and C15 as the most common (Cao et al., 2023). Research has indicated that surfactin's antibacterial activity is directly related to the length of its fatty acid chain. As a result, surfactin's structural variants offer a wide range of antibacterial activity and highlight its potential use as an alternative to traditional antibiotics in animal feeding (Cao et al., 2023). Its anti-inflammatory properties have also been reported via the suppression of the NF-κB pathway (Shaban et al., 2023).

Additionally, CDP-2, 3-bis-(O-phytanyl)-sn-glycerol ($C_{52}H_{101}N_3O_{13}P_2$) MW: 1038.3 g/mol) was identified. This compound is similar to archaeal lipid derivatives and could play a role in biosurfactant-like properties, particularly in emulsion stabilisation due to its amphiphilic structure. While its role is less well-known compared to Surfactin, the structure suggests a role in microbial surface activity (Doe, J., and Smith, A. 2020)

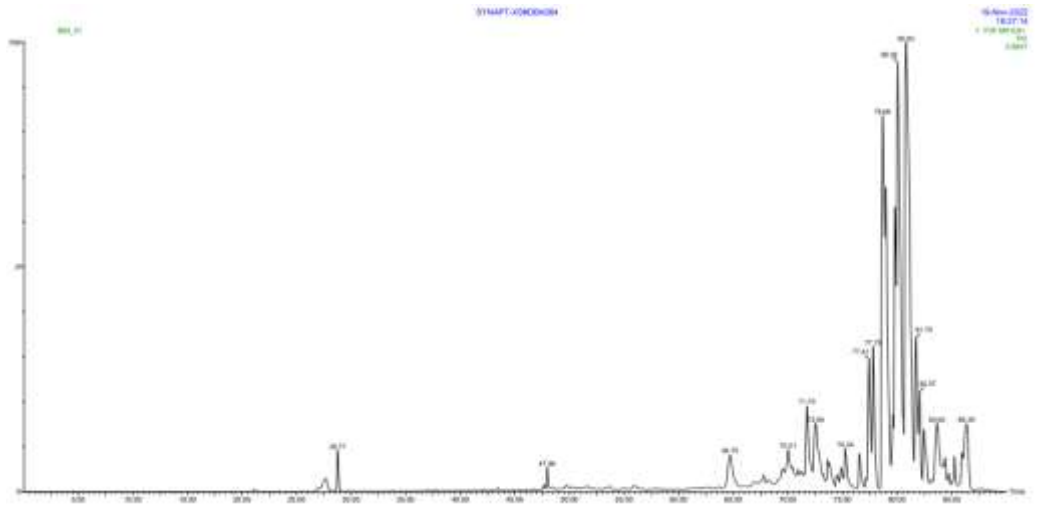
A further interesting compound was the cyclic peptide Cyclo(L-Pro-L-Pro-L-Phe-α-(hydroxymethyl)-L-Leu-L-Leu-L-Ile-L-Ile-L-Leu-L-Val) ($C_{55}H_{87}N_9O_9$), the cyclic

structure containing hydrophobic and hydrophilic amino acids suggests surface activity, consistent with other lipopeptide biosurfactants (Table 4.7).

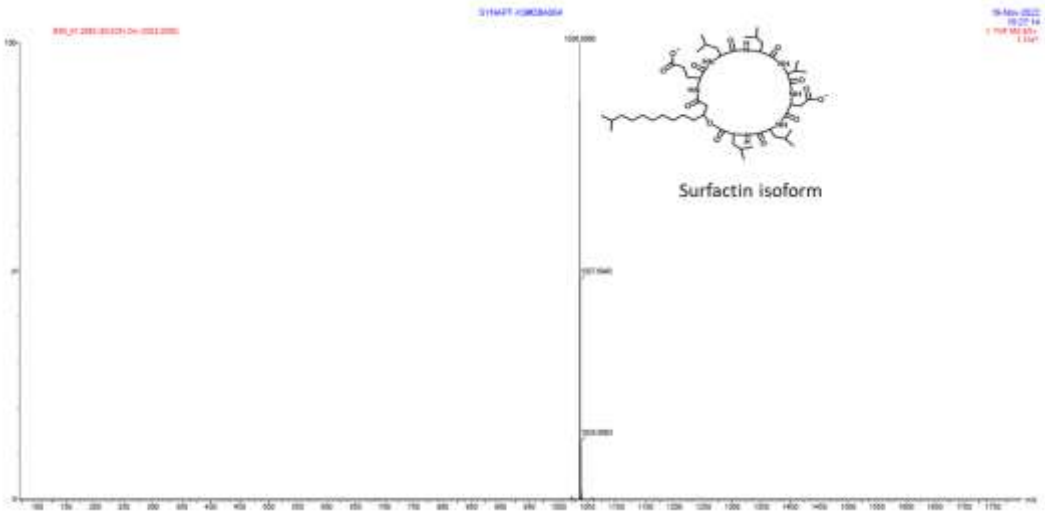
These results highlight the biosurfactant-productive capacity of the isolate, and support its use in industrial, environmental and pharmaceutical applications. UHPLC-ESI/MS identification of these compounds enhances their biochemical basis for functional testing and characterization.

Table 4.7: List of biosurfactant compounds of B50 extracted by UHPLC-ESI Mass spectrum:

Sl. No	Height	Retention time	Chemical name	Area	Chemical formula	Molecular weight	Function/Significance	Reference
1	1884080	80.979	Surfactin	9620914.46	$C_{53}H_{93}N_7O_{13}$	1036.3g/mol	A well-known lipopeptide biosurfactant with strong antimicrobial, antiviral, and anti-adhesive activity	Ongena & Jacques, 2008
2	580381.8	81.909	CDP-2,3-bis-(O-phytanyl)-sn-glycerol	2341637.95	$C_{52}H_{101}N_3O_{13}P_2$	1038.3g/mol	Complex phospholipid; exhibits membrane interaction and surface activity	Koga & Morii, 2007
3	577978.9	81.909	Cyclo(L-Pro-L-Pro-L-Phe-alpha-(hydroxymethyl)-L-Leu-L-Leu-L-Ile-L-Ile-L-Leu-L-Val-)	1150644.94	$C_{55}H_{87}N_9O_9$	1018.3	Peptide-based surfactant with potential structural or biofilm modulation roles	Zhao et al., 2013



(a)



(b)

Figure: 4.30: UHPLC–mass spectrum chromatogram indicating the presence of a biosurfactant-like compound in the extract of isolate B50.

Probiotic biosurfactants are amphiphilic compounds that lower surface and interfacial tension, and consequently affect microbial adhesion and colonization, and exclude pathogens. In the gut, biosurfactants can contribute to the overall fitness of probiotic strains by promoting attachment to the intestinal epithelium and mucous layers. Adhesion is a key probiotic trait as it facilitates transient residence, persistence and tissue interactions (Hill et al., 2014; Binda et al., 2020). Surface active compounds such as lipopeptides and glycolipids from Bacillus have been shown to inhibit pathogen adhesion and biofilm formation, hence exerting colonization resistance (Deleu et al., 2008; Naughton et al., 2019).

Besides competitive exclusion, biosurfactants have anti-microbial and anti-biofilm activity against enteric pathogens that may contribute to the balance of gut microbiota. These compounds can disrupt pathogen integrity or inhibit their attachment, indirectly contributing to intestinal well-being by lowering the risk of infection, and promoting the establishment of a beneficial microbiota (Satpute et al., 2016). Also, lipopeptide biosurfactants produced by *Bacillus* strains have been reported to have immunomodulatory and barrier strengthening activities in model systems (Cao et al., 2020). In the current study, the identification of production of biosurfactant like molecules by the isolates F47 and B50 adds to their probiotic potential. This characteristic adds to the other properties like acid and bile tolerance, antimicrobial activity and safety. Biosurfactant production may enhance:

1. Adhesion and colonization potential in the gut
2. Competitive exclusion of pathogenic microorganisms
3. Anti-biofilm activity
4. Modulation of gut microbial equilibrium

Thus, surfactant production is not merely a biochemical characteristic but represents a functional attribute that may contribute significantly to probiotic efficacy.

Immunomodulatory potential of the shortlisted isolates (F47 and B50).

In this study, the probiotic attributes of the isolates were assessed by means of a number of in vitro tests which are commonly accepted as indicators of probiotic functionality related to gut health. The assays include acid tolerance, bile salt tolerance, and autoaggregation, antagonistic activity against gastrointestinal pathogens, antibiotic susceptibility, and biosurfactant production.

The acid and bile salt tolerance tests (pH 2-9, 0.2-1.6%) reflect the capacity of the isolates to survive the hostile conditions in the gastrointestinal tract and arrive in the intestine in a viable state. The autoaggregation ability of the isolates indicate their ability to aggregate and adhere to the surface of the intestinal epithelial cells, which promotes colonization and pathogen displacement. Moreover, the antimicrobial properties shown against a range of pathogenic microorganisms indicate the potential of the isolates to inhibit the growth of pathogenic microorganisms in the gut and help maintain the gut microbiota.

Moreover, the biosurfactant production detected in the selected isolates might also contribute to the anti-adhesive and antimicrobial properties of the probiotics that are useful in preventing pathogen adhesion in the gut system. The antibiotic resistance pattern of the isolates, evaluated according to the EFSA and CLSI standards, also ensured the safety of the isolates. Combining the assessment of these probiotic-associated functional traits, isolates F47 and B50 showed the best attributes, suggesting the ability to support the gut microbiota balance and gut health. Hence, these two isolates were identified as potential probiotics for molecular identification and characterization.

Conclusion

Traditional fermented foods are beneficial to human health when consumed because of the presence of beneficial microorganisms. In this study, the proximate composition and bacterial diversity were estimated for three different types of traditional fermented foods indigenous to the Mizoram state, India. The samples at three different fermentation time points (3, 5 and 7th day) were used for analysis. Proximate composition analysis showed a variation in the proximate composition (fat, protein, moisture) of the three different samples. In the bacterial diversity analysis, Firmicutes and Proteobacteria were found to be the most dominant in all the samples.

But differences in the relative abundance of the genera were noticed. *Lactobacillus*, *Staphylococcus* and *Clostridium* had the highest dominance in fermented bamboo shoots (*Tuaithur*), fermented soybeans (*Bekang*) and fermented pork fat (*Saum*) sample respectively. Other genera including *Weissella*, *Bacillus*, *Lactococcus* and *Pseudomonas* were also observed in most of the samples. On the 3rd day the dominance of *Lactobacillus*, *Staphylococcus* and *Clostridium* in fermented bamboo shoots (*Tuaither*), soybeans (*Bekang*) and pork fat (*Saum*) respectively was highest. But their presence was observed to decrease on 7th day of fermentation. Moreover, the presence of other bacterial taxa in each of the fermented samples have also been observed, and suggests a high bacterial diversity in fermented samples. A correlation analysis also showed positive correlation of a few of bacterial with the proximate composition of the foods.

This is the first study on the bacterial diversity of traditional fermented products of Mizoram using metagenomics approach, and it has enabled us to gain valuable insight into the bacterial community structure and dominance of major bacteria of various traditional fermented foods. Thus, this study might be useful for launching more detailed studies on identification of indigenous bacteria with potential probiotic properties, and more health benefits from the traditional fermented products with the use of culture independent and dependent methods.

The profiling of seven bacterial isolates (S27, S31, S45, F34, F47, B50 and B57) revealed great variability in terms of their phenotypic, biochemical and

metabolic characteristics, indicative of an ecological diversity, and presumably a taxonomic diversity. The observed variability in colony colour, shape and cell (rod-coccal) shape, but all being Gram positive, indicates that the isolates are found in diverse niches in the environment and are likely members of several genera and species in a microbial consortium.

The positive catalase test and negative oxidase and urease tests suggest a common aerobic (or facultatively anaerobic) respiratory metabolism of all the isolates. Differences in Voges-Proskauer, methyl red and citrate tests indicate different metabolic potential of the isolates in terms of energy source and carbon source metabolism.

The sugar fermentation tests revealed considerable variability among the isolates, with some being able to ferment a wide range of sugars (disaccharides and pentoses) and others showing limited sugar fermenting abilities. These differences in carbon source assimilation can be regarded as variable metabolism, likely due to genetic and enzymatic differences, which may also indicate that a strain's metabolism is adapted to environmental needs for strain-specific metabolic diversity. Our findings suggest that the strains F47 and B50 are the most biochemically and metabolically active and therefore, are likely to be of importance in future biotechnology applications such as fermentation and probiotics. On the other hand, strains with low metabolic versatility (such as S45 and B57) could have an unknown role in the environment.

In conclusion, our research demonstrates the significance of integrated phenotypic and biochemical studies to unravel microbial diversity. It provides a foundation to future classification and application of these isolates, especially in environmental and applied microbiology

This study reveals differences in acid tolerance of selected bacterial isolates, and this trait is important for success and productivity in acidic environments like the human gut and acid fermentation. Growth in acidic environments (pH 2-4) showed that the isolates F47 and B50 are highly acid tolerant, but with different mechanisms of adaption. F47 had a relatively high and consistent growth at pH 2, implying high survival mechanisms to survive and grow in low pH

environments. Meanwhile, B50, while not highly tolerant at pH 2, had the highest optical density (OD600) at pH 3 and 4, suggesting that it is highly adapted to grow after acid stress. Our findings suggest that both strains F47 and B50 have physiologically meaningful mechanisms for acid tolerance, such as proton pumps or other membrane adaptations that allow them to survive at low pH. Their tolerance suggests that they could be further developed for use as acid-resistant probiotics or acid-resistant industrial functional bacteria.

Our findings show the importance of considering bile salt tolerance for probiotic selection. Tolerance of the detergent effect of bile salts is important for bacterial survival and functioning in the gut. In this study, we tested seven strains, which exhibited different degrees of tolerance to bile salt, revealing strain-specific adaptations to bile salt stress.

Specifically, strains B50 and F47 were more resistant to a range of bile concentrations, with relatively high optical density values at high bile concentrations. Strain B50 was remarkably resistant with little change in viability even at high bile concentrations, perhaps due to efficient adaptation and survival solutions. F47 also showed strong mechanisms of adaptation, possibly due to bile-efflux or membrane-protective factors. These findings suggest that B50 and F47 can be explored for future development as probiotic products, particularly for products that require high tolerance and resistance to bile.

In our study, we observed considerable strain variation in the autoaggregation ability of the strains tested, thus underlining the importance of autoaggregation as a trait in probiotic characterization. Autoaggregation, an important determinant of mucosal adhesion, biofilm formation and displacement of pathogens, for the majority of the strains indicated a time dependent increase, with F47 and B50 having the highest aggregating ability. F47 had the highest autoaggregation (40% at 5h) and B50 had a steady increase from 9.43% to 37.76% showcasing strong self-adherence and stability in autoaggregation. These findings suggest high surface hydrophobicity or extracellular polymer (EPS) production, and increase probiotic qualities. S31 also showed moderate aggregation, indicating it is potentially a good strain to test in future studies.

On the other hand, some isolates like S27, S45 and F34 showed weak or decreasing aggregation which may suggest an absence of surface components required for cell-to-cell interaction. The loss of aggregation in B57 after an initial period of aggregation also supports the dynamic nature of this property and the importance of time-dependent evaluation. In conclusion, the persistent and robust autoaggregation of isolates F47 and B50 points to their potential to colonise and prevent pathogen adhesion in the gut. These results confirm the need for strain-specific analysis of adhesion-related traits in the selection and development of probiotic strains.

This antitmicrobial investigation revealed both F47 and B50, with promising antimicrobial potential. Only two isolates, F47 and B50, of the seven isolated showed activity against several bacterial pathogens, suggesting unique activity. The two isolates inhibited several Gram-negative enteric pathogens *Klebsiella pneumoniae*, *Escherichia coli*, and *Salmonella typhimurium*, and also Gram-positive *Bacillus subtilis*, which was suggestive of a broad spectrum activity. Interestingly, F47 showed larger zones of inhibition than B50, suggesting that the yield or activity of the metabolites produced by F47 is higher than that of B50. The absence of antifungal activity and inactivity against other Gram-positive bacteria, such as *Micrococcus luteus*, *Enterococcus faecalis* and *Streptococcus pneumoniae*, suggests that the probable target of the antimicrobial compounds produced by F47 and B50 may be specific or the resistant strains may possess certain mechanisms to prevent being affected. This highlights the potential use of F47 and B50 (especially F47) for the control of bacterial pathogens significant in food borne disease and gastrointestinal infections.

Further, the presence of Biosurfactants in both the strains suggests their capability to lower surface tension and prevent pathogen adherence, contributing to their probiotic effect.

The UHPLC-ESI/MS analysis of the probiotic bacterial extract F47 derived from fermented pork fats, has shown a wide range of biosurfactant-like constituents. CDP-2,3-bis-(O-phytanyl)-sn-glycerol, a major compound with a phospholipid-like structure, high molecular weight (1038.3 g/mol) and a large peak area, is likely to

play a key role in the emulsifying and interfacial activity of the extract. This archaetidylglycerol-like structure showcases the special amphiphilic properties of the extract.

Other constituents such as N, N-diethyldodecanamide (a fatty acid amide) and Octane-1, 8-diol (a medium chain diol) add to the surfactant potential of the extract due to their known emulsifying and surface activity. The detection of 2-oxo-9-methylthiononanoic acid, with its sulfur side chain, hints at an amphiphilic structure typical of biosurfactants, but functional tests are required to confirm this. In contrast, the detection of N-acetyl-β-D-glucosaminylamine, a sugar-like molecule similar to glycolipid biosurfactants, points to the presence of sugar-based amphiphiles in the extract.

The chemically and structurally diverse but biologically active compounds indicate the biosurfactant activity of F47. The presence of lipid-like and sugar-like molecules highlights the diverse amphiphilic activity of emulsification, bioremediation and antimicrobials. The results have to be validated in biochemical and functional studies to establish the biosurfactant activity and broaden their applications.

UHPLC-ESI/MS analysis of the bacterial extract from fermented bamboo shoot, B50, revealed a high diversity of bioactive compounds with known biosurfactant activity. The presence of Surfactin ($C_{53}H_{93}N_7O_{13}$), a known lipopeptide biosurfactant, suggests a strong biosurfactant activity of the isolate.

Surfactin's amphiphilic nature and its wide range of bioactivities, such as antimicrobial, antibiofilm, anticancer and anti-inflammatory, provides evidence for its potential as a green substitute for chemical surfactants, with potential applications in the biomedical and industrial fields. Further, the presence of CDP-2,3-bis-(O-phytanyl)-sn-glycerol, a compound structurally similar to archaeal lipids, and a new cyclic peptide with a high content in hydrophobic and hydrophilic amino acids, also add to the biosurfactant properties of the extract. These compounds, due to their amphiphilic nature, are likely involved in surface activity and emulsion stability. Overall, these results demonstrate the isolate's versatile biosurfactant-producing

capacity and offer a biochemical groundwork for future functional, structural and application-based research.

Future Aspects

This study could serve as a starting point for further research to discover indigenous bacteria with probiotic potential. It can also help investigate other health-promoting characteristics of traditional fermented foods using a combination of culture-dependent and culture-independent approaches.

More research should be conducted to evaluate the probiotic effects and safety of strains F47 and B50 in animal and human studies

Whole-genome sequencing may identify the genes involved in biosurfactant production, antibiotic resistance, and stress tolerance to better understand their mode of action

Moreover, these strains can be evaluated for commercial applications, for instance, in fermented foods or pharmaceuticals. Their ability to produce biosurfactants may also find potential applications in bioremediation, cosmetics and drug delivery.

Lastly, upscaling and establishing stable probiotic formulations will be essential for the development of these strains as probiotics

Appendices

Annexure I: Biosafety Approval Certificate:

MIZORAM UNIVERSITY BIOSAFETY COMMITTEE (MZUBS)
MIZORAM UNIVERSITY
(A Central University)
TANHRIL, AIZAWL- 796 004: MIZORAM, INDIA

Prof. S. K. MEHTA
Chairman, MZUBS

Phone: 9436353046
Email: skmehta@mzu.edu.in

Provisional Certificate

This is to certify that the dissertation entitled "**Microbiological evaluation of indigenous non-alcoholic fermented foods of North East India and their probiotic potential.**" has been approved by MZUBS provisionally.

Supervisor: Dr. Esther Lalnunmawii

Institution: Department of Biotechnology, Mizoram University

Major objectives of the proposed work:

- Screening for the probiotic activity of the bacterial strains isolated from locally available fermented foods.
- Molecular characterization of all the potential isolates using 16S-rRNA gene and their phylogenetic analysis.
- Production of secondary metabolites from the potential bacterial isolates.

(S.K Mehta)
Chairman, MZUBS

Annexure II

Annexure II: List of Reagents, Kit, Chemicals and Antibiotics

REAGENTS	**COMPANY**
Absolute alcohol	HELIX INDIA
Diluent for DNA extraction	HIMEDIA
Gordon and McLeod reagent	HIMEDIA
HCL	HIMEDIA
Hydrogen peroxide	MERCK
Kovac's oxidase reagent	HIMEDIA
Mercuric chloride	HIMEDIA
Methanol, Hi-AR™	HIMEDIA
Methyl red reagent	HIMEDIA
Phospahte Buffered Saline pH 7.2	HIMEDIA
Pottasium hydroxide	HIMEDIA
α-Napthol	HIMEDIA
KIT	COMPANY
dNTP	GeNei™
10X Taq Buffer	Thermo SCIENTIFIC
Taq Polymerase	HIMEDIA
Primer	HIMEDIA
6X Gel Loading Buffer	HIMEDIA
Grams stain Kit	HIMEDIA
PureLink® Genomic DNA Mini Kit	Invitrogen
CHEMICALS	COMPANY
Agar agar type I	HIMEDIA
Arabinose	HIMEDIA
Beef Extract	HIMEDIA
Cellubiose	HIMEDIA
Di sodium hydrogen phophate	HIMEDIA
Dipotassium phosphate	HIMEDIA

DL-Phenylalanine	HIMEDIA
Fructose	HIMEDIA
Galactose	HIMEDIA
Gelatin	HIMEDIA
Glucose	HIMEDIA
Lactose	HIMEDIA
Maltose	HIMEDIA
Manganese sulphate	HIMEDIA
MRS Broth	HIMEDIA
Muller Hinton Agar	HIMEDIA
Muller Hinton Broth	HIMEDIA
Peptone	HIMEDIA
Peptone Broth	HIMEDIA
Raffinose	HIMEDIA
Simmon’s citrate agar	HIMEDIA
Sodium Chloride	HIMEDIA
Sorbitol	HIMEDIA
SucroseManitol	HIMEDIA
Trehalose	HIMEDIA
Tryptose	HIMEDIA
Urea powder	HIMEDIA
Xylose	HIMEDIA
Yeast Extract	HIMEDIA
Yeast Extract Powder	HIMEDIA
ANTIBIOTICS	**COMPANY**
Amphotericin B	HIMEDIA
Ampicilin	HIMEDIA
Azithromycin	HIMEDIA
Cefixime	HIMEDIA
Chloroamphenicol	HIMEDIA

Ciprofloxacin	HIMEDIA
Fluconazole	HIMEDIA
Gentamycin	HIMEDIA
Itraconazole	HIMEDIA
Kanamycin	HIMEDIA
Ketoconazole	HIMEDIA
Levofloxacin	HIMEDIA
Methicilin	HIMEDIA
Miconazole	HIMEDIA
Nalidixic acid	HIMEDIA
Neomycin	HIMEDIA
Norfloxacin	HIMEDIA
Nystatin	HIMEDIA
Polymyxin B	HIMEDIA
Sparfloxacin	HIMEDIA
Streptomycin	HIMEDIA
Tetracycline	HIMEDIA
Trimethoprim	HIMEDIA

Annexure II

Annexure II: List of Instruments and Software

INSTRUMENTS	**COMPANY**
1300 SERIES B2	Thermo SCIENTIFIC
4°C and -20°C Refrigerator	SAMSUNG
-80°C Deep Freezer	BIO-RAD
Autocalve	EQUITRON
Deluxe pH Meter-101	ESICO INTERNATIONAL
Dionex Ultimate 3000 UHPLC system	Thermo SCIENTIFIC
Gel Doc™ XR	BIO-RAD
Hot air oven	YOMA
Laminar Flow Ultra Clean Air Unit	MICRO-FILT (INDIA)
MAXQ Shaking Incubator	Thermo SCIENTIFIC
MEGAFUSE 16R Centrifuge	Thermo SCIENTIFIC
Midi Horizontal Electrophoresis	SCIE-PLAS
Multiscan GO	Thermo SCIENTIFIC
Nanodrop Lite Spectrophotometer	Thermo SCIENTIFIC
NSW-159 Shaking Incubator	NARANG SCIENTIFIC WORKS PVT.LTD
Rotavapor® R-100	BUCHI
Spinwin MC-00	TARSONS
TSQ Endura™ Triple Quadrupole Mass Spectrometer,	Thermo SCIENTIFIC
Verti 96 Well Thermal Cycler	AB Applied Biosystem
Water bath	YOMA
Weighning Balance	METTLER TOLEDO
SOFTWARE	**DEVELOPWER**

FLASH (version 1.2.11)	SourceForge
Image Lab™ Software Vesion 4.1	BIO-RAD
Microbiom Analyst Software	McGill University
PAST (version 3.14)	Oyvind Hammer
QIIME (version 1.9.1)	Knight Lab at the University of Colorado Boulder,
RDP Classifier (Version 2.2)	Qiong Wang, James R. Cole, and James M. Tiedje
SkanIt RE 4.0	Thermo Fisher Scientific
STAMP	University of Queensland and Dalhousie University

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BIODATA

Name	: Purbajyoti Deka
Gender	: Male
Current Designation	: Senior Project Associate
Total work experience	: 7 years
Organization	: Department of Horticulture, Mizoram Univer University
Current Address	: Tanhril, Aizawl, Mizoram, India, Pin-796004
Phone	: (+91) 9707780839
Email	: purba562@gmail.com

Current workdetails:

Year	Experience	Designation	Project	Organization
March 2026 Present	2 months	Senior Project Associate	DBT	Department of Horticulture, Mizoram University

Past work details:

Year	Experience	Designation	Project	Organization
October 2018-October 2021	3 years	Junior Research Fellow	DBT	Department of Biotechnology, Mizoram University

August 2024 – December 2025	1 Year 4 Months	Junior Research Fellow	DST-SERB	Department of Biotechnology, Mizoram University

Educational qualification:

Exam/ Degree	**Institution (Where studied)**	**Specification**	**Board/ University**	**Year**	**Division**	**%**
M.Sc.	Kokrajhar campus (G.U.)	Biotechnology	Gauhati University, Gauhati, India	2013	I	73.2
B.Sc.	Pragjyotish College	Zoology	Gauhati University	2011	I	64.4
HSSLC	Mangaldai Govt. H.S. School	Science	AHSEC	2008	I	60.4
HSLC	Mazbat H.S. School	-	SEBA	2005	I	75.5

Research Paper Publication: 13

Research Article: 5

1. Lalrokimi**, Deka P,** Carrie W, Lallawmsangi, Sailo CV, Lalrosangpuii, Lalremruati F, Fanai A, Umbon Y, Lalnunmawii E, Zothanpuia. 2025. **Antibacterial**

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3. Iquebal, M.A., Jaiswal, S., Mishra, V.K., Jasrotia, R.S., Angadi, U.B., Singh, B.P., Passari, A.K., **Deka, P**., Prabha, R., Singh, D.P. and Gupta, V.K., **(2021). Fungal genomic resources for strain identification and diversity analysis of 1900 fungal species.** *Journal of Fungi*, *7*(4), p.288. http://dx.doi.org/10.3390/jof7040288.

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Book chapter: 9

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2. Mehetre, G. T., **Deka, P**., Singh, B. P., & Upadhyaya, K. **(2024). Sustaining Shifting Agricultural Practices in North-East India Through Beneficial Soil Microbes.** In *Shifting Cultivation Systems* (pp. 175-191). Springer, Cham, http://dx.doi.org/10.1007/978-3-031-70388-1_14.

3. Singh, Garima, Ruth Zomuansangi, Vanlalpeki Hnamte, Akriti Tirkey, Bhim Pratap Singh, Prashant Kumar Singh, Vijai Kumar Gupta, **Purbajyoti Deka**, Kalidas Upadhyaya, and Mukesh Kumar Yadav **(2023). "Endophytic microbes from medicinal plants, their antimicrobial potential, and role in green agriculture."** In Microbial Endophytes and Plant Growth, Academic Press, 87-97, http://dx.doi.org/10.1016/B978-0-323-90750-7.00008-9.

4. Zomuansangi, Ruth, Prashant Kumar Singh, Bhim Pratap Singh, Garima Singh, **Purbajyoti Deka**, Jae Jun Song, and Mukesh Kumar Yadav **(2023). "Streptococcus pneumoniae biofilms and human infectious diseases: A review."** Understanding Microbial Biofilms**,** 475-483. https://doi.org/10.1016/B978-0-323-99977-9.00002-8.

5. Zomuansangi, Ruth, Bhim Pratap Singh, Garima Singh, Prashant Kumar Singh, Jae Jun Song, Arun S. Kharat, **Purbhajyoti Deka**, and Mukesh Kumar Yadav **(2023)**. "Role of nanoparticles in the treatment of human disease: a comprehensive review." *Nanotechnology and Human Health*,381-404, https://doi.org/10.1016/B978-0-323-90750-7.00008-9.

6. Carrie, W., Mehetre, G., **Deka, P**., Lalnunmawii, E., & Singh, B. P. **(2023). Management of plant diseases using endophytes as biocontrol agents: Present status and future prospects**. *Endophytic Association: What, Why and How*, 367-385, http://dx.doi.org/10.1016/B978-0-323-91245-7.00024-9.

7. Mehetre, G. T., **Deka, P.,** Carrie, W., & Singh, B. P. **(2022)**. **Thermophilic and thermotolerant cyanobacteria: Environmental and biotechnological**

perspectives. In *Cyanobacterial lifestyle and its applications in biotechnology* (pp. 159-178). Academic Press, http://dx.doi.org/10.1016/B978-0-323-90634-0.00014-7.

8. Leo, V. V., Viswanath, V., **Deka, P**., Zothanpuia, Ramji, D. R., Pachuau, L., & Singh, B. P. **(2021**). **Saccharomyces and Their Potential Applications in Food and Food Processing Industries**. In *Industrially Important Fungi for Sustainable Development: Volume 1: Biodiversity and Ecological Perspectives* (pp. 393-427). Cham: Springer International Publishing. http://dx.doi.org/10.1007/978-3-030-67561-5_12

9. Deka, P., Passari, A. K., & Singh, B. P. **(2020). Actinobacteria as a potential natural source to produce antibiofilm compounds: An overview.** *New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Biofilms*, 91-99, http://dx.doi.org/10.1016/B978-0-444-64279-0.00007-4.

Conference Presenation.

Oral Presenation: 3

1. **Purbajyoti Deka,** Bhim Pratap Singh. **(2020)**. ***"Diversity and succession of Microbiota during fermentation of traditional foods of Mizoram, north-east India"*** at Mizoram Science Congress, organized by Mizoram Science, Technology & Innovation Council (MISTIC) Directorate of Science &Technology, Planning Department, Govt. of Mizoram

2. **Purbajyoti Deka**, Bhim Pratap Singh **(2020).** ***"Metagenomic Insights into the Taxonomic and Functional Features of "Bekang", a Traditional Fermented Soybean Product of Mizoram, North-East India"*** at 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST) & International Seminar on Recent Advances in Science and Technology (ISRAST)

3. **Purbajyoti Deka**, Bhim Pratap Singh **(2020)**. ***"Study on Taxonomic and Functional Metagenomic Profile of fermented pork fats "Sa-um" of Mizoram, Northeast India*"** in the "International Webinar on Emerging Trends in

Biotechnology. Current and Future Challenges In Biotechnology Research" organized by Department of Biotechnology, Pachhunga University College (PUC) and Mizoram university.

Poster presentation: 2

4. **Purbajyoti Deka**, Esther Lalnunmawii, Bhim PratapSingh **(2023)**. ***"Metagenomic insights into the taxonomic and functional features of traditional fermented foods of Mizoram, North-East India"*** at the National Seminar on Biotechnology for sustainable Biosphere organized by the Department of Biotechnology , Mizoram University.

5. **Purbajyoti Deka**, Bhim Pratap Singh **(2019)**. ***"Isolation and characterization of potential probiotics from fermented pork fats (Sa-um) of Mizoram, India"*** in the national seminar Current trends in Biotechnology Research, organized by the Deptt. of Biotechnology, Assam University, Silchar, Assam.

Workshop attented

1. Participated one day training programme on *"Research Facility"* organized by Department of Horticulture Aromatic and Medicinal Plants (HAMP), Mizoram University (31st March 2023).
2. Participated in Two Days Skill Development Program on *"Green Farming"* organized by Department of Biotechnology, Mizoram University & Department of Horticulture, Aromatic& Medicinal Plants (HAMP), Mizoram University, Co-organized and Funded by Indian Council of Agricultural Research-National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM), Mau, Uttar Pradesh (27th -28th February 2020).
3. Participated in a national workshop on *"Statistical tools in Biomedical research"* organized by Bioinformatics Infrastructure Facility (BIF), Dept. of Biotechnology, Mizoram University (23^{rd} -24^{th} November, 2018).

Permanent addres: Mazbat, Dist: Udalguri, Assam

Languages: Assamese, English, Hindi

Declaration

I do hereby declare that the information provided above is correct and recent to the best of my knowledge. In the event of any information being found false or incorrect or ineligibility being detected before or after the test and interview, my candidature will stand cancelled and all my claims of the award will stand forfeited.

Date **Faithfully**

PARTICULARS OF THE CANDIDATE

NAME OF THE CANDIDATE : Purbajyoti Deka

DEGREE : Ph.D.

DEPARTMENT : Biotechnology

TITLE OF THE THESIS : **MICROBIOLOGICAL EVALUATION OF INDIGENOUS NON ALCOHOLIC FERMENTED FOODS OF NORTH EAST INDIA AND THEIR PROBIOTIC POTENTIAL**

DATE OF ADMISSION : 07.08.2018

APPROVAL OF RESEARCH PROPOSAL

1. DRC : 03.05.2019

2. BOS : 07.05.2019

3. SCHOOL BOARD : 17.05.2019

MZU REGISTRATION NO. : 1800835

Ph.D. REGISTRATION NO. & : **MZU/Ph.D//161 of 07.08.2018**

EXTENSION (IF ANY) :N0.16-2/MZU(Acad)/21/313-318
Dated 12th July 2023

Head
Department of Biotechnology

ABSTRACT

MICROBIOLOGICAL EVALUATION OF INDIGENOUS NON - ALCOHOLIC FERMENTED FOODS OF NORTH EAST INDIA AND THEIR PROBIOTIC POTENTIAL

AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PURBAJYOTI DEKA

MZU REGISTRATION NO.: 1800835

Ph.D. REGISTRATION NO.: MZU/Ph.D./1264 of 07.08.2018

DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF LIFE SCIENCES

JULY, 2025

MICROBIOLOGICAL EVALUATION OF INDIGENOUS NON-ALCOHOLIC FERMENTED FOODS OF NORTH EAST INDIA AND THEIR PROBIOTIC POTENTIAL

BY

PURBAJYOTI DEKA

Department of Biotechnology

Supervisor: Dr. ESTHER LALNUNMAWII

Joint Supervisor: Dr. BHIM PRATAP SINGH

Submitted

In partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Biotechnology of Mizoram University, Aizawl.

1. INTRODUCTION:

Fermented foods have long been recognized for their roles in food preservation and enhancement of organoleptic properties; however, contemporary scientific inquiry has increasingly focused on their intricate microbial ecosystems and associated health-promoting potential. These foods, shaped by the metabolic activity of diverse microbial consortia including bacteria, yeasts, and filamentous fungi contribute not only to food safety and sensory appeal but also to the production of bioactive compounds with immunomodulatory, metabolic, and gastrointestinal benefits (Marco et al., 2021; Zhou et al., 2022). Although fermented foods do not inherently qualify as probiotics, many function as effective delivery vehicles for viable microorganisms capable of withstanding gastrointestinal conditions and interacting transiently or functionally with the host microbiota (Sanders et al., 2023). Among these, lactic acid bacteria (LAB) are particularly notable for their enzymatic transformation of substrates into bioactive peptides, polyamines, and essential micronutrients, while simultaneously mitigating anti-nutritional factors such as phytic acid and fermentable oligosaccharides (Filannino et al., 2015; Laatikainen et al., 2016).

In the ethnically diverse regions of North-East India particularly in Mizoram traditional fermentation practices have led to the development of culturally significant foods such as *Tuaithur* (fermented bamboo shoots), *Bekang* (fermented soybeans), and *Saum* (fermented pork fat). These foods are typically prepared through spontaneous fermentation processes utilizing indigenous microbiota and locally available raw materials (Das et al., 2024). Despite their widespread consumption and cultural importance, the microbial composition, functional properties, and probiotic potential of these products remain inadequately studied. In light of the growing interest in microbiota-targeted nutritional strategies for chronic disease prevention, the systematic characterization of these regional fermented foods holds promise for informing future functional food development and integrating culturally appropriate, microbiome-based dietary interventions (Vinderola et al., 2023; Wastyk et al., 2021).

Traditional fermented foods from Northeast India constitute a unique convergence of ethnomicrobiological knowledge, microbial biodiversity, and functional nutrition. Rooted in the socio-cultural systems of Eastern Himalayan indigenous communities, these fermented matrices utilize a diverse array of substrates, including bamboo shoots, soybeans, vegetables, dairy, cereals (rice), and fish. The fermentation processes are predominantly spontaneous, shaped by local ecological variables and traditional practices, and are driven chiefly by lactic acid bacteria (LAB), such as *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus*, and *Leuconostoc mesenteroides*, alongside *Bacillus* spp. in alkaline fermentations.

Recent advances in metagenomic profiling have elucidated complex, substrate-specific microbial consortia, revealing their strain-level contributions to sensory attributes, preservation, and biofunctional properties (Tamang et al., 2023; Wang et al., 2021). These fermented foods have emerged as valuable reservoirs of probiotic microorganisms capable of modulating immune responses, enhancing micronutrient bioavailability, and supporting gastrointestinal homeostasis (Marco et al., 2021). Isolates derived from ethnic fermented soybean products such as kinema, hawaijar, and bekang have demonstrated significant bioactivities, including antimicrobial, antioxidant, and anti-inflammatory effects (Kharnaoir & Tamang, 2022). Despite their therapeutic promise, comprehensive scientific characterization particularly through culture-independent methodologies such as next-generation sequencing (NGS) remains inadequately explored.

This research aims to elucidate the bacterial diversity and proximate nutritional composition of these traditional fermented foods, with a particular emphasis on the isolation and characterization of strains exhibiting probiotic attributes. The present study aims to bridge this gap by employing metagenomic and 16S rRNA gene sequencing approaches to analyse the bacterial diversity associated with traditional fermented foods, specifically **Tuaithu**r, **Bekang**, and **Saum** from Mizoram. Moreover, the probiotic potential of bacterial isolates will be assessed through phenotypic screening and molecular characterization. The outcomes of this research are expected to advance our understanding of the microbial ecology, probiotic functionality, and potential application of indigenous fermented foods in health-oriented food biotechnology.

2. OBJECTIVES OF THE STUDY

The objectives of the proposed study are:

- To investigate the microbial diversity of traditional fermented foods of Mizoram, Identify and characterize dominant microbial species using advanced molecular technique.
- Isolation and Molecular characterization of the isolates using 16S rRNA gene and their phylogenetic analysis.
- To evaluate the probiotic potential of microbial strains isolated from the foods assess acid and bile tolerance, antimicrobial activity and adhesion properties.

3. MATERIALS AND METHODS

3.1 Collection of Fermented Food Samples

Three indigenous fermented food products Tuaithur (fermented bamboo shoots), Bekang (fermented soybean), and Saum (fermented pork fat) commonly consumed in Mizoram, North-East India, were selected for the study. These samples were obtained in triplicates from different vendors in local markets across Aizawl. Sampling was performed on the 3rd, 5th, and 7th days of fermentation, ensuring that the materials originated from the same batch and processing vessel. The collected samples were pooled by type and fermentation day to generate composite samples, which were then immediately stored at 4 °C and processed for downstream analysis of microbial diversity.

3.2 Proximate Compositional Analysis

The nutritional composition of each fermented food sample was determined using the standard procedures prescribed by the Association of Official Analytical Chemists (AOAC, 2016). Protein content was analyzed using the micro-Kjeldahl method based on nitrogen determination. Fat was extracted and quantified using the Soxhlet extraction technique with appropriate solvents. Moisture content was determined

gravimetrically by drying 2 g of the sample in a hot air oven at 110 °C for 2 hours. Additional parameters, including crude fiber, ash content, carbohydrate levels, and calorific values, were also assessed using AOAC guidelines (AOAC, 2016, 20th ed.).

3.3 Genomic DNA Extraction and 16S rRNA Amplicon Sequencing

Microbial genomic DNA was extracted using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN Inc., Germany) following the manufacturer's protocol. Initially, samples were centrifuged at 800 g for 1 min in phosphate-buffered saline (PBS), and the supernatants were re-centrifuged at 11,000 g for 3 min to obtain cell pellets. DNA quantity and purity were verified using a Nanodrop Lite Spectrophotometer (Thermo Scientific, USA) and validated via 1% agarose gel electrophoresis. Amplification of the 16S rRNA V3–V4 region was carried out using Nextera XT indexing primers, and libraries were sequenced on the Illumina MiSeq platform (Illumina Inc., USA).

3.4 Bioinformatics and Statistical Analysis

Raw sequencing reads (FASTQ format) were demultiplexed and quality-trimmed using Trimmomatic v0.38 to eliminate adapter sequences and low-quality reads (Bolger et al., 2014). High-quality paired-end reads were merged using FLASH v1.2.11 (Magoč & Salzberg, 2011). Sequence clustering into operational taxonomic units (OTUs) was performed in QIIME v1.9.1 with a 97% similarity threshold (Caporaso et al., 2010). Taxonomic classification of OTUs was achieved using the Ribosomal Database Project (RDP) classifier (Wang et al., 2007) against the Greengenes database (version 13_8). Alpha diversity indices (Chao1, Shannon index) and compositional analysis were calculated in QIIME. Multivariate analyses, including principal component analysis (PCA) and canonical correspondence analysis (CCA), were performed using STAMP (Parks et al., 2014) and PAST v3.14 (Hammer et al., 2001), respectively.

3.5 Isolation of Bacterial Strains

Bacteria were isolated following the method of Poornachandra Rao et al. (2015). Briefly, 10 g of each fermented sample was homogenized in 90 mL of 0.85% phosphate-buffered saline (PBS) and serially diluted (10^{-1} to 10^{-5}). Dilutions were pour-plated on Luria Bertani (LB) agar and incubated anaerobically at 37°C for 24 hours. Morphologically distinct colonies were sub-cultured on fresh LB agar plates to obtain pure cultures. These isolates were maintained in 15% glycerol stocks at −20°C for long-term preservation.

3.6 Morphological and Biochemical Characterization of Isolates

Preliminary identification of isolates was based on Gram staining, cell morphology, and catalase activity. Further biochemical characterization included methyl red test, Voges–Proskauer test, catalase test, citrate utilization, oxidase activity, urease test, phenylalanine deaminase test, gelatin liquefaction test, and carbohydrate fermentation capabilities, as described in standard protocols (Holt et al., 1994; Cappuccino & Sherman, 1996; Ni et al., 2015).

3.7 16S rRNA Gene-Based Molecular Identification

3.7.1 Genomic DNA Extraction and Visualization

Genomic DNA was extracted from overnight-grown isolates using the PureLink Genomic DNA Mini Kit (Invitrogen, USA). DNA integrity was evaluated using 0.8% agarose gel electrophoresis with 1X TAE buffer. Gels were stained with ethidium bromide and visualized under UV illumination.

3.7.2 PCR Amplification and Sequencing

The 16S rRNA gene was amplified using universal primers PA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and PH (5′-AAG GAG GTG ATC CAG CCG CA-3′) (Qin et al., 2009). PCR was performed in a 25 µL reaction volume, and amplified products were resolved by 1.5% agarose gel electrophoresis. Purified amplicons were

sequenced, and the resulting sequences were analyzed using BLAST against the NCBI database for taxonomic assignment.

3.8 Evaluation of Probiotic Potential

3.8.1 Acid and Bile Salt Tolerance

Isolates were cultured in LB broth adjusted to various pH levels (2–9) to evaluate acid tolerance, with OD_{600} readings recorded after 24 h incubation at 37°C (Dowarah et al., 2018; Chen et al., 2022). Bile tolerance was tested by supplementing LB broth with increasing concentrations of bile salts (0.2%–1.6%), followed by similar monitoring of optical density.

3.8.2 Auto-Aggregation Assay

Auto-aggregation properties were assessed following the method of Zommiti et al. (2017). Cell suspensions in PBS were incubated at 37°C, and optical density was measured at regular intervals over 5 hours. The percentage of auto-aggregation was calculated based on the decline in OD_{600} over time.

3.9 Safety Assessment of the Isolates

3.9.1 Antimicrobial Activity

Antimicrobial properties of the isolates were tested using the agar well diffusion method (Sim et al., 2010). Culture supernatants were placed in wells on Mueller-Hinton agar plates inoculated with pathogenic indicator strains. Zones of inhibition were measured after 24 hours of incubation at 37°C.

3.9.2 Antibiotic Susceptibility Test

Antibiotic resistance profiling was conducted using the disc diffusion method on LB agar with standard antibiotic discs, following CLSI guidelines (CLSI, 2016; Singh et al., 2012). The results were interpreted as sensitive, intermediate, or resistant based on the diameter of inhibition zones.

3.10 Biosurfactant Production and Extraction

3.10.1 Oil Displacement Assay

To detect biosurfactant production, the oil displacement method was employed as described by Joe et al. (2020). Culture supernatants were added to the surface of crude oil layered on water, and the diameter of the resulting clear zone was measured after 30 seconds.

3.10.2 Biosurfactant Extraction

Selected isolates were inoculated into MRS-Lac broth and incubated for 72 hours. The culture was acidified to pH 2 using 3N HCl and left overnight at 4°C to precipitate biosurfactant, following the modified method of Kim et al. (2004). Extracts were subsequently purified with dichloromethane and evaporated using a rotary vacuum evaporator.

3.11 Biosurfactant Characterization

Crude biosurfactants were analyzed by ultra-high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (UHPLC-ESI–MS) using a Hypersil Gold C18 column. A linear acetonitrile–formic acid water gradient was applied, and ionization spectra were recorded in the positive mode within the m/z range of 200–2000.

4. RESULTS AND DISCUSSION

The present research comprehensively examined the proximate compositional dynamics and microbial community structures of three traditional fermented food products of Mizoram **Tuaithur** (fermented bamboo shoots), **Bekang** (fermented soybean), and **Saum** (fermented pork fat) evaluated at three fermentation intervals (3rd, 5th, and 7th day). Proximate composition analysis revealed that Tuaithur possessed the highest moisture content (86.93%), whereas Saum exhibited an exceptionally elevated fat content (93.6%). Bekang, on the other hand, was

characterized by its superior protein (19.36%) and carbohydrate (11.03%) concentrations, underscoring its enhanced nutritional potential. Variations observed in macronutrient levels over the fermentation period were marginal but consistent with enzymatic transformations driven by microbial metabolism, as documented by McFeeters (2004) and Sharma et al. (2020).

High-throughput sequencing of the 16S rRNA gene generated a total of 3,424 operational taxonomic units (OTUs), with bacterial communities predominantly comprising members of the phyla **Firmicutes** and **Proteobacteria** across all samples. Analysis of alpha diversity metrics, including Shannon and Chao1 indices, indicated that fermented bamboo shoots harbored the highest bacterial richness and diversity, corroborating findings reported by He et al. (2020). Beta diversity assessments using principal coordinate analysis (PCoA) and hierarchical heatmap clustering further validated that each food type supported a distinct microbial consortium, with fermentation duration exerting a comparatively lower impact on intragroup diversity.

At the genus level, **Lactobacillus** predominated in Tuaithur (91.64%), whereas **Staphylococcus** and **Bacillus** were found to be the most abundant genera in Bekang. In Saum, the bacterial population was dominated by **Clostridium** and **Sutterella**. The notable abundance of *Clostridium* and *Staphylococcus*, both of which include species with pathogenic potential, underscores significant food safety considerations, consistent with previous reports by Keisam et al. (2019) and Fetsch and Johler (2018). Canonical correspondence analysis (CCA) further demonstrated a strong positive correlation between *Staphylococcus* and the higher protein and carbohydrate contents observed in Bekang, suggesting a possible functional role of microbial populations in modulating nutritional attributes during fermentation.

Subsequent culture-based screening led to the identification of two potent bacterial isolates such as **F47 (Bacillus tequilensis)** and **B50 (Bacillus velezensis)** which exhibited considerable probiotic potential. Both strains displayed high tolerance to acidic pH and bile salts, substantial auto-aggregation capacity, and broad-spectrum antimicrobial activity against key foodborne pathogens including

Escherichia coli, *Klebsiella pneumoniae*, *Bacillus subtilis*, and *Salmonella typhimurium*. UHPLC-ESI/MS analysis of the isolates confirmed the biosynthesis of bioactive surface-active metabolites, notably **Surfactin** and **CDP-2,3-bis-(O-phytanyl)-sn-glycerol**, both of which are recognized for their antimicrobial and emulsifying properties (Qin et al., 2025; Koga et al., 2000). Moreover, antibiotic susceptibility testing revealed that the isolates were sensitive to the majority of clinically relevant antibiotics, thereby meeting the safety criteria outlined by FAO/WHO (2002) for probiotic strains.

In summary, this study offers an integrative understanding of the nutritional and microbiological dynamics underpinning traditional Mizo fermented foods. The findings not only elucidate the structural diversity of indigenous microbial consortia but also identify promising bacterial candidates with functional probiotic and biosurfactant-producing capacities, which may have potential applications in food biotechnology, health, and industrial fermentation.

5. CONCLUSION:

The present investigation constitutes the first metagenomic characterization of bacterial community dynamics and proximate compositional attributes in traditional fermented foods indigenous to Mizoram, India. High-resolution microbial and biochemical analyses revealed *Firmicutes* and *Proteobacteria* as the predominant bacterial phyla across all samples, with notable taxonomic heterogeneity observed at the genus level, varying according to both food matrix and fermentation timepoints. Among the cultivable isolates, strains F47 and B50 exhibited pronounced functional phenotypes, including enhanced tolerance to acidic and bile salt conditions, robust auto-aggregation capacity, and broad-spectrum antibacterial activity traits consistent with effective probiotic candidates. Ultra-high performance liquid chromatography coupled with electrospray ionization mass spectrometry (UHPLC-ESI/MS) further elucidated the biosurfactant repertoire of these strains, identifying amphiphilic compounds such as Surfactin and CDP-2,3-bis-(O-phytanyl)-sn-glycerol with established surface-active and bioactive potential. Collectively, these findings underscore the functional and industrial relevance of microbial consortia associated

with traditional fermentation practices, and establish a foundational framework for the bioprospecting and development of next-generation probiotics and biofunctional agents.

6. SCOPE OF THE RESEARCH STUDY

The present research undertakes a comprehensive investigation into the microbial diversity, probiotic potential, and biosafety profiles of selected indigenous non-alcoholic fermented foods of Mizoram, namely *Tuaithur* (fermented bamboo shoots), *Bekang* (fermented soybeans), and *Saum* (fermented pork fat). These traditional fermented products, prepared through spontaneous, uncontrolled fermentation by local ethnic communities, represent complex microbial ecosystems that remain largely unexplored in terms of taxonomic and functional characterization.

This multidisciplinary study integrates microbiological, molecular, biochemical, and bioinformatic methodologies to achieve the following scientific objectives:

- To determine the proximate nutritional composition of the selected fermented food matrices at various stages of fermentation, thereby establishing a correlation between nutrient profiles and microbial succession.
- To elucidate the structure and composition of bacterial communities using both culture-dependent techniques and high-throughput 16S rRNA gene amplicon sequencing, enabling the identification of both culturable and unculturable microbial taxa.
- To screen and functionally characterize isolated bacterial strains for probiotic attributes, including tolerance to acidic and bile environments, auto-aggregation capacity, antimicrobial activity against pathogenic microorganisms, and antibiotic susceptibility patterns.
- To assess the biosafety of potential probiotic strains through in vitro assays, ensuring their non-pathogenicity and suitability for future application in food and therapeutic formulations.
- To evaluate the ability of selected isolates to produce biosurfactants, and to characterize the physicochemical properties of these surface-active

compounds, with an emphasis on their prospective utility in pharmaceutical, agricultural, and biotechnological industries.

The outcomes of this research aim to address critical gaps in the scientific understanding of the microbial ecology and functional potential of traditional Mizo fermented foods. By documenting the probiotic attributes and biosafety of indigenous microbial isolates, this study contributes to the development of safe, efficacious, and culturally rooted functional foods, while also advancing the global repository of probiotic and biotechnologically relevant microbial strains.

7. References:

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