Exploration and documentation of bacteria in some fermented foods and beverages of Nagaland, India

By

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DECLARATION

I, Miss Lydia Yeptho bearing Ph.D. Registration No. Ph.D./BOT/00073 w.e.f. 24/8/2017, hereby declare that the subject matter of my Ph.D. thesis entitled "Exploration and documentation of bacteria in some fermented foods and beverages of Nagaland, India" is the record of original work done by me, and that the contents of this thesis did not form the basis of award for any degree to me or to anybody else to the best of my knowledge. This thesis has not been submitted by me for any Research Degree in any other University/ Institute.

This is further certified that the Ph.D. thesis is submitted in compliance with the UGC Regulation 2016 dated May 05, 2016 (Minimum Standard and Procedure for Award of M.Phil. /Ph.D. Degree). Further, it is certified that the content of the thesis is checked for 'Plagiarism' with licensed software 'URKUND' and satisfies with norms of 'University Grants Commission', Govt. of India. This thesis is being submitted to the Nagaland University for the degree of 'Doctor of Philosophy in Botany'.

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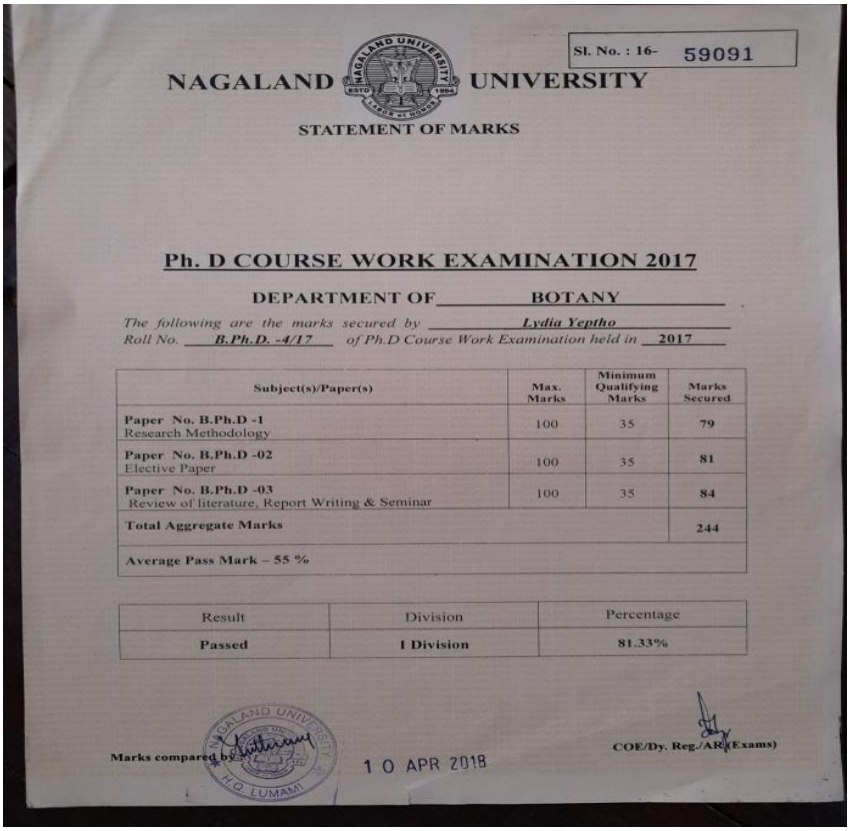
Ph.D. Coursework Mark sheet and Certificate



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STATEMENT OF MARKS

Ph. D COURSE WORK EXAMINATION 2017

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The following are the marks secured by ***Lydia Yeptho***
Roll No. ***B.Ph.D. -4/17*** *of Ph.D Course Work Examination held in* **2017**

Subject(s)/Paper(s)	Max. Marks	Minimum Qualifying Marks	Marks Secured
Paper No. B.Ph.D -1 Research Methodology	100	35	**79**
Paper No. B.Ph.D -02 Elective Paper	100	35	**81**
Paper No. B.Ph.D -03 Review of literature, Report Writing & Seminar	100	35	**84**
Total Aggregate Marks			**244**
Average Pass Mark – 55 %			

Result	Division	Percentage
Passed	**I Division**	**81.33%**

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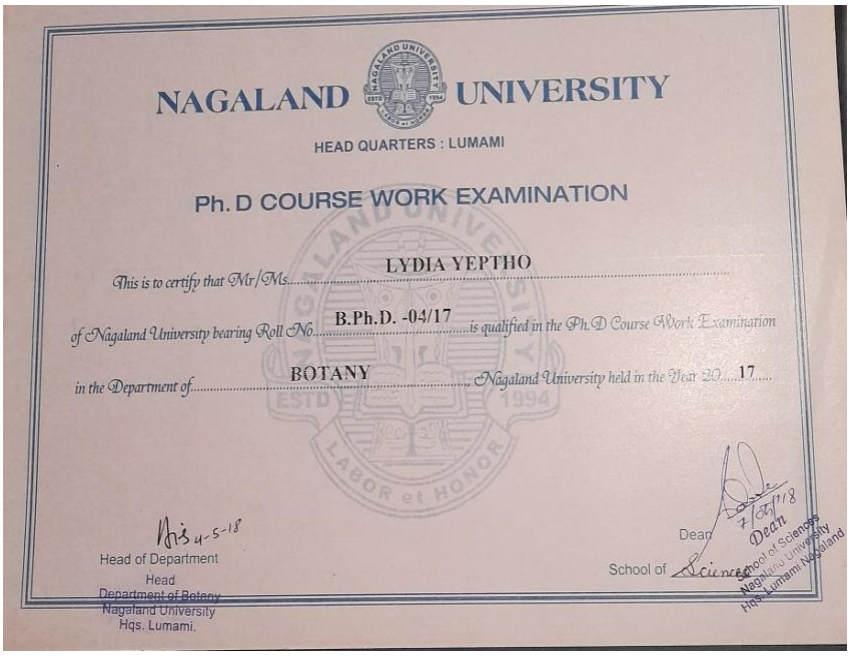

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Abbreviations

Abbreviation	Expanded form
-	Negative
+	Positive
%	Percentage
^{o}C	Degree Celsius
µg	Microgram
µl	Microlitre
µm	Micromolar
***p*H**	Potential of hydrogen
N	Normal
M	Molar
G	Gram
ml	Milliltre
mg	Milligram
hr	Hour
min	Minute
mM	Millimolar
ng	Nanogram
nm	Nanometer
ABS	Absorbance
ACE	Angiotensin Converting Enzyme

AOAC	Association of Official Analytical Chemist
AF	Alcoholic fermentation
AAF	Acetic acid fermentation
ASK	*Ashikumna*
bp	Base pair
BSA	Bovine Serum Albumin
CVD	Cardiovascular disease
COG	Clusters of Orthologous Groups
CNF	Cooked non-fermented
DBA	Dry Bastenga
DNA	Deoxyribonucleic acid
DNSA	Dinitrosalicyclic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
FW	Fresh weight
FPF	Fresh Pork Fats
GA	Glufosinate ammonium
GAE	Gallic Acid
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HVR	Hyper Variable Region
IC50	Half-maximal Inhibitory Concentration
IDF	International Dairy Federation

ITS	Internal Transcribed Spacer
JAP	*Jangpangngatsu*
KAT	*Katsing*
KAPC	*Katsing* Positive Control
KEGG	Kyoto Encyclopedia of Genes and Genomes
KES	*Kese*
LAB	Lactic Acid Bacteria
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NaOH	Sodium hydroxide
NGS	Next Generation Sequencing
OTA	Ochratoxin A
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PCR-DGGE	Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QE	Quercetin
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SAV	Shanxi aged vinegar
SRA	Sequence Read Archive

SS	Starch saccharification
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
T2D	Type 2 diabetes
VBNC	Viable-but- Non culturable
WHO	World Health Organisation
WGS	Whole Genome Sequence
ZU	*Zusem*
ZUPC	*Zusem* Positive Control

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Chapter - 1

Introduction and review of literature

The historical milestones of scientific discoveries have helped shape our understanding of the biology of the microorganisms that drive fermentation. The scientific rationale behind fermentation started with the identification of microorganisms in 1665 by Van Leeuwenhoek and Hooke after which the discovery of metabolic activity of yeast in fermentation by Louis Pasteur (1857) sparked the interest of researchers from every field during the nineteenth century. Fermentation is considered as one of the oldest biotechnological methods for producing foods with desirable attributes such as prolonged shelf-life and favorable organoleptic properties (Smid and Hugenholz 2010). Biochemically it is a metabolic process that derives energy from organic compounds without involving an exogenous oxidizing agent. The fermentation scientist uses an environment that is capable of being controlled to a limited degree (i.e., the fermentor) to

develop a control strategy that will modify inputs to the fermentor system to achieve the desired outputs. In its natural habitat, the microbe will respond to environmental stimuli such as excess nutrients by synthesizing enzymes and biomass capable of exploiting the resource as effectively as possible. However in the fermentor system, the microbe will be inoculated into the fermentation medium and will thus attempt to colonize this environment through rapid growth. The role of the fermentation control strategy is to provide control of environmental effectors such as temperature, aeration, *p*H, water activity and dissolved oxygen that are considered as the optimum conditions for growth and colonization (Jagani et al. 2010).

History of fermented foods

Fermentation drives the ancient old interest in illuminating the cultural cuisines across the globe. The origin of fermentation showcases a shaded image with historians tracking it to about 7000 BC. During those times, leavened bread, cheese, beer and wine were known to the west regions since Neolithic times. Later around 6000-1500 BC fermented food products like yoghurt, sauerkraut, vinegar, butter and pickles along with traditional alcoholic beverages were known to men in East Asian regions (Cavalieri et al. 2003; McGovern 2004; Vouillamoz et al. 2006). Sāttvic food symbolizes food for longevity, prosperity, intelligence, strength, health, and happiness, which includes fruits, vegetables, legumes, cereals, and sweets. Rājasic food symbolizes activity, passion, and restlessness, which include hot, sour, spicy, alliaceous plants including onions and garlic and salty foods. *Tāmasic* foods are considered to be intoxicating and unhealthy, that generally causes dullness and passivity. The ethnic food culture of Nagas maybe categorize as a blend of *sattvic* and *rajasic* food since most of the ethnic food products of Nagaland are

fermented, sundried or smoked using unique techniques of preservation and the culinary culture sequels acquired taste to supplement the organoleptic and palatability of the ethnic food products. The ancestry of fermented foods and beverages of Nagaland are associated with stories of origin, myths and taboos that heralds the past culture. The history of alcoholic fermentation can be traced back to oral traditions using rice as the principal component for alcoholic fermentation. Alcoholic rice beer preparation was common to all the tribes using unique starter cultures and methods of preparation with specific vernacular names. Besides, alcoholic fermentation of wild and domesticated fruits is also common within the local community.

The fermented foods in India are categorized into 10 major groups according to Tamang, 2020 based on the substrates used i.e plant based and animal based fermented foods which are listed below:

1. Fermented cereal foods
2. Fermented non- soybean legume foods
3. Fermented soybean foods
4. Fermented vegetable foods
5. Fermented dairy foods
6. Fermented/ sundried/ smoked fish products
7. Fermented/ sundried/ smoked meat products
8. Amylolytic starters for production of alcoholic beverages and
9. Alcoholic beverages fermented cereal foods
10. Miscellaneous fermented products: fermented tea, crabs, fruits, etc

Microorganisms in fermented foods and their metabolic pathways

The microbiology, biochemistry and nutrition of fermented foods and beverages have been studied by several researchers across the globe. Fermented foods are the hub of consortia of microorganisms, since they are either present as natural indigenous microbiota in uncooked plant or animal substrates, utensils, containers, earthen pots, and the environment (Franz et al. 2014), or as starter culture(s) containing functional microorganisms (Holzapfel 1997) which modify the substrates biochemically, and organoleptically into edible products that are culturally and socially acceptable to the consumers (Platt 1994; Steinkraus 1997; Tamang and Samuel 2010). Microorganisms convert the chemical composition of raw materials during fermentation, which enrich the nutritional value in some fermented foods, and impart health-benefits to the consumers (Steinkraus 2002; Farhad et al. 2010). Microbial community comprising the acetic acid bacteria, lactic acid bacteria, non- lactic acid bacteria, gram-negative bacteria, filamentous mold, and alcohol producing yeasts dominates the Indian fermented foods and alcoholic beverages (Tamang et al. 2007; Chettri and Tamang 2015; Tamang et al. 2016a; Sha et al. 2017; Shangpliang et al. 2018; Sha et al. 2018; Sha et al. 2019). The Lactic Acid Bacteria (LAB) comprising of the genus *Lactobacillus, Streptococcus* and *Leuconostoc* dominates the yoghurt and cheese production by creating an acidic environment that denatures proteins and solidifies it. Homolactic and heterolactic fermentation are the 2 types of fermentation based on the end product result. Yoghurt production is a classical example of homolactic fermentation performed by *Lactobacillus delbrueckii* and *Streptococcus thermophiles* where lactic acid is the end product. Heterolactic fermentation produces a mixture of ethanol, lactic acid and/or acetic acid and CO_2 via the pentose phosphate

pathway. *Leuconostoc mesenteroides* is an important heterolactive fermentation bacteria that causes souring in pickles and sauerkraut. Additionally, LAB is also an important component of our gastrointestinal tracts promising their role in probiotics (Enrica 2012). Alcoholic fermentation is another important fermentation process catalyzed by *Saccharomyces cerevisiae* for production of alcoholic beverages and production of bread and confectionary products.

Fermentation pathway deviates from following the normal respiratory chain reactions and the alternative electron acceptor chain that produces large amounts of NADH. The Krebs cycle and pyruvate dehydrogenase reaction remains nonoperational during anaerobic conditions, thus fermentation recycles the NADH by converting pyruvate produced by the phosphotransferase system into various fermentation products. The common fermentation pathways based on the end product substrate can be grouped into the following types listed below (Gray et al. 1966; Wimpenny 1969; Smith et al. 1983; Spencer et al. 1985):

1. Lactic acid fermentation *(Streptococcus, Lactobacillus)* e.g. Sauerkraut, yogurt
2. Propionic acid fermentation (*Propionibacterium, Bifidobacterium)* e.g. Swiss cheese
3. Mixed acid fermentation (*Escherichia, Shigella,)* e.g. Vinegar, cosmetics, pharmaceuticals.
4. Butyric acid fermentation (*Clostridium butyricum)* e.g. Butter.
5. Butanediol fermentation (*Klebsiella, Enterobacter)* e.g. Chardonnay wine.
6. Alcoholic fermentation (*Candida, Saccharomyces)* e.g. Beer, bread.
7. Acetone-butanol-ethanol fermentation *(Clostridium acetobutylicum)* e.g. Commercial solvents, gasoline alternative.

Role of fermentation in food processing

1. Microbial role in maintaining a healthy gut microflora in mammals (Savage 1986; Hespell 1988; Mackie and White 1990).
2. Production of secondary metabolites such as antibiotics, pre and probiotics, pigments through fermentation (Franco and Coutinho 1991; Calegari-Santos et al. 2016; Harms et al. 2017; Sadh et al. 2018).
3. Production of amino acids, proteins, enzymes, vitamins, fatty acids and surface- active compounds (Kim and Park 2019).
4. Exploitation of hyperthermophilic microrganisms for special metabolic capabilities (Adams 1993; Kelly and Adams 1994; Torregrosa Crespo 2017).
5. Use of non- conventional yeast eg *Yarrowia lipolytica* for production of oils and fats (Carsanba et al. 2018).
6. Derivations of bio products such as biofuels, biogas and biodiesels (Dos Santos Vieira et al. 2019).
7. Developing biosorption and bioremediation processes using microbes or their productive strains to treat waste water and degraded lands (Aracil-Gisbert et al. 2017).
8. Use of microbial enzymes in applied biotechnology (Vittaladevaram 2017).
9. Production of lignocellulose for use as major carbon source by bacteria, filamentous fungi and yeast (De Paula et al. 2019; Hosseini Koupaie 2019).
10. Use of extremophilic microorganisms for high catalytic efficiency in industrial processes (Esclapez Espliego et al. 2018).
11. Preservation of food through formation of inhibitory metabolites such as organic acid (lactic acid, acetic acid, formic acid, propionic acid), ethanol, bacteriocins, etc., often

in combination with decrease of water activity (by drying or use of salt) (Ross et al. 2002; Gaggia 2011).

12. Improving food safety through inhibition of pathogens (Adams and Nicolaides 2008).
13. Improving the nutritional value (Boekel et al. 2010; Poutanen et al. 2009).
14. Organoleptic quality of the food (Lacroix et al. 2010; Sicard and Legras 2011).

Metagenomic studies in food fermentation

Metagenomics studies the DNA of microorganisms directly from the environment without obtaining a pure culture and the data serves to represent the original sample. Microbiological study encompasses the structure and function of microbe community, the mechanisms of internal communities and interrelationships, the relationship between microbes and their host or environments. The application of metagenomics for food fermentation is gaining popularity with the rise of NGS due to affordability, speed and quality of data. The rise of Next-Generation Sequencing (NGS) technologies has revolutionized the field of microbial ecology by providing comprehensive description of the microbial DNA content in a given sample. NGS can be briefly categorized into “whole genome sequencing” (WGS) and “metagenomics”, where NGS is applied to a biological sample generating sequences of multiple (if not all) microorganisms in that sample.

Metagenomics provide insights on food safety, quality improvement, predicting the presence or emergence of pathogens and spoilage microorganisms based on changes observed in entire microbial communities, as well as the potential to characterize unknown microbiota. Obtaining a pure culture was the standard culturing laboratory technique practiced by traditional microbiologist and it provided less than 1% information on microbial diversity (Torsvik et al. 1990). Although significant efforts on culturing of as-

yet- uncultured microbes seems to be increasing, techniques inclined towards the culture-independent methods provides more holistic genetic information. However these methods are not without error, and may mislead the interpretation of results. For instance, for proper accession of nucleic acids, the cell lysis of microorganisms must be homogenized and the reagents plays a crucial role in determining accurate results (Bag et al. 2016; Knudsen et al. 2016). Another factor can be the inability to detect the lesser no of microorganisms in a sample swarmed by a dominant microbe. A study on a traditional cereal fermented food of Africa, showed the presence of *Clostridium perfringens* and *Bacillus cereus* in the clone library, however the PCR-DGGE profile failed to detect it from the main bands (Oguntoyinbo et al. 2011). Though discrepancy of results still exists between classical approach and use of molecular tools, metagenomic analysis using bioinformatics pipelines still remains a reliable approach to study and generate metagenomic datas pertaining to fermented food samples. Metagenomics in food fermentation thus reveals the identity of phylogenetically distant related species that maybe non-viable in laboratory conditions and their activity influences the physiological properties of similar food characteristics (Cocolin et al. 2013). The organoleptic properties of any fermented food product is also influenced by the dynamics of microbial population, the food composition, and the interaction between the microbiome and the fermenting food matrix which produces change in physicochemical conditions. As fermentation progresses, the no of microorganisms decreases and consequently a dominant microflora emerges that maybe viable-but- nonculturable (VBNC) and/or non-viable states of microorganisms and metagenomic studies helps in assessing the removal of toxinogens

from a food substrate (Hammes and Tichaczek 1994), monitors the survival and render protection from pathogens (Adams and Mitchell 2002, Adams and Nicolaides 2008).

Scientific findings on the beneficial role of fermented foods.

Foods produced by fermentation have a reduced risk of contamination while being rich in anti-microbial end-products, such as organic acids, ethanol, and bacteriocins. Unlike those present in the starting materials fermented foods also include the new and desirable tastes and textures. Beyond these characteristics, the outcomes of fermentation and the contributions of microbes can provide additional properties beyond basic nutrition. These benefits might extend to immediate physiological responses, as advocated in fermented milk, consumption showed improved glucose metabolism and reduced muscle soreness (Iwasa et al. 2013). Other long-term prospective studies showed reduced risk of cardiovascular disease (CVD), type 2 diabetes (T2D), and overall mortality from frequent yogurt consumption (Muthu et al. 2013; Chen et al. 2014; Tapsell 2015; Eussen et al. 2016). Similarly, health benefits claims of fermented foods have been proposed, against arthritis and sclerosis, although clinical data have not yet been reported (Baroja et al. 2007). Lastly, the emerging new axis of microbiota-gut-brain research, indicates that fermented food consumption can alter mood and brain activity (Hilimire et al. 2015).

Lactose- intolerant individuals typically tolerates cheeses and yogurt as lactose originally present in the milk is fermented and yogurt, in particular is generally well tolerated by lactose-maldigesters due to the in vivo release of b-galactosidase by yogurt cultures (Savaiano 2014). In plant and vegetable fermentations, the growth of LAB enhances conversion of phenolic compounds such as flavonoids to biologically active metabolites via expression of glycosyl hydrolase, esterase, decarboxylase, and phenolic

acid reductase (Filannio 2015).The subsequent reaction of these metabolites with anthocyanidins results in formation of pyranoanthocyanidins or 3-deoxypyranoanthocyanidins (Bai et al. 2014). The B vitamins including folate, riboflavin, and B12 are synthesized from various non-vitamin precursors by certain bacteria in plant and dairy foods (Russo 2014; Chamlagain et al. 2015). Additionally, certain secreted proteins and exopolysaccharides produced during food fermentations might serve as anti-oxidants (London et al. 2014; Hong et al. 2015), prevent adhesion of pathogens to the intestinal mucosa (Chen et al. 2014), or confer immune-stimulatory (Makino et al. 2016) or hypocholesterolemic activities (Martoni et al. 2015). The ingestion of fermented foods potentially increases the numbers of microbes in the diet by up to 10 000-fold (Lang et al. 2014), and consuming 'living' fermented foods on a daily basis could be equivalent to introducing new, albeit transient microbes into the indigenous, intestinal microbiota (Ple et al. 2015). Such diets contrast with the highly processed and sanitized foods that limit microbial exposures. The hygiene (or diversity) hypothesis proposes that such microbial exposures are essential for the normal development of immune system and neural function (Stefka et al. 2014). Fermented foods, thus show tremendous potential as a practical vehicle in which to provide established probiotic strains to people in low-income countries (Kort et al. 2015; Mpofu et al. 2014).

Food studies on status of fermented foods

Gastronomy of South Asia, North Asia, Far-East Asia and the Indian subcontinent excluding Western and Northern India consists of boiled rice with fermented and non-fermented food products, meat, fish, vegetables and pickles while in the West Asian continent, Europe, North America, Australia, New Zealand, the Western and Northern part of India, wheat/barley-based breads/loaves comprise a staple diet followed by milk and fermented milk products, meat, and fermented meats (Tamang and Samuel 2010). Sorghum/maize porridges, on the other hand, are the main courses of diet with many fermented and non-fermented sorghum/maize/millets, cassava, wild legume seeds, meat, and milk products in Africa and South America. Studies on Shanxi aged vinegar (SAV), a well-known vinegar used in China for more than 3000 years showed that microorganisms and their metabolites change along with the successive stages of fermentation (Wu et al. 2012; Nie et al. 2017). The stress conditions naturally imposed to yeasts during grape juice fermentation showed that Glufosinate ammonium (GA), a widely used herbicide inhibits glutamine synthetase (Matallana and Aranda 2017) and this inhibition starves the internal amino acid causing the activation of different nutrient sensing pathways and inhibiting the growth of yeast to further prolong the quality of wine (Vallejo et al. 2017). Studies on the probiotic properties of a *Bifidobacterium* strain and its functional features and fermentation behavior in rice gruel revealed that the isolate MKK4 may help in human wellness to combat life-style related diseases (Ray et al. 2017). Studies on the preparation process, microbial, and chemical compositions of the 2 main types of chhurpi found in Ladakh -soft and hard (sun dried) showed that yeast, mold, lactic acid bacteria, and *Bifidobacterium* were the major participating microbes. The amount of riboflavin

(162.71 mg/g), thiamine (64.48 mg/g), and vitamin C (23.53 mg/g) were higher in soft chhurpi compared to the hard chhurpi. It also contained a high amount of protein, carbohydrates and low amount of fats, ethanol and methanol (Panda et al. 2016).

Fermented soybean foods supplement the local diet with inexpensive, high digested plant protein content and promote health benefits. *Kinema* is a soybean fermented food similar to *Natto* of Japan, Chinese *Schuidouchi* and Korean *Chungkukjang.* Analogous products are found in other North-Eastern States of India, known by different names, like *Hawaijar* in Manipur, *Turangbai* in Meghalaya, *Akhuni/ Axone* in Nagaland, *Bekang* in Mizoram and *Peruyan* in Arunachal Pradesh (Jeyaram et al. 2008). The methanolic extract of *Kinema*, fermented using *Bacillus subtilis*, and cooked non-fermented (CNF) soybean were evaluated by four in vitro methods, namely stable DPPH - scavenging activity, Fe^{3+}-reducing power, Fe^{2+} chelating activity, and activity in linoleic acid emulsion system and it was concluded that *Kinema* may be exploited as a functional food to alleviate oxidative stress. Data revealed enhanced free radical-scavenging activity, metal-chelating ability, reducing power and lipid peroxidation inhibitory activity. All these antioxidant activities of *Kinema* were significantly higher ($P < 0.05$) than those of soybean, suggesting the role of fermentation in enhancing these attributes (Moktan and Sarkar 2008).

Study on the comparative biochemical analysis of some foreign liquors such as beer, gin, vodka etc against the indigenous rice beverages from certain tribes of Assam showed higher content of proteins, carbohydrates and fats. The protein and carbohydrate content was found to be as high as 6.2 mg/ml and 13.2 mg/ml in *Jumai* while free amino acid content was found to be highest in *Judima* (0.24 mg/ml). The least protein and

carbohydrate content was found to be in beer kingfisher with values as 0.11 mg/ml and 1.22 mg/ml respectively while the free amino acid content remains more or less the same (0.08 mg/ml and 0.09 mg/ml) (Arjun 2015). Studies on *Hawaijar,* a non-salted traditional fermented food of Manipur revealed *Bacillus* to be the pre-dominant organism and preparation by traditional method showed that *B. subtilis* fails to inhibit *B. cereus* and becomes inactive in stored food which allows the spores of *B. cereus* to germinate and produce enterotoxin. Also consumption as fresh salads with vegetables, chillies and salt raises the risk of food poisoning (Nout et al. 1998). Studies on *Ngari* prepared from *Puntius sophore (Ham.),* revealed drastic change in structural bacterial community with respect to the raw material. The most dominant bacteria was found to be *Staphylococcus cohnii* (38.0%), including *Tetragenococcus halophilus* (16.8%), *Enterococcus faecium* (7.2%), *Bacillus indicus* (6.3%) and *Staphylococcus carnosus* (3.8%) (Devi and Jeyaram 2015). Bamboo based food products provides rich nutrient composition of carbohydrates, proteins, minerals, fibres, vitamins, phytosterols, phenols and very less fats (Nirmala et al. 2007). Study on the microbiology, biochemistry and technology of bamboo shoot revealed that the chemical composition of commonly edible bamboo shoots such as *Bambussa polymorpha, B. pallida* had high water content and low hydrocyanic acid content. Hydrogen cyanide content (mg/gm) of the different regions of the shoot showed highest in tips, followed by middle portion and the base (Choudhury et al. 2011). Study on the natural microbial flora of bamboo shoot also showed the presence of *Lactobacillus plantarum, L. brevis, L. casei, L. fermentum, L. curvatus, Leuconostoc mesenteroides, L. fallax* and *Tetragenococcus halophilus* as predominant and various lactic acid bacteria played dominant role in imparting flavor, taste and aroma (Nongdam 2015). The study to

evaluate the nutrient components and antioxidant capacities of bamboo shoot (*Phyllostachys praecox*) by three methods of cooking namely boiling, steaming and stir-frying for 5-10 mins showed that stir- frying is more suitable for bamboo shoots as it could obtain the maximum retention of antioxidant capacities (Zhang et al. 2011).

With the increasing trend on studies of fermentation and their products thereof, it is also imperative to study the safety of these products. Adedeji et al. evaluated two commonly consumed traditional condiments (*Iru* and *Ogiri*) and their respective raw seeds (locust bean and melon) and found out that it was contaminated with potentially pathogenic species such as *Alcaligenes faecalis, Bacillus anthracis, Proteus mirabilis* and *Staphylococcus sciuri* subsp. *sciuri (*Adedeji 2007). Studies on 51 *Doenjang* samples showed contamination with *Bacillus cereus* and the isolated strains were tested were positive for diarrheal toxin genes (Park et al. 2016). Common pathogens found in African fermented foods includes *Bacillus cereus, Escherichia coli, Salmonella, Staphylococcus aureus, Vibrio cholera, Aeromonas, Klebsiella, Campylobacter and Shigella* sp (Gadaga et al. 2007). In a survey on several types of food products on sale in Portugal some fermented foods (ready-to-eat) were found positive for the presence of *Listeria monocytogene* (Mena et al. 2004). Besides pathogens, mycotoxins such as Ochratoxin A (OTA) have been found to contaminate cocoa beans through fermentation (Dano et al. 2013). Fermentative sausages have also reported to produce biogenic amines mainly produced by fermentative microbial population (Suzzi and Gardini 2003).

Status of fermented foods in Nagaland, India.

The knowledge on the role of microbial application on fermented foods and beverages of Nagaland is at its nascent stage with few fermented foods studied so far and little of its microbiota known. Studies on indigenous rice beer of Nagaland, *Zutho*, containing 5.0 % (v/v) ethanol subjected to analytical and microbiological characterization showed the presence of a strain of *Saccharomyces cerevisiae*, found to be suitable for ethanol fermentation as brewing yeast. This product was also found to be similar with respect to its taste (sour), smell (fruity aroma) and its unique aroma to those of sprouted rice *sake* and Japanese *sake* (Teramoto et al. 2002). The dominant microorganisms in *Axone* (fermented soybean) includes *Bacillus subtilis, B.licheniformis* ,*B.cereus* (Jamir and Deb, 2018), *Aeromonas hydrophila, A. eucranophila, A. salmonicida, B. coagulans, B. pantothenticus, B lentus and B. stearothermophilus, Erwinia ananas, Enterobacter* sp, *Klebsiella oxytoca, K. pneumonia, Hafnia alvei, Salmonella enterica ser Typhimurium, Morganella morganii, Pseudomonas* sp., *Providencia rettgeri* and *Proteus sp*. (Singh *et al.* 2014). Its nutritional value contains moisture (%): 50, *p*H: 8, protein (g/100g): 42.1, crude fibre (g/100g): 1.61, reducing sugars (%): 29.7 (Jamir and Deb, 2018). Other fermented foods such as *Tsutuocie* (fermented cucumber) and *Bastenga* (fermented bamboo shoot) were found to be dominated by *Bacillus subtilis* whereas *Bacillus licheniformis* formed the predominant microorganism in *Anishi* (fermented taro leaves) and *Hungrii* (fermented mustard leaves) (Deb and Jamir, 2020).

The microbial repertoire in fermented foods and beverages includes complex interaction of microbes and their metabolic activities. The principal function of this study is to link the food culture and culinary values of a community to underlying mechanisms

of scientific rationale driving it and administering knowledge on nutritional food consumption implicit to human health. The subsequent chapters will further discuss in detail the general features of food habits, edible plants, herbs and spices, raw materials used for food fermentation, method of preparation, organoleptic properties of the foods and the culinary heritage linked with the foods among the tribal communities of Nagaland. The proximate composition and nutritional values of some fermented foods and beverages of Nagaland will also be highlighted with unfolding of the microbial profile in *Katsing* (fermented rice beer) and *Zusem* (fermented bamboo shoot) using metagenomic application.

Chapter - 2

Documentation of the ethnic food habits, fermented foods and beverages of Nagaland, India

Introduction

Nagaland is the 16th state of the Indian Union, established on December 1, 1963 lies between 25^{o}06'N and 27^{o}04'N latitude and 93^{o}20'E and 95^{o}15'E longitude and covers an area of 16,579 sq. Km (Deorani and Sharma 2007). It is exquisitely rich in flora and fauna and the world's tallest *Rhododendron* is found in Mt. Japhu and *Cymbidium tigrinum*, a species of orchid was first discovered in Nagaland. Nagaland supports a rich history of tribes where 16 tribes are recognised as major tribes and except the difference in language, all Naga tribes have similar yet unique traditions and practices. Nagas are well known for their feasts of merit, colourful customs and majority of the Nagas are Christians, practice jhum cultivation and live in villages. Each village consists of several clans and scores of dialects co-exists both among the various tribes and within a specific tribe. The family organization is patriarchal and endogamy is considered to be the preferred mode of marriage. While irrigation is the main occupation of the Nagas, dexterity is especially seen among the woman in the society where they are regarded with respect and honour. A

central feature of Naga life is the essence of celebration of series of festivals throughout the year where each tribe has its own folklore and song that expresses all the exuberant concern for life.

Cultural cuisine of local and traditional foods before modernization and industrialization provided a cultural identity among the societies (Jordana 2000). The continuous interaction of cultures with the local ecosystems resulted in the traditional food systems harbouring indigenous knowledge over generations (Kuhlein 2009). Traditional foods form culturally accepted local products of a particular culture in a community (Kuhnlein and Receveur 1996) and reflects the cultural identity, sensitivity and health perception of a community. Ethnic people produce ethnic or traditional foods that are culturally and socially accepted to the consumer by using raw materials that are locally available (Tamang 2010a, 2010b, 2010d). With the onset of human civilization, the dietary cultures of tribal communities across the world have been shaped by the indigenous food products (Tibor 2007). Also ethnic food serves to meet the hunger and provides cure as medicine (Shin and Jeong 2015; Thapa and Tamang 2015). The knowledge on the of use of edible plants, their processing and conservation for consumption and use as medicines accounts to the increasing and cumulative learning by the societies having a close connection with nature (Singh et al. 2007).

Ethnobiology or food gastronomy reflects the interactions between the environment and local societies which results in bio cultural heritage of traditional fermented beverages that help sustain the local traditional foods and implements food sovereignty (Nabhan 2010; Pieroni et al. 2016). Indigenous societies during colonisation have suffered great discredit which is especially true in the case of fermented beverage

hence documenting the cultural relevance is important to recuperate the traditional fermented beverages in already existing local contexts (Madej et al. 2014). To recuperate the local gastronomic condition, that seems to be lost in the present world, food- and- wine culture paves way to an experienced progressive marginalisation that unites a community (Fontefrancesco 2015, 2018). The food tradition in this sense is becoming a dream object of a new modern western as described by Ferguson (McKay 2011). Traditional gastronomy plays the dual nature of prolonging the dynamics of present consumption and tends to idealize the local feebly to promote economic source for the community. Thus, it can be safely categorised as embeddedness for socio- cultural rooting, embeddedness to redistribution of local economic resources, and the strength of social ties strengthened with great strength of actors (Granovetter 1973, 1985). In this context, Barthes suggested the dynamics related to traditional food gastronomy as an idea to express the beliefs and opinions of the individual and social ethical orientation equally (Barthes 1961). However, it is important to analyse the history and cultural roots of a food product and also to analyse the value chains and productive structure of these products (Fontefrancesco 2020). The traditional knowledge on the ethnic food preparation among the Nagas is generally confined to the women folks or elders in the society and the knowledge is passed thereon. The food products are prepared at household level and the raw materials are gathered from the jungle, fields or cultivated in kitchen gardens. The degree of diversity on method of food preparation and products varies between certain villages and individual perspective however they have expertise in the art of food processing and these foods in turn forms an essential part of their cultures and customs.

2.2. Methodology

Data collection, documentation and sample survey was done across 9 districts in Nagaland namely Longleng, Kohima, Zunheboto, Phek, Mokokchung, Tuensang, Mon, Dimapur and Wokha. Purposive sampling (Patton 1990) and snowball sampling (Coleman 1958-1959) were adopted to document the traditional method of preparation and the raw materials used. Selected households from each district were either personally or telephonic interviewed to collect the data on gastronomy, edible plants, herbs and spices, the culinary and the ethical values of traditional dishes.

2.3 Results and Discussion

2.3.1 Ethnic food habits and food preparation

Most of the Nagas live in small villages and its land supports a considerable amount of crops like corn, pulses, fibres, potatoes, tobacco, oilseeds, sugarcane, millets and rice besides the abundance of wild fruits and vegetables. Traditionally the Nagas lived a hunter- gatherer lifestyle but the transition influenced mainly by religion and education has led to sedentary phase with changes in beliefs and palatable flavour profile. Food is reflected as a part of our rich culture and the range of fermented food products produced and consumed serves as a gateway to understand our ethnic and unique food habits prepared at its best. Distinct cuisines separate a certain tribe from others while most food overlaps between the various tribes. A typical traditional Naga meal is cooked rice, meat or vegetable cooked with a fermented food product supplemented with chutney in a traditional wooden plate. A cup of red tea at the end of the meal wraps up the repast. The traditional and culinary knowledge are passed down to younger generations mostly by mothers to their daughters by cooking together with them. The method of preparation also varies according to individual perceptions expounding the diversity of traditional knowledge. Meat and meat products are either fermented, sundried or smoked over kitchen hearth. Entrails are a delicacy among the Nagas, prepared in unique ways with varieties of spices and condiments. Tubers like tapioca (*Manihot esculenta*) and sweet potato (*Ipomeae batatas*) and yams are traditionally consumed by covering it with hot ash near the fireplace which is used as a source of carbohydrate particularly in rural villages of the Eastern Naga tribes. Cultivated cereals like Job's tears (*Coix lacryma-jobi*), finger millet (*Eleusine coracana*), and maize *(Zea mays)* are used as traditional snacks. Various

herbs and leafy vegetables are a part of the regular diet. Leaves of *Zanthoxyllum* and *Allium* are used widely to enhance the flavour profile of the curries. Some commonly available species of bamboo harvested as edible shoots are *Bambussa bambos, Bambusa balcooa, Bambussa tulda, Chimonobambusa callosa, Dendrocalamus hamiltoni, Dendrocalamus hookeri, Dendrocalamus giganteus* and *Melocanna baccifera*. Young shoots are harvested during the month of late May to October and are consumed as fresh, fermented overnight with ash and cooked the following day, or made into pickles. Other methods include fermenting the shoots by pounding or shredding into pieces and collecting the brine which is used as a condiment for curries. *Bastenga* is the common name of fermented bamboo shoot in Nagaland, however it is known by various other names according to the language of each tribe. Originally *'Bas'* means 'smelly' and '*Tenga'* means 'sour' in Nagamese which is the most common language spoken in Nagaland. *Bastenga* is of two types-wet *Bastenga* and dried *Bastenga*. The wet *Bastenga* is off-white in colour, whereas dried *Bastenga* is golden brown and both types give off a pungent smell. Besides food, consumption of varieties of indigenous drinks is also very popular among the Nagas. Fermented rice beverages are common alcoholic beverages prepared by the various Naga tribes closely relate to their use in religious ceremonies, social gatherings and festivals. During ancient times, alcoholic rice beverages were served to all guests, irrespective of gender and status however with the blend of religion and western culture it has now being replaced by tea or green teas and herbal teas. Preparation of fermented fruit beverage is also very common among the Naga tribes. The fruits from wild as well domesticated fruit trees are used for preparation of these beverages, some of the fruits commonly used are listed in Table 2.1.

Table 2.1. Common fermented fruit beverages of Nagaland with the local name, substrate used, scientific name and family

Local name	Substrate used	Scientific name	Family
Bell zü (Sumi tribe)	Passion fruit	*Passiflora edulis*	Passifloraceae
Angaras zü (Sumi tribe)	Pineapple	*Ananas comosus*	Bromeliaceae
Aucho zü (Sumi tribe)	Banana	*Musa paradisiaca*	Musaceae
Chepo dzü (Angami tribe)	Wild apple	*Docynia indica*	Rosaceae
Kholethi zü (Sumi tribe)	Indian gooseberry	*Phyllantus emblica*	Phyllanthaceae
Kothal zü (Sumi tribe)	Jackfruit	*Artocarpus heterophyllus*	Moraceae
Khola dzü (Angami tribe)	Nepali hog plum	*Choerospondias axillaris*	Anacardiaceae

Table 2.2. Fermented food products, local name, parts used as substrate used and the sensory properties of some common fermented foods of Nagaland

Substrate used	**Local name**	**Sensory property**
Mustard leaves	*Ahequ* (Sumi tribe)	Dry; fermented; curry
Tender bamboo shoots	*Bastenga* (Nagamese)	Fermented; pungent smell; curry
Seeds of soybean	*Axone* (Sumi tribe)	Sticky; dry; flavored; curry
Crab and Sesame seeds	J*angpangnatsu* (Ao tribe)	Semi- solid, flavored; curry
Taro leaves	*Anishi* (Ao tribe)	Dry; fermented; curry
Pork fats	*Thevocie* (Angami tribe) / *Ashikumna* (Sumi tribe)	Semi-solid, flavored; curry
Cucumber fruits and leaves	*Cutocie* (Angami tribe)	Liquid, flavored; curry

Table 2.3. Edible plants: Herbs, spices and condiments listed with their scientific name, family, parts used and their sensory property as utilised by the Nagas of Nagaland, India

Product	**Scientific name**	**Family**	**Parts used**	**Sensory property**
Slipper gourd	*Cyclanthera pedata*	Cucubitaceae	Fruits	Dry; yellow coloured; curry
Chinese onion	*Allium chinense*	Amaryllidaceae	Bulbs	Dry; curry
Garlic	*Allium sativum*	Amaryllidaceae	Bulbs	Dry; curry; chutney
Dried taro stem	*Colocasia esculenta*	Araceae	Stem	Dry; chutney
Hooker chives	*Allium hookeri*	Amaryllidaceae	Leaves	Dry; curry
Fern	*Diplazium esculentum*	Athyriaceae	Leaves	Dry; curry
Shallot	*Allium ascalonicum*	Amaryllidaceae	Bulbs	Dry; curry
Dried roselle	*Hibiscus sabdariffa*	Malvaceae	Pods and young leaf	Dry; chutney
Dried goosefoot	*Chenopodium album*	Amaranthaceae	Seeds	Dry; sour; red;
Perilla seeds	*Perilla frutescens*	Lamiaceae	Seeds	Dry; curry
Winged	*Zanthoxylum* sp	Rutaceae	Seeds and	Dry; curry

prickly ash			leaves	
Caper vine	*Stixis suaveolens*	Resedaceae	Fruits	Dry; pickle
Chinese sumac	*Rhus chinensis*	Anacardiaceae	Fruits	Dry; curry
Tree tomato	*Solanum betaceum*	Solanaceae	Fruits	Red to orange; sour; chutney
Bitter tomato	*Solanum aetheopicum*	Solanaceae	Fruits	Pink; pungent; side dish; curry
Indian night shade	*Solanum indicum*	Solanaceae	Fruits	Bitter; curry, boil; chutney
Ginger	*Zingiber officinale*	Zingiberaceae	Rhizome	Pungent; curry ; chutney
Red ginger	*Zingiber officinale var. rubra*	Zingiberaceae	Rhizome	Pungent; curry
Turmeric	*Curcuma longa*	Zingiberaceae	Rhizome	Pungent; curry; chutney
King chilly	*Capsicum chinense*	Lauraceae	Fruits	Pungent;curry; chutney
Cardamom	*Elettaria cardamomum*	Zingiberaceae	Seeds	Dry; boil; tea
Himalayan knotweed	*Koenigia polystachya*	Polygonaceae	Leaves	Sour; side dish
Indian bay leaf	*Cinnamomum*	Lauraceae	Leaves	Curry; tea; side

	tamala			dish
Holy basil	*Ocimum tenuiflorum*	Lamiaceae	Leaves and flowers	Pungent; curry; chutney
Sawtooth corainder	*Eryngium foetidum*	Apiaceae	Leaves	Curry; chutney
Black pepper	*Piper nigrum*	Piperaceae	Fruit	Dry; curry; chutney
Mint	*Mentha arvensis*	Lamiaceae	Leaves	Curry; chutney
Bird's eye chilli	*Capsicum annuum*	Solanaceae	Fruits	Pungent; curry; chutney
Heart leaf	*Houttuynia cordata*	Saururaceae	Leaves and roots	Chutney; curry
Black-Galangal	*Alpinia nigra*	Zingiberaceae	Stem	Bitter; boil; side dish
East Indian glory bower	*Clerodendrum glandulosum*	Lamiaceae	Leaves	Bitter; boil; side dish
Passion fruit	*Passiflora edulis*	Passifloraceae	Leaves	Bitter; boil; side dish
Okinawan spinach	*Gynura bicolor*	Asteraceae	Leaves	Boil; slippery; side dish
Indian pennywort	*Centella asiatica*	Apiaceae	Leaves	Bitter; boil; raw; side dish

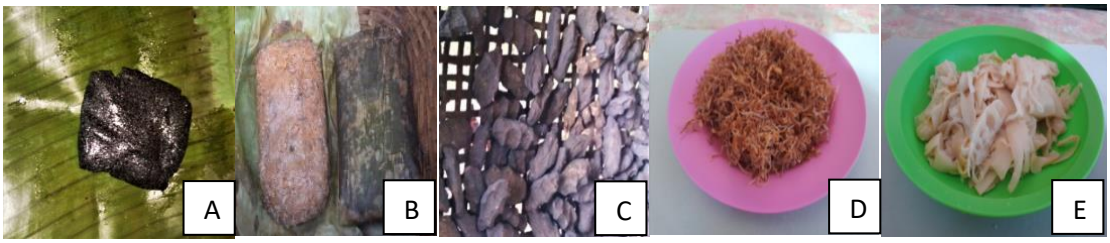

Figure 2.1(A-E): Common fermented foods of Nagaland. A: Fermented crab (*Jangpangngatsu*), B: Fermented soybean (*Axone*), C: Fermented taro leaves (*Anishi)*, D: Fermented bamboo shoot (dry *Bastenga*), E: Fermented bamboo shoot (wet *Bastenga*).

Figure 2.2(A-L): Common edible plants, herbs and spices of Nagaland. A: *Allium ascalonicum,* B: *Allium chinense,* C: *Hibiscus sabdariffa,* D: *Allium sativum,* E: *Colocasia esculenta,* F: *Zanthoxylum* seeds, G: *Chenopodium album,* H*: Perilla frutescens,* I: *Rhus chinensis,* J*: Diplazium esculentum,* K: *Brassica campestris,* L: *Elsholtzia communi.*

2.3.2. Ethnobiology of the traditional alcoholic rice beers of Nagaland, India

Nagas primarily are agriculturalist and it is only with the recent trends of development, the society has seen a transition from agriculturalist to fragile development of economy. The genesis of most of the alcoholic rice beverages is uncertain but since rice is the staple food of the Nagas, it can be ascertained that the local custom of preparing traditional rice beverages has developed through diversified class of starter cultures unified by rice. This exemplifies diversity of traditional epistemology shaped by adaptations to local bio-cultural context, raw materials used, fermentation process, storage processing, and sensory properties. Rice beer was used as the principal beverages served during festivals, religious ceremonies and customary rites. It was the hero drink served around the hearth, accompanied by songs and folklores which is the common form of preserving oral traditions. *Zutho* (Fig 2.3T) which seems to be the most popular rice beer of Nagaland was principally used as an energy booster drink which provided immunity and also served to commemorate the arrival of a new born baby. The use of rice beers was common during marriage ceremonies, for example, *Aji*, (Fig 2.3D) was used to serve to all the guests and relatives during betrothal ceremony. At present, most of the rice beers are no longer served during festivals, however *Zou- ngao* (Fig 2.3H) is still served during Gan-ngai festival celebrated by the Rongmei Naga tribe. The popularity and use of *Aji, Katsing* (Fig 2.3K), *Khe* (Fig 2.3P) and *Zou- ngao* seems to be confined within the tribes and this research work serves as the first of its kind to document the traditional method of preparation of the four rice beers. Table 2.4 below discusses in detail about the 5 alcoholic rice beverages of Nagaland.

Table 2.4. Details on the various alcoholic rice beers of Nagaland and the tribes unique to it, along with their culinary heritage

Product	**Starter culture**	**Tribe**	**Sensory properties**	**Culinary heritage**
Aji	Seeds of *Solanum indicum* + unhusked rice grains	Sumi	White, low alcoholic	Served to visitors, particularly during engagement party of a Sumi couple. Used traditionally during Tuluni and *Ahuna* festivals.
Katsing	Roasted *Chenopodium* sp seeds	Ao	Milky white, low alcoholic	The solid part is sweetened and served as snack, served to visitors.
Khe	Bark of *Senegelia pennata* + unhulled rice grains	Angami	Light yellow, strong alcoholic	Less common and preferred by men, sold commercially at household level and not common for use in festivals or religious customs.
Zou-ngao	Pounded unhulled rice	Rongmei	White, moderate alcohol content	Used as a celebratory drink to commemorate the arrival of a new born baby. Popular beverage served during social gatherings or village body meetings. It is still used during *Gan – ngai* festival which is celebrated on the first week of January.
Zutho	Pounded unhulled rice	Angami	White, low-alcoholic	Used during *Sekhrenyi* festival, most popular drink of Nagaland. Traditionally used as a substitute of tea and sold commercially in Kohima district.

Figure 2.3(A- T): Pictorial representation of the various fermented indigenous rice beverages of Nagaland, India. A: Plant of *Solanum violaceum* B: fruits of *Solanum violaceum*, C: germinated rice grains. D: *Aji*, E-H: Pictorial representation on the traditional method of preparation of Z*ou-ngao* by Rongmei tribe of Nagaland. (P.C. Mercy Gangmei, 2020), I and J: *Chenopodium* sp. K: *Katsing*, L: rice grains soaked in water, M: bark of *Senegalia pennata.* N and O: *Khekhrei.* P and Q: *Khe* at a local vendors place, R: rice soaked in water, S: germinated rice grains, T: *Zutho*

2.3.3. Study of social dimensions on status quo of *Zutho*, a fermented rice beer of Nagaland, India

Traditional method of preparation of *Zutho*

Flowchart as follows

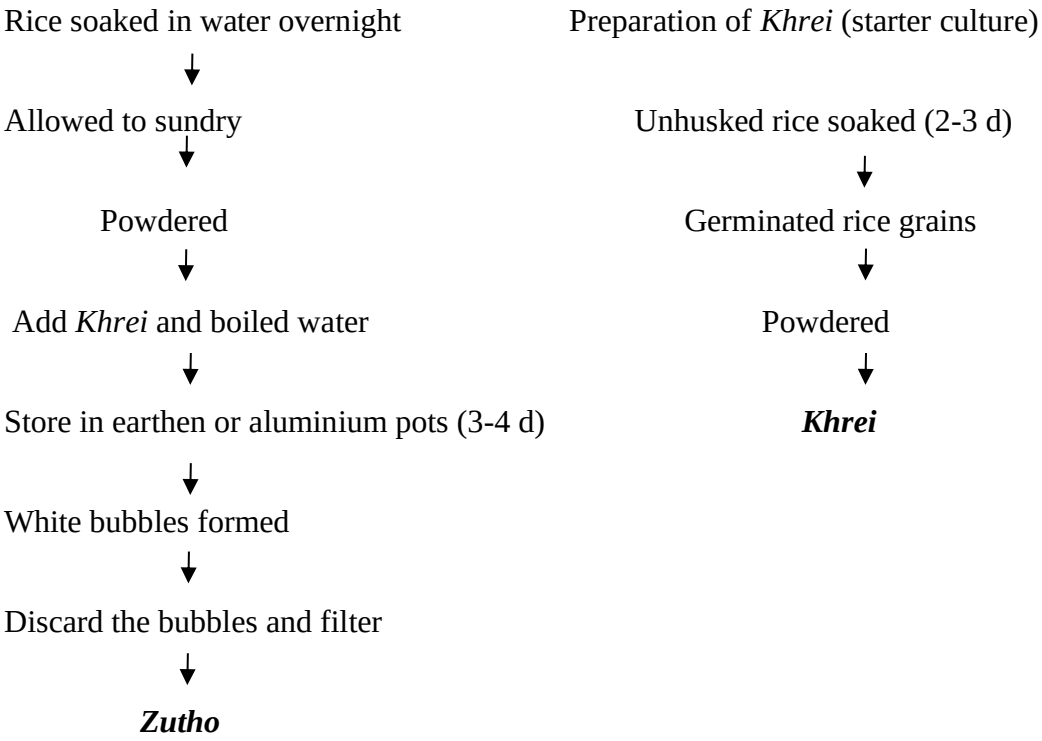

Figure 2.4(A-F): Pictorial representation on the raw materials used for preparing *Zutho* by Angami Naga at Bara Basti, Kohima village. A: rice soaked in water, B: *khrie* (germinated rice grains), C: rice and *khrie* after 3 days of fermentation, D: *Zutho*, E: earthern pots used for brewing *Zutho*, F: *mesüpu* (*Lageneria* pots).

2.3.4. The social and cultural context

The beginning on significant use of *Zutho* can be traced back to several decades by folklores instrumental in elucidating its role. On tracing the origin of *Zutho*, many respondents failed to point the exact conundrum of discovery but the beliefs merges to the popularity of terrace rice cultivation and hence brewing of beers by the women folks. One respondent stated "During the world war II, lack of food resources prompted us to look for means to prolong the food resources available and since rice was available in plenty, we brewed and stored it in earthen pots (Fig: 2.4E) or *mesüpu* (Fig: 2.4F), *mesüpu* (*Lagenaria* pots) was also used for serving the drink, that way food (rice used for brewing *Zutho*) as well as the beverage provided energy". Since Angami Nagas were previously agriculturalist, local varieties of rice (Fig: 2.4A) were available in plenty and hence brewing *Zutho* was easy. Fermentation (Fig: 2.4C) usually takes about a week and *Khrie* (Fig: 2.4B) is used as the starter culture. Another respondent recalls "We use to drink *Zutho* thrice a day, once in the morning, afternoon and before going to bed and they also served to visitors, irrespective of age or status". Among the few persons interviewed, Mrs Dziesetou-ü Sogotsu from Nerhema village, Kohima vividly cherishes those time when serving *Zutho* was a marked of respect to the elders. She states "today, *Zutho* no longer holds the ecstatic feeling it once had as younger generations seldom care about courtesy of serving the elders first". Life as recalled by most elderly respondents was simple and included communities working in the fields and coming home to bonfires. Since agriculture was the main activity it required vigour, hence *Zutho* provided energy and strength. Another respondent states "when a child was born, one of the parents dips their finger into *Zutho* and feds few drops to the baby as a sign of welcoming the child into the

family". Hence *Zutho* played a pivotal role in marking auspicious days and celebrations among the Angami Nagas.

At present, rice beer is only prepared and sold by few families and instead teas have replaced the homes of the Angamis. Of late, healthy teas like green tea, herbal teas etc are more common which shows that the younger generations are geared driven towards health consciousness and *Zutho* is only remembered as a product of cultural pride that tenaciously gritted the stability of the past community. Conflicts as such remains difficult to answer as the lineage and ancestral heritage remains strongly tied and interwoven into the community and is seen to be built and intimately linked into the family structure, making it difficult to disentangle these processes at the community level. Today most of the younger generations do not practice agriculture and farming but strives to excel in other professions causing a shift from rural to urban or semi-urban areas. When asked if they are aware on the significance or use of *Zutho* in the society, many recalls it to be a forbidden drink and hence cared less to know about its traditional role in the present culture. Many youngsters especially the females had never tasted *Zutho* and showed no interest in consuming it as it was forbidden by their parents. The ignorance of traditional cultures and ethos or lack of communication between the older and the younger generations can be indirectly blamed to have caused shift in the lifestyles and societal organization of the Angamis. The advancement of modernism particularly education may have caused a shift in progress. Today, it is a rare sight to observe community gathering except during festivals, holidays or special occasions and people seem to be busy with works under the sun. However the Angamis still carry the heart of hospitability, hard work and honesty that defines them.

2.3.5. Religion

Christianity has played a key role in causing transition of culture among the Angamis. Most Nagas are Christian and the state thrives on the slogan "Nagaland for Christ". Angami Nagas are no exception and every child is born into a Christian family, thus determining a child's religion by birth. Angeline Lotsuro, suggested that Nagas are nominal and external Christians (Lotsuro, 1991) and to this day most Nagas remains so. This fissiparous tendency is supported through personal testimonies of religious enthusiasm or through custom changes in marriage rituals and change in taboos associated with markings of birth and death. Angami Nagas traditionally followed animism and their folklores and legends tell about numerous deities. The spirits are called by distinct names unique to each village but when compared, the spirits is described very similar to each other. Also the rituals and ceremonies associated with eulogizing songs and exuberant dances all points to the worship of deities. They believed in a Creator who brings forth everything in its season and thus before starting their day in the fields, *Zutho* was spilled on the ground as a sign of worship offering. Also a child's journey to practicing farming is marked with a day of rest where the child witnesses the elders working and drinks *Zutho.* With the onset of Christianity and the acceptance of Nagas to it, many superstitions associated with observing the rituals and rites had been abolished. For instance the religious significance associated with consuming *Zutho* during *Sekrenyi*, one of the festivals of the Angamis had observed significant changes. *Sekrenyi* (S*ekre* meaning sanctification and *thenyi* meaning festival) is a sanctification festival where rice beer was the hero drink served. During the preparation of *Zutho*, a small quantity is specifically set aside for the male performers and during this period, they remain chaste. Their dancing

marks the beginning of the celebration of *Sekrenyi* and *Zutho* is served to all, irrespective of age, status or gender. However, at present, consuming *Zutho* is considered to violate the sacred belief that Christianity believes in honoring a person's physical body as a temple of God. Another reason on discouraging the use of *Zutho*, suggests that alcohol consumption is considered as sin and may provoke debauchery and harm the body, or cause conflicts within the family due to inappropriate use of alcohol. Embracing Christianity still paves way to study and preserve the ethnicity of a culture yet their ethnicity may not be defined by the traditional way of life and food but also by the beliefs that sustained them and the impact of religion on them. Traditionally the Angamis were fecund people and to this day they remain so, proving the powerful nature of humans to preserve their tradition which holds intrinsic values of life.

2.4. Conclusion

Documentation on the traditional knowledge places importance on oral traditions to continue to preserve the legacy and culture and narrate the traditions of the old which may succumb to rising changes of modern lifestyle or ignorance. However the likelihood for the demand of preserved or processed foods and limited interest in traditional foods among the urban population may lessen the knowledge of traditional gastronomy in Nagaland. The state, at present, celebrates the unique identity and food culture of each tribe that adds to the culinary diversity in the state and sustains the traditional heritage of the indigenous people. Nutrition and health benefit claims supplements the diet and lifestyle rooted in rich ancestral traditional knowledge. At present, socio-economic lifestyles and consumption patterns reveals the continuing diet culture of traditional foods which is sometimes limited by supply of natural ingredients in the urban areas. Most of the Nagas still practice the art of agriculture by maintaining kitchen gardens in the backyards or adopting modern techniques such as pot culture to continue their legacy as agriculturalist. The supermarkets in urban areas are swarmed by local food products brought in from rural areas and this rise in demand for organic foods is influenced by knowledge on health issues underlying diseases that may arise due to large consumption of synthetically produced food. These in turn boosts the economic livelihood of the people as the food items are sold at high prices in the market. The hotels and restaurants across the state are mostly run by the local population producing ethnic dishes that creates a placebo abode of home cooking for travellers. Thus, the traditional gastronomy of Nagaland prolongs the dynamics of the present culture which is evident in their daily plethora of preparing, serving, gifting of food items that glues the society at individualistic

level ultimately uniting and defining a community interwoven by similar yet unique food flavour profiles and indigenous heritage.

Culture acts as a catalyst in the evolutionary process of humans whereas religion or belief reveals the most profound of meanings that humans carry – their rationale, their origins, and their purpose. Culture links with religion to build a person and the level of values and norms fixed by culture and religion is often what is highlighted in human rights discourse. It is observed that ancient customs and ethos have evolved with the change in lifestyle and adoption of Christianity among the Nagas. This is particularly true in the case of indigenous rice beverages. Alcoholic rice beverages which served as energy drink of the past is considered a taboo today and the transition caused may attribute to the use or misuse of it by the younger generation or that it no longer is needed to meet its requirements. It is also observed that in the past, drinking alcoholic rice beverages was common even among the women and children irrespective of age or status. It can also be ascertained that the upcoming generations are losing their link with the rich cultural heritage of their ancestors, held by cultural and religious practices acting as stereotype to align the perceptions of old to the optimality of the new traditions.

Chapter - 3

Proximate analysis and nutritional values of some fermented foods and beverages of Nagaland, India

Introduction

Fermented foods forms a daily part of the dietary culture of the people of Nagas and the ethnic foods and beverages mirrors the celebration of festivals, religious ceremonies, social events and house visitations. Culinary skills blended with cultural legacy have created a wide range of indigenous food products with unique flavor profiles that sustains the traditional food habits. It is estimated that one third of all food consumed by humans are fermented (Katz 2013) and as such their beneficial roles pertaining to bioactive components and phytochemicals have been investigated by many researchers. Foods produced by fermentation have a reduced risk of contamination while being rich in anti-microbial end-products, such as organic acids, ethanol, and bacteriocins. Unlike those present in the starting materials fermented foods also include the new and desirable tastes and textures and promote human health in ways not directly attributable to the starting food materials. Many studies support the beneficial roles of fermented foods in promoting human health (Baroja et al. 2007; An et al. 2013; Iwasa et al. 2013; Muttu et al. 2013; Chen et al. 2014; Hillmire et al. 2015; Tapsell, 2015; Eussen et al. 2016). Few studies

have reported the antagonistic nature of fermented foods, one study showed that fermented fish causes thiamine deficiency (Stuetz et al. 2016) which links to the potential danger of beri – beri within the rice consuming populations (Koshy et al. 2018).

When a stable DPPH radical accepts an electron from an antioxidant compound, the violet color of the DPPH radical turns transparent and this can be measured colorimetrically. Thus substances capable of performing this reaction are considered as antioxidants and radical scavengers (Dehpour et al. 2009). Phenols and flavonoid compounds are reported to act as scavengers of free radicals and singlet oxygen in biological systems providing protective factors against heart diseases and cancer (Jorgensen et al. 1999). Phenol is the most diverse secondary metabolite in the plant kingdom and its antioxidant activity attributes to singlet oxygen quenchers, hydrogen donors and their redox properties (Rice-Evans et al. 1995). The metabolic activity in our body naturally balances the dynamics between the free radicals generated and the antioxidants required to scavenge it creating a homeostatic balance. This scavenging activity protects our body from detrimental effects and disease conditions like atherosclerosis, hypertension, Alzhemier's disease, cancer, diabetes mellitus and inflammatory conditions primarily considered to be caused due to the imbalance between prooxidant and antioxidant homeostasis (Gilgun- Sherki et al. 2002; Islam et al. 2013). Flavonoid is one such compound that possess tremendous antioxidant properties and confer resistance to diseases and infections (Wang et al. 2018). They are divided into classes such as anthocyanins, flavonols, flavonones and isoflavones (Scalbert, 2000). Quercetin belonging to class flavonol is reported to be the major flavonoid with an antioxidant, anti-inflammatory, anti-diabetic, and anti-proliferative activities assisting in

ameliorating mental and physical health (Stockmoava, 2013). Another class of compound linked with nutritional, antimicrobial and sensorial properties of vegetables and fruits are phenolic acids (Robbins 2003). The chemical composition of phenol is composed of hydroxybenzoic acids and hydroxycinnamic acids. Studies have shown that the hydroxycinnamic acid has higher antioxidant potential than their corresponding hydroxybenzoic acid as the number and position of hydroxyl groups on the molecule determines the antioxidant activity (Andreasen 2001).

Plant based products are natural sources of phytochemicals- phenols and flavonoids being the most common phytoconstituents responsible for antioxidant function (Scalbert et al. 2005). Most of the fermented foods of Nagaland are derivatives of plants or plants based products imbibed with indigenous knowledge. Therefore, in this chapter, the proximate biochemical and nutritional composition of fermented foods of Nagaland viz *Ashikumna* (fermented pork fats) of Sumi tribe, *Kese* (fermented bamboo shoot) of Angami tribe, dry *Bastenga* (common dried fermented bamboo shoot) of Lotha tribe, *Zusem* (fermented bamboo shoot) and *Jangpangngatsu* (fermented crab) of Ao tribe was studied. The presence of various components which might contribute to the unique characteristics and nutritional aspects of fermented foods and beverages was studied. The comparative evaluation has been done against its negative and positive control based on the composition of total carbohydrates, reducing sugar, proteins, ash and crude fiber content, phenolic and flavonoid content and the total antioxidant capacity. It is the first report on the biochemical parameters of these foods and intends to popularize scientific knowledge about fermented food diet to establish healthy and safe food consumptions among the indigenous people of Nagaland.

3.2. Materials and Methods

3.2.1. Collection and preparation of samples.

3.2.1.1. *Ashikumna* (ASK) is fermented pork fat unique to Lazami of Sumi tribe. The sample was collected from a household in Kohima and pork fats (FPF) was bought from the market, washed with distilled water and stored in the refrigerator at 4^{o}C until further analysis. Fresh pork fats (FPF) served as the negative control for *Ashikumna.*

3.2.1.2. *Zusem* (ZU) is fermented bamboo shoot unique to the Ao nagas. It is prepared preferably by adding ash and charcoal of *Phyllantus emblica* barks or simply using the ash and charcoal of any firewood with lukewarm water, and allowed to ferment for 24- 48 hours. The bamboo variety, *Dendrocalamus hamiltonni* shoots were collected from Lumami village, Zunheboto for the preparation of *Zusem* following the traditional method at home and a control set following the same method was performed in the laboratory using laminar air chamber. The ash and charcoal of *Phyllantus emblica* barks were used for home as well as laboratory preparation (ZUPC) and tender fresh bamboo shoots (FBS) were taken as negative control.

3.2.1.3. *Kese* (KES) is fermented bamboo shoot unique to the Angami Nagas. The sample was collected from Bara Basti, Kohima village.

3.2.1.4. Dry *bastenga* (DBA) is dried bamboo shoot famous among the Lotha Naga tribe. It was collected from Wokha district of Nagaland.

3.2.1.5. J*angpangngatsu* (JAP) is fermented crab prepared with roasted sesame seeds. The sample was collected from Changki Village, Mokokchung.

3.2.1.6. *Katsing* (KAT) was bought from Chuchuyimpang village, Mokokchung, collected in sterile bottle and kept at 4^{o}C until further analysis. Positive control (KAPC) was prepared in the laboratory by autoclaving 250 gms of sticky rice with 500 ml of distilled water for 15mins, after cooling 50 gms of roasted *Chenopodium* seeds and 250 ml of distilled water was added. It was allowed to ferment for 5 days at 35- 37^{o}C after which it was kept 4^{o}C until further analysis.

3.2.2. Quantification of carbohydrates: Carbohydrates content was estimated using the anthrone method (Heidge 1962). 100 mg FW of the sample was boiled in 2.5N HCL for 3 hours. To 1 ml of the sample, 4ml of anthrone reagent was added, heat for 8 minutes in a boiling water bath and cooled rapidly. The absorbance was taken at 630 nm and glucose was taken as standard.

3.2.3. Quantification of reducing sugar: Reducing sugar was estimated using 3, 5-dinitrosalicylic acid (DNSA) reagent (Miller 1959) and ethanol was used for extraction procedure. To 2 ml of extract, 2 ml of DNSA reagent was added and mixture was boiled for 9 mins followed by cooling to room temperature and addition of 0.5 ml of potassium sodium tartrate. The absorbance was measured at 540nm and glucose was taken as the standard.

3.2.4. Quantification of non- reducing sugar: the reducing sugar value was subtracted from the carbohydrate value and this constituted the non- reducing sugar value of each sample studied.

3.2.5. Quantification of protein: Protein estimation was measured following Bradford method (Bradford 1978). Phosphate buffer was used for extraction. To 1m of sample, 5 ml

of dye was added, homogenized by shaking and allowed to stand for 5 mins. The absorbance was taken at 595 nm and Bovine Serum Albumin (BSA) was taken as standard.

3.2.6. Crude fiber: Crude fiber was determined following AOAC 978.10 (AOAC 2005). One gram of dried samples was subjected to defatting with petroleum ether. 200 ml of 0.25N Sulphuric acid was added to the sample and boiled for 30 min. It was then filtered with No. 1 Whatman filter paper. The filtrate was then again boiled with 200 ml of 0.313N NaOH solution for 30 min followed by filtration and washed subsequently with hot distilled water till the colour becomes translucent. The residue was then removed and transferred to pre-weighed aching dish (W1) and dried for 2 h at 130±2ºC and then cooled. The aching dish was cooled and weighed (W2). It was ignited for 4 hours at 600°C and after cooling in desiccator, it was again reweighed (W3) and the crude fiber content determined using the formula:

$$\text{Crude fiber (g/100g)} = \frac{\text{Loss in weight on ignition (W2-W1) - (W3-W1)}}{\text{Original weight of sample}} \times 100$$

3.2.7. Ash content: Total ash content was determined following AOAC 942.05 with slight modification (AOAC 2005). 1 gram of the powdered sample (W3) was transferred to a pre- weighed crucible (W1) and combusted at 600°C for 4 hours (W2) and allowed to cool in a desiccator. The total ash content was determined using the formula

$$\text{Ash \%} = \text{W2-W1/W3} \times 100$$

3.2.8. Moisture content: Moisture content was estimated following AOAC 930.15 with modification (AOAC 2005). 30g of sample was taken in a pre-weighed dish plate and

placed in the oven for ~16 h at 70±1°C till a constant weight was achieved. The moisture content was determined by using the formula:

$$\text{Moisture content (\%)} = \frac{\text{Loss of weight}}{\text{Weight of the sample}} \times 100$$

3.2.9. Determination of *p*H using *p*H meter: Five gram of sample was blended with 10 ml of distilled water in a homogenizer and the p*H* of the slurry was determined directly using a digital *p*H meter.

3.2.10. Determination of alcoholic content using hydrometer: The percentage of alcohol by volume and weight was measured using Pro Series Triple Scale Hydrometer.

3.2.11. Total Phenolic assay: The total phenolic content was determined by using the Folin- Ciocalteu assay (Singleton and Rossi 1965) with modification. Ethanolic extraction was followed for the study. To 3 ml of sample solution, add 0.5 ml of Folin- Ciocalteu reagent and mix by shaking. After 5 minutes, 1.5 ml of 20% Sodium Carbonate was added and absorbance was taken after an hour at 510 nm and gallic acid was used as standard.

3.2.12. Determination of total flavonoid content (TFC): Total flavonoid content was determined following technique of Sahreen and Khan (Sahreen et al. 2010) with slight modification. Methanolic extraction of dried sample was performed for the study. To 0.3 ml of extract, 3.4 ml of 30% methanol, 0.15 ml of 0.5M sodium nitrite and 0.15 ml of 0.3M aluminum chloride were added. The mixture was then allowed to stand for 5 min and then 1 ml of 1M NaOH was added. The absorbance was measured at 510 nm and standard curve was prepared using Quercetin and expressed as mg Quercetin equivalents (QE) / 100 mg of extract.

3.2.13. DPPH radical scavenging assay: DPPH radical scavenging assay is a widely used method to evaluate the free radical scavenging ability of natural compounds. This assay is based on the measurement of the scavenging ability of antioxidant substances towards the stable radical. The free radical scavenging activity of the extracts was examined in vitro using DPPH radical as described by Aoshima, (Aoshima et al. 2004) with modification. 1.0 ml of various concentrations of methanolic extracts (2-10 mg/ml) was mixed with 1.0 ml of 0.8 mM DPPH solution. The mixture was shaken and left to stand for 30 min in the dark and the absorbance was measured at 517 nm against a reagent blank (methanol). Ascorbic acid was used as standard. The inhibition percentage for scavenging DPPH radical was calculated according to the equation

% Decolorization = (ABS control - ABS sample/ABS control) x 100

Where ABS control= absorbance of control at 517 nm

ABS sample= absorbance of sample at 517 nm

3.2.14. Calculation of IC_{50} value: IC_{50} values which shows 50% inhibition was calculated using regression analysis in MS excel.

3.2.15. Statistical analysis: Data are reported as mean ± standard deviation of three determinations.

3.3. Results and Discussion

Table 3.1. Proximate analysis of fermented bamboo shoot products of Nagaland, India. ZU – *Zusem*, ZUPC - *Zusem* Positive control, FBS - fresh bamboo shoot, KES – *Kese* and DBA - dry *Bastenga*

Proximate analysis	ZU	ZUPC	FBS	KES	DBA
Moisture content (%)	95.29 %	95.76 %	92.66 %	92.68%	91.98 %
Crude fibre (mg/100mg)	13.89	14.70	9.6	15.95	15.1
Ash (%)	11.2 %	17.7 %	13%	12.2 %	7.5 %
*p*H	5.1	5.4	6.9	4.4	4.1
Carbohydrates (mg/100mgof FW)	2.729 ± 0.002	2.872 ± 0.611	3.474 ± 0.096	0.109 ± 0.01	2.355 ± 0.585
Reducing sugar (mg/100mg of FW)	1.08 ± 0.001	1.125 ± 0.001	2.205 ± 0.003	0.038 ± 0.001	0.945 ± 0.004
Non- reducing sugar (mg/100mg of FW)	1.649 ± 0.002	1.747 ±0.590	1.269 ± 0.07	0.071± 0.01	1.41 ± 0.023
Proteins (mg/100mg of FW)	0.061 ± 0.005	0.054 ± 0.004	0.084 ± 0.004	0.028 ± 0.005	0.106 ± 0.011
Phenol (mg/100mg FW)	0.169 ± 0.008	0.156 ± 0.02	1.003 ± 0.7	1.959 ± 0.9	1.026 ± 0.1
Flavonoid (mg/100mg of DW)	5.525 ± 2.72	4.151 ± 1.044	6.733 ± 0.356	8.035 ± 1.175	2.583 ± 0.911

Table 3.2. Proximate biochemical analysis of fermented pork fats (ASK), its negative control (FPF) and fermented crab (JAP) of Nagaland, India

Proximate analysis	ASK	FPF	JAP
*p*H	4.2	5.3	7.1
Carbohydrates (mg/100mgof FW)	0.757± 0.001	0.681± 0.044	2.62 ± 0.43
Reducing sugar (mg/100mg of FW)	0.431± 0.003	0.481± 0.008	0.864±0.004
Non- reducing sugar (mg/100mg of FW)	0.326±0.001	0.200± 0.076	1.756±0.065
Proteins (mg/100mg of FW)	0.021± 0.008	0.012± 0.001	0.148 ± 0.09
Phenol (mg/100mg FW)	0.019± 0.005	0.014± 0.005	0.356±0.006
Flavonoid (mg/100mg of DW)	7.626 ± 1.53	17.59± 0.285	14.461±5.09

Table 3.3. Proximate nutritional composition of KAT (*Katsing)* against its positive control KAPC

Proximate analysis	KAT	KAPC
*p*H	2.9	3.9
Carbohydrates (mg/100ml)	631.85 ± 0.975	512.7 ± 0.579
Reducing sugar (mg/100ml)	382.5 ± 0.018	396 ± 0.006
Non reducing sugar (mg/100ml)	249.35 ±0.712	116 ± 0.321
Alcoholic content (%)	7 %	5 %
Proteins (mg/100ml)	32.28 ± 0.494	8.928 ± 1.037
Phenol(mg/100ml)	1.427 ± 0.105	1.086 ± 0.335
Flavonoid (mg/100ml)	377.5 ± 4.376	353.95 ± 4.765

Figure 3.3(A-D): Figure representing the raw materials required for preparation of *Katsing*. A: Plant of *Chenopodium*, B: *Chenopodium* seeds, C: Positive control, D: *Katsing*

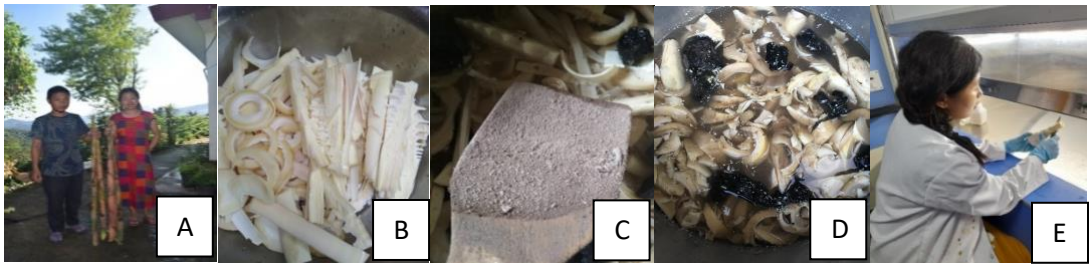

Figure 3.4(A-E): A-D: traditional method of preparation of *Zusem* with *Dendrocalamus hamiltonii,* E- preparation of positive control inside the laminar air cabinet

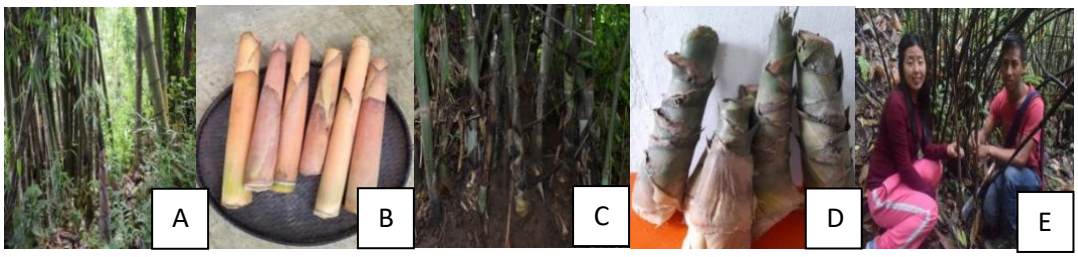

Figure 3.5(A-E): Some other species of bamboo found in Nagaland used for preparing *Bastenga*

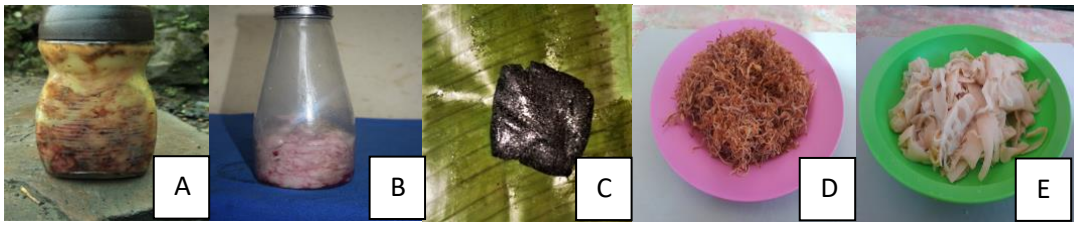

Figure 3.6(A–E): Photoplates of some fermented food samples. A: *Ashikumna,* B: pork fats (negative control of *Ashikumna*), C: fermented crab, D: dry *Bastenga*, E: *Kese*

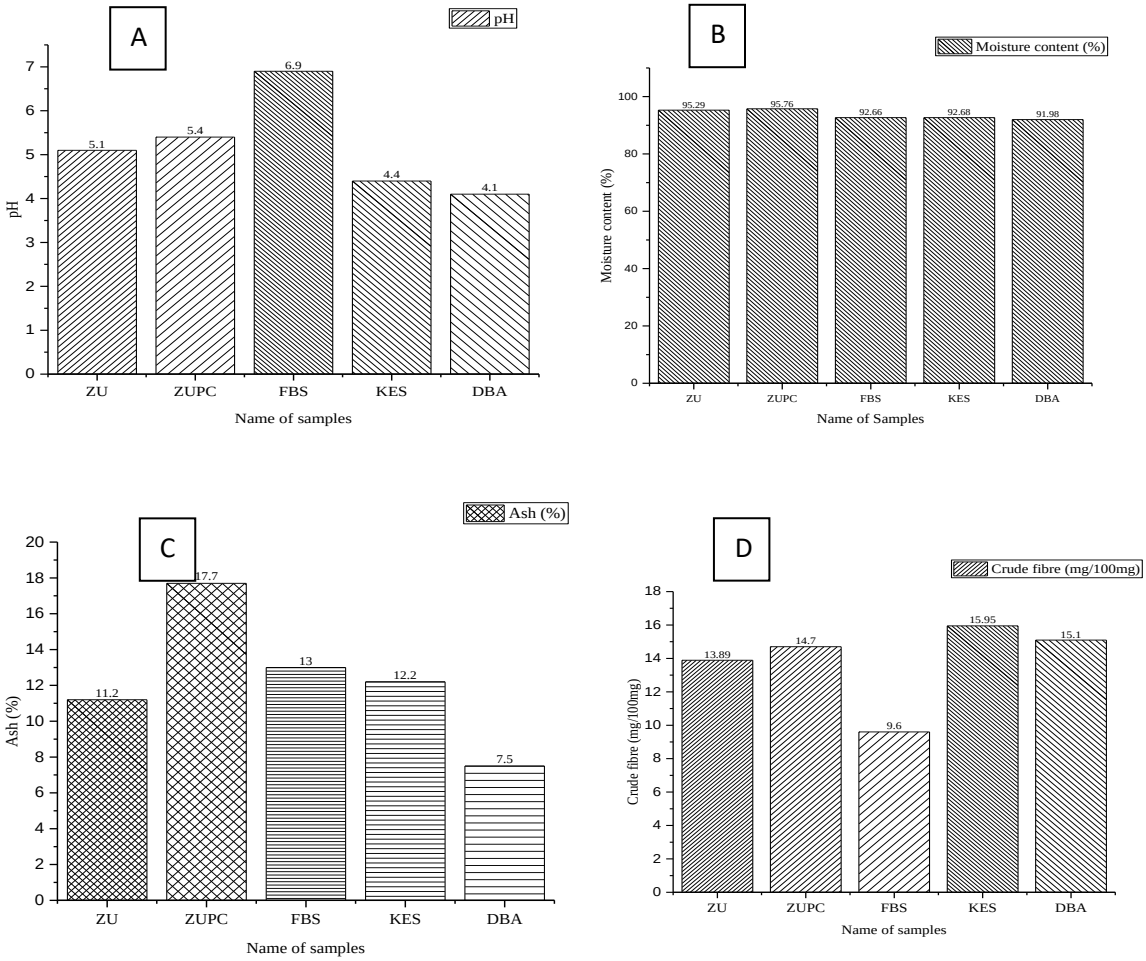

Figure 3.7(A–D): Various biochemical parameters of ZU (*Zusem*), ZUPC (*Zusem* positive control), FBS (fresh bamboo shoot), KES (*Kese*) and DBA (dry *Bastenga*). A- *p*H value of ZU, ZUPC, FBS, KES, DBA. B- Moisture content (%) of ZU, ZUPC, FBS, KES, DBA. C- Ash content (g/100g) of ZU, ZUPC, FBS, KES, DBA. D- Crude fibre content (g/100g) of ZU, ZUPC, FBS, KES, DBA

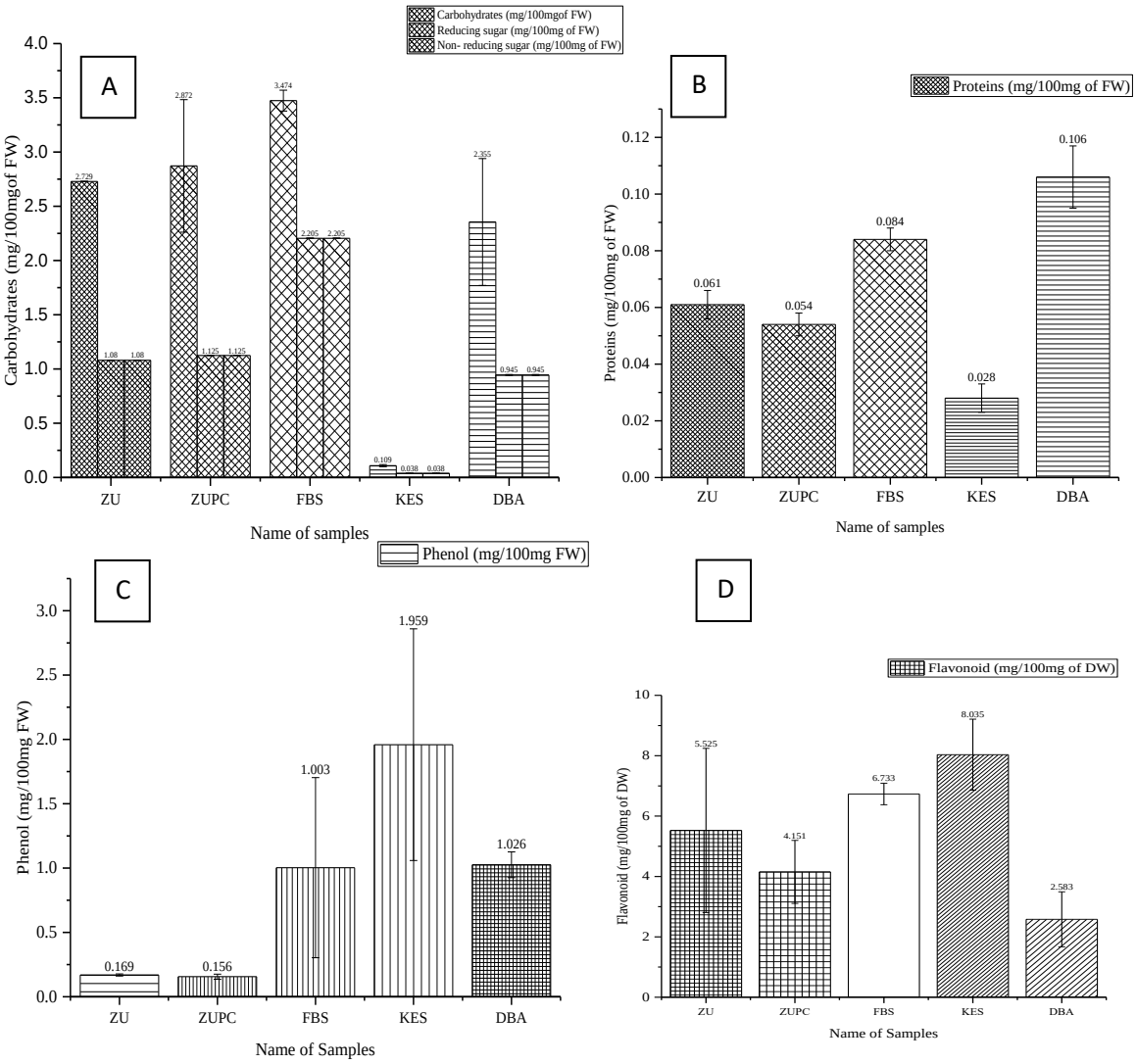

Figure 3.8(A–D): Various biochemical parameters of ZU *(Zusem*), ZUPC (*Zusem* positive control), FBS (fresh bamboo shoot), KES (*Kese*) and DBA (dry *Bastenga*). A- Carbohydrates, reducing sugars and non- reducing sugars content of ZU, ZUPC, FBS, KES, DBA. B- Protein content (mg /100 mg) of ZU, ZUPC, FBS, KES, DBA. C- Total phenolic content (TPC mg GAE/ 100mg FW) of ZU, ZUPC, FBS, KES, DBA. D- Total Flavonoid Content (TFC mg Qe/ 100mg DW) of ZU, ZUPC, FBS, KES, DBA

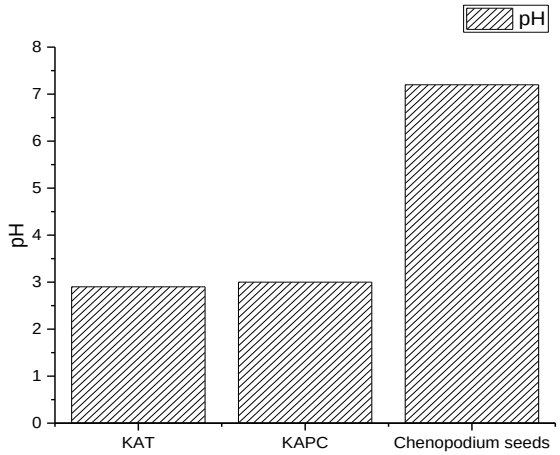

Figure 3.9: *pH* content of KAT (*Katsing),* KAPC (*Katsing* Positive control) and *Chenopodium* seeds

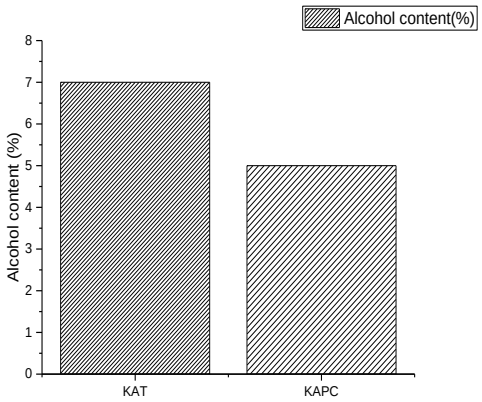

Figure 3.10: Alcohol content of KAT (*Katsing)* and KAPC (*Katsing* Positive Control)

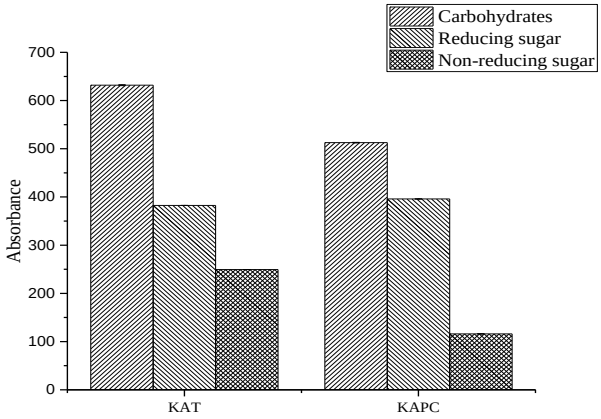

Figure 3.11: Carbohydrates, reducing sugars and non- reducing sugars content of KAT (*Katsing)* and KAPC *(Katsing* positive control)

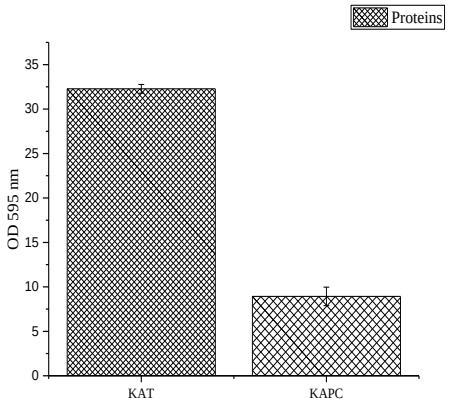

Figure 3.12: Protein content (mg /100 mg) of KAT (*Katsing)* and KAPC *(Katsing* positive control)

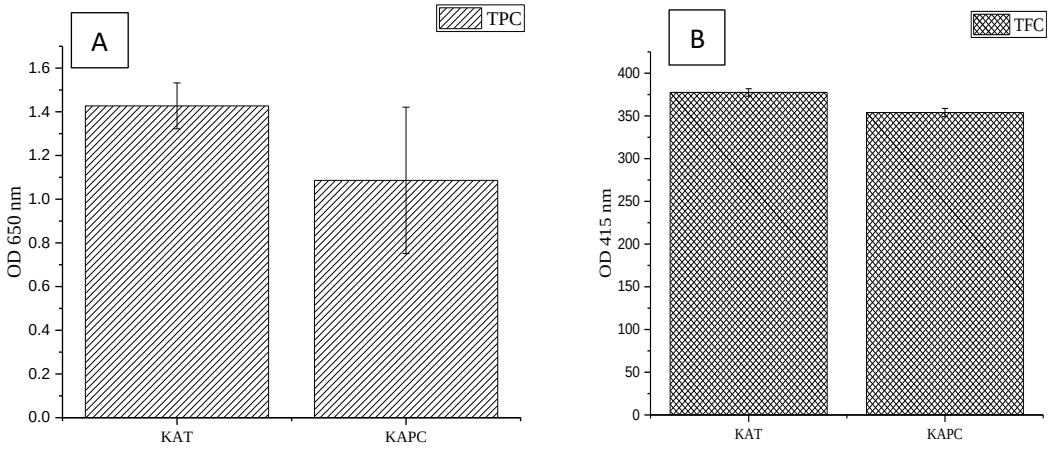

Figure 3.13(A-B): A-Total phenolic content (TPC mg GAE/ 100ml FW) of KAT (*Katsing)* and KAPC *(Katsing* positive control) and Total Flavonoid Content (TFC mg Qe/ 100ml DW) of KAT (*Katsing)* and KAPC *(Katsing* positive control)

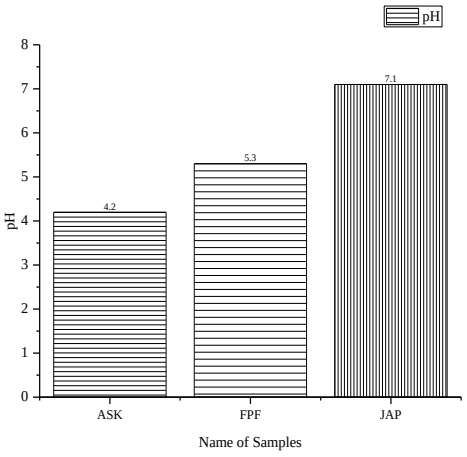

Figure 3.14: *pH* content of ASK (*Ashikumna),* FPF (pork fats negative control) and JAP (fermented crab)

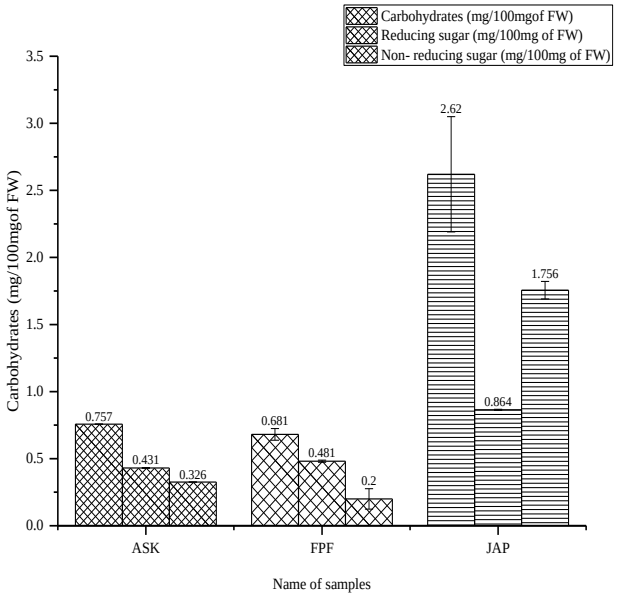

Figure3.15: Carbohydrates, reducing sugars and non- reducing sugars content of ASK (*Ashikumna),* FPF (pork fats negative control) and JAP (fermented crab)

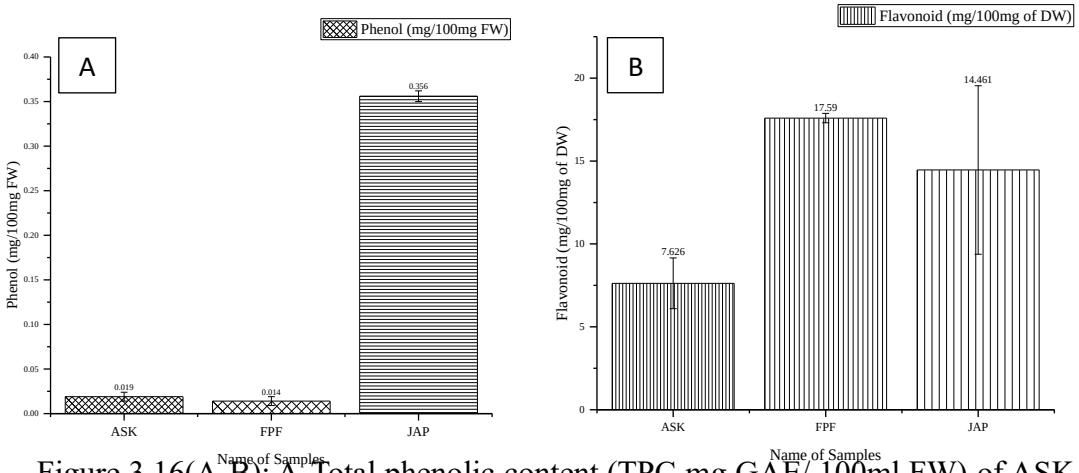

Figure 3.16(A-B): A-Total phenolic content (TPC mg GAE/ 100ml FW) of ASK (*Ashikumna),* FPF (pork fats negative control) and JAP (fermented crab) and Total Flavonoid Content (TFC mg Qe/ 100ml DW) of ASK (*Ashikumna),* FPF (pork fats negative control) and JAP (fermented crab)

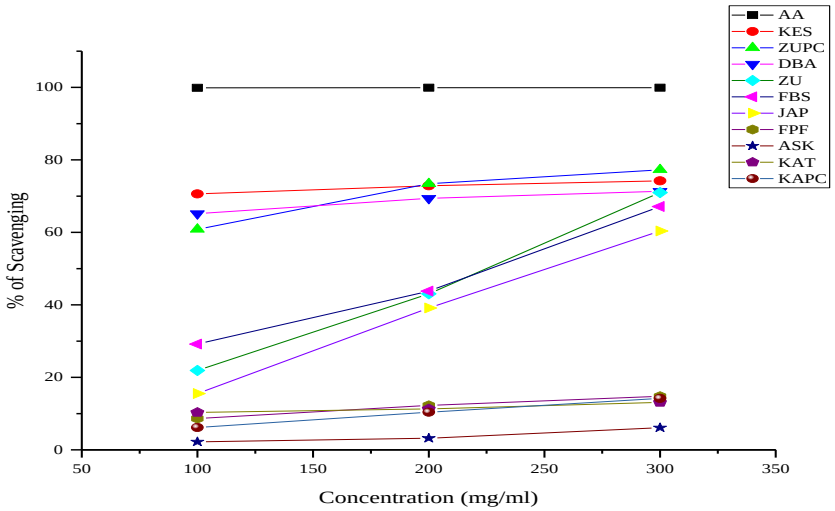

Figure 3.17: DPPH radical scavenging activity (%) of different extract concentration against Ascorbic acid on DPPH radicals. AA- Ascorbic acid, KES - *Kese*, ZUPC - *Zusem* positive control, ZU - *Zusem*, FBS - fresh bamboo shoot, JAP- *Jangpangngatsu,* FPF - fresh pork fats , DBA - dry *Bastenga* ,KAPC – *Katsing* Positive control, KAT- *Katsing* and ASK – *Ashikumna*

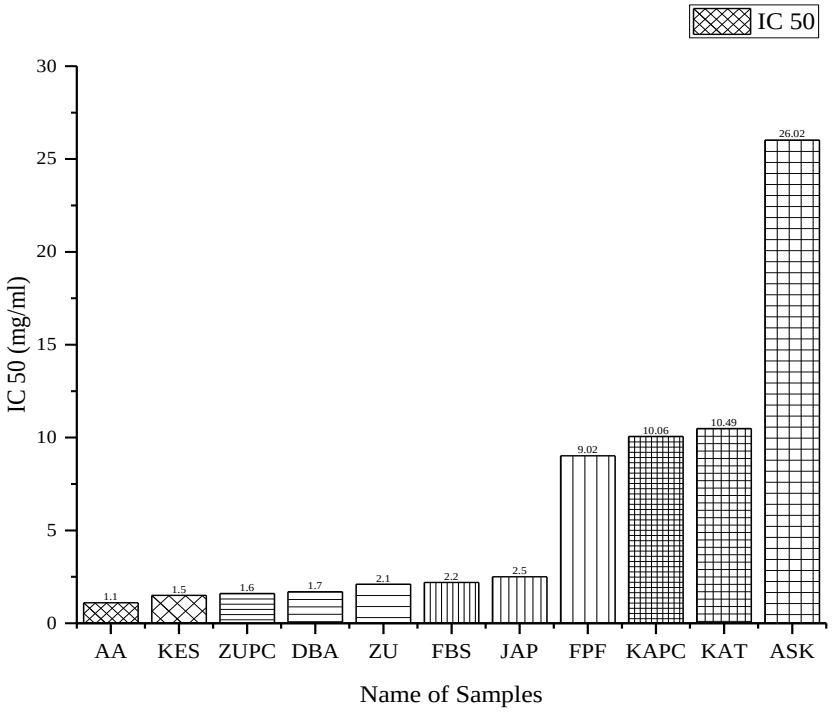

Figure 3.18: the comparison of IC 50 values of the fermented foods against Ascorbic acid. AA- Ascorbic acid, KES - *Kese*, ZUPC - *Zusem* Positive control, DBA - dry *Bastenga,* ZU- *Zusem* , FBS - fresh bamboo shoot, JAP- *Jangpangngatsu,* FPF - fresh pork fats, KAPC – *Katsing* Positive control, KAT- *Katsing* and ASK – *Ashikumna*

3.3.1. General biochemical characteristics on fermented bamboo shoots of Nagaland

Details on the proximate composition of fermented bamboo shoots products of Nagaland have been discussed in detail in Table 3.1. All the fermented bamboo shoots (ZU, ZUPC, KES, DBA) were found to be acidic in nature with *p*H ranging between 4.1- 5.4 except for the negative control (FBS) which was found to be neutral in nature (*p*H 6.9). Crude fiber (mg/100mg) was found to be highest in KES (15.95) while the carbohydrate (mg/100mgof FW) and protein content (mg/100mg of FW) was found to be the lowest in the particular sample (0.109 ± 0.01, 0.028 ± 0.005) respectively. FBS (3.474 ± 0.096) showed the highest carbohydrate (mg/100mgof FW) value while DBA (0.106 ± 0.011) showed the highest protein content (mg/100mg of FW) among the samples studied. Reducing sugar (mg/100mg of FW) and non- reducing sugar (mg/100mg of FW) exhibited the highest value in FBS (1.08 ± 0.001, 1.649 ± 0.002) and lowest in KES (0.038 ± 0.001, 0.071±0.01) respectively. The total phenolic (mg/100mg FW) and flavonoid (mg/100mg of DW) content was also found to be highest in KES (1.959 ± 0.9, 8.035 ± 1.175) which corresponds to its high antioxidant activity. It was also observed that in fermented bamboo shoots, differences in terms of physical and bio-chemical properties were observed in the sample against its negative control. The probable reason for this variation might be attributed to differences in the edible bamboo species used for preparing the bamboo shoot product. Our results also highlighted the acidic nature of the fermented foods which may aid in preventing the growth of harmful microbes. The high moisture in the bamboo shoots renders its short shelf life, thus fermented bamboo shoots must be stored decently in airtight containers, kept in cool and dry place. The presence of ash and protein in minimal amount in the fermented bamboo shoot fits its use as a balanced nutritional food.

3.3.2. General biochemical characteristics on fermented beverage of Nagaland (*Katsing)*

The World Health Organization (WHO) guidelines define one unit of alcohol as the equivalent of 8g of ethanol and the "responsible" or "low", level of risk for men as "3 units per day and 21 per week" and for women as "2 units per day and 14 per week" spread throughout the week (including 2 alcohol free days per week). Our result revealed that the average alcoholic content of *Katsing* was 5 % which can be considered a safe level of consumption. It was also observed that, even though the same method was adopted for preparation of positive control, differences in terms of alcoholic content and bio- chemical properties appeared. The plausible reason for this variation might be attributed to differences in the variety of rice used in preparing the starters, their ratio and also the duration of the product stored. *p*H value of *Katsing* also indicates its acidic nature and low alcoholic content makes it a desirable drink even among the womenfolks as the ancestral Naga culture forbids women intoxicated with wine. Biochemical analysis presented in Table 3.3 presents a detailed discussion on the proximate composition of *Katsing* against its positive control. All the biochemical parameters studied revealed a higher value of *Katsing* against its positive control.

3.3.3. General biochemical characteristics on other fermented foods of Nagaland, India

The details of fermented pork fats (ASK), its negative control and fermented crab (JAP) are discussed in Table 3.2. The *p*H of JAP (7.1) revealed its neutral nature with low protein content (0.148 ± 0.09 mg/100mg of FW) and high flavonoid content (14.461 ± 5.079 mg/100mg of DW). ASK (Figure 3.6A) showed negligible difference against its negative control in all the biochemical parameters observed, with slightly higher values

inclined towards fermented product except for flavonoids. It can be ascertained that fermentation of pork fats reduces its *p*H but undergoes moderate biochemical and physiochemical changes based on the parameters studied.

3.3.4. Antioxidant capacity and IC50 values of fermented foods and beverage of Nagaland, India

DPPH radical scavenging activity of various fermented foods is denoted in Figure 3.17. All the extracts showed different levels of DPPH radical scavenging activity over the range of 100-300 mg/ml concentration and the IC50 value of KES - *Kese*, ZUPC - *Zusem* positive control, ZU - *Zusem* , FBS - fresh bamboo shoot, JAP- *Jangpangngatsu,* FPF - fresh pork fats , DBA - dry *Bastenga* ,KAPC – *Katsing* Positive control, KAT- *Katsing* and ASK – *Ashikumna* was found to be 26.02, 1.7, 2.1, 2.5, 2.2, 9.02, 1.6 and 1.5 mg/ml respectively (Figure 3.18). KES exhibited the strongest DPPH radical scavenging activity which corresponds to the highest phenolic content as well. The extracts radical scavenging activity were effective in the order KES> ZUPC> DBA> ZU> FBS> JAP> FPF> KAPC> KAT> ASK. Ascorbic acid was used as standard at the concentration of 100-300 mg/ml and the IC50 value was found to be 1.1 mg/ml. Standard and all the extracts showed a dose dependent inhibition on the DPPH radicals.

3.4. Conclusion

Fermentation is exclusively a beneficial application to food and beverage production and the diversity of plant and animal products employed for the art of fermentation supports the abundance of rich natural resource and traditional knowledge possessed by the tribal community. Our study revealed that fermented foods of Nagaland are a potential source of nutrition or natural antioxidants owing to their biochemical compositions like phenol and flavonoid contents which are known as good antioxidants. In food fermentations raw materials are converted to products through the use of biocatalysts and for widely used plant substrates for example, breeding to reduce toxic or anti- nutritional components or to increase protein or vitamin content would be useful. Also many indigenous fermented foods are produced by spontaneous or natural fermentation, dominated by specific micro-organism. Isolation and characterization of predominant organisms is essential albeit isolation should not be confined to the dominant organisms because other microbes found in lower numbers might have an important function in the process. Alternatively or additionally, it would be valuable to identify microorganisms that can synthesize important ingredients for example essential amino acids, vitamins for populations where malnutrition is a problem or the production of enzymes to utilize recalcitrant waste as substrates. These foods can be further exploited for their health-giving attributes as they are highly valued for their promising prebiotic and probiotic prospects. Understanding the interaction between diet and human gastrointestinal microbiota by the rational choice of food-fermenting microbes can aid in improving the nutritional status of fermented foods. The elucidation of the microbial origin of flavors in fermented foods and the relationship between microflora and the organoleptic

properties or the inability to synthesize toxins and other undesirable secondary products may further aid in promoting the traditional foods. However, flavor and colour must be generated to meet local population preferences and the introduction of new processes or products should take into account the sensory requirements of target social groups. The safety and shelf life of fermented products may also be improved through the development of organisms that produce alcohols, antibiotics, or other substances that can inhibit the growth of undesirable organisms. However, training in basic microbiology, biochemical engineering, and the new techniques of molecular biology for personnel and less developed countries can be implemented to improve the traditional fermented processes. In modernizing the production of traditional fermented foods, appropriate and affordable technology should emphasize on the process changes and take into account the role of the tribal communities associated with it and how they will benefit from the modifications. However in depth studies are required to investigate the marginal and safe limit of fermented food intake, their medicative properties in promoting nutritional quality of foods and detecting any toxic oxidation products. At present, the fermented foods and beverages of Nagaland inflates the culinary diversity, vitalizes the local economy and hearten the livelihood of the Nagas pre-eminent by the food culture.

Chapter - 4

Metagenomic microbial profile of *Zusem* (fermented bamboo shoot) and *Katsing* (fermented rice beer)

Introduction

For ages, humans have relied on the process of fermentation as a means of food preservation, and in most parts of the world, it occurs spontaneously. Fermentation maybe alcoholic or lactic acid fermentation or a combination of both depending on the raw substrates used and the final product obtained. The microbiome represents the sum total of all microbial species revealing the genetic and functional characteristics and interactions between the organisms (Di Mucci et al. 2018). The microbiota of any food product is a result of complex interaction of individual microorganisms or groups that influences the organoleptic properties of any fermented food product influenced by the dynamics of microbial population, the food composition, and the interaction between the microbiome

and the fermenting food matrix which produces change in physicochemical conditions. Metagenomic analysis is the umbrella term revealing different microbial populations, dominant microbiota, metabolic potential and the functional role of microbes directly from the extracted DNA of a sample (Langille et al. 2013). It is a potential tool of choice to in food fermentation studies as it assesses the survival of pathogens, toxinogens or spoilage species over fermented food elaboration process (Adams and Mitchel 2002; Adams and Nicolaides 2008).

Bamboos are popular due to their rich nutrient composition of carbohydrates, proteins, minerals, fibres, vitamins, phytosterols, phenols and very less fats (Nirmala et al. 2007). The use of fermented bamboo shoot as a versatile food component is prevalent only in the North Eastern part of India nestled by tribal peoples. Any edible bamboo species is harvested during its season of availability largely gathered from the forest. The tender shoots are preferred for the preparation of fermented product and the methods of preparation sometimes overlap between the different tribes or regions of the states, however each method has unique style of preparation and preservation. Studies on natural microbial flora of fermented bamboo shoots revealed Lactic Acid Bacteria (LAB) as the dominant species present particularly, *Lactobacillus plantarum, L. brevis, L. casei, L. fermentum, L. curvatus, Leuconostoc mesenteroides, Leuc. fallax, Leuc. citreum, Lactococcus lactis*, *Enterococcus durans* and *Tetragenococcus halophilus* (Tamang et al. 2009; Das and Deka, 2012; Nongdam, 2015; Badwaik et al. 2015; Takur et al. 2015; Thakur and Tomar, 2016; Thakur et al. 2016). The LAB are believed to impart characteristic flavor, taste and aroma to fermented bamboo shoots by releasing organic acids and other metabolites that degrades phytic acid, play the dual nature of protease as

well as lipolytic activity within the cell machinery and aids in cell hydrophobicity (Sonar Halami 2014).

Rice is the staple food of the Nagas and fermentation of rice beverage with unique starter cultures were popular among the tribal community. Ancient customs delineate agricultural practices with celebration of festivals and feasts throughout the year and thus rice beer consumption were cardinal to its tradition and cultural heritage. The diversity of fungi involved in fermented rice beverage belong to the genera *Aspergillus, Penicillium, Rhizopus, Amylomyces, Mucor, Neurospora, Monascus* and *Actinomucor* (Campbell-Platt and Cook 1989). *Saccharomyces cerevisiae* seems to be the dominant microorganism that drives the fermentation of alcoholic beverages (Dangi et al. 2017). Other microbes include *Candida glabrata, Meyerozyma guilliermondii, Ogataea parapolymorpha Wickerhamomyces ciferrii, Debaryomyces hansenii,* and *Dekkera bruxellensis* (Bora et al. 2016). Assessing the metabolic versatility in fermented rice beverages still remains to be fully discovered and characterized and thus metagenomic tools illuminates knowledge linking food processing techniques and dynamic microbial ecology indicative of pathogenic microbes (Weimer et al. 2016).

Indigenous foods and beverages of Nagaland complement the vast majority of tribes found in the state and these foods in turn structures the population diet. Rice is the staple food of the Nagas and as such fermented rice beers with different texture and taste complements the ethnicity of tribal people. The objective of this study was to explore the usefulness of culture – independent method in detecting the bacterial community in *Zusem* and fungal repertoire of *Katsing. Zusem* is an indigeneous fermented bamboo shoot while *Katsing* is a traditional fermented rice beverage, both of which are unique to the Ao Nagas

of Nagaland. Metagenomic anlaysis in our study provides an opportunity to survey the complete microbial profile of a food product at a particular stage to understand the dynamic balance between microbes and the food substrate. This study to our knowledge is the first to report the bacterial community of *Zusem* and the fungal repertoire of *Katsing*, and provides foundational resource for further studies investigating the metabolic and functional potential of microorganisms and the use of these products as a potential nutritional beverage.

4.2. Materials and methods

4.2.1. Methodology of *16S V3 V4* metagenome sequencing

4.2.1.1. DNA extraction and sequencing: The DNA was extracted using DNeasy PowerSoil Kit (Catalog: 12888-100, Qiagen) and DNA concentration was determined by using Qubit™ dsDNA BR Assay Kit (Catalog: Q32853, Thermo Fisher Scientific). The readings were taken in a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and the QC status of the sample showed intact amplification at approximately 450 bp.

4.2.1.2. Library Preparation: 20 ng of DNA was used to amplify *V3&V4* hyper variable regions of *16S* gene using KAPA HiFi HotStart ReadyMix PCR Kit (Catalog: 0370, KAPA BIOSYSTEMS) following the Klindworth method (Klindworth et al. 2013). The PCR involved an initial denaturation of 95°C for 5 min followed by 25 cycles of 95°C for 30s, 55°C for 45s and 72°C for 30s and a final extension at 72°C for 7 min and holding at 4^0C. The amplification was confirmed by loading 3 uls of the PCR product on 2% agarose gel and the presence of a band at ~456 bp. The PCR products were further purified using 0.9X AMPure XP beads (Catalog: A63881, Beckman Coulter) to remove unused primers and eluted in 10 uls of 0.1X TE buffer. Further unique P7 (AGATCGGAAGAGCACA CGTCTGAACTCCAGTCA) and P5 (AGATCGGAAGAGCGTCGTGTAGGGAAAGA GTGT) barcoding was performed by additional 8 cycles of PCR by taking 4 uLs of the PCR product. The same cycle of purification was repeated and the final library was eluted in 15 uLs of 0.1X TE buffer.

4.2.1.3. Library Quantification: The library concentration was determined in a Qubit.3 Fluorometer (Catalog: Q33216, Life technologies using The Qubit™ dsDNA BR (Broad

Range) Assay Kit (Catalog: Q32853, ThermoFisher Scientific). Dye and the buffer was diluted at 1:200 ratio and 1 ul of the library mixed with the dye mix was incubated at RT for 2 minutes and the readings were taken in the Qubit.3 Fluorometer (Catalog: Q33216, Life technologies).

4.2.1.4. Library Validation: The library quality assessment was done using Agilent D5000 ScreenTape System (Catalog: 5067- 5588, Agilent) in a 4150 TapeStation System (Catalog: G2992AA, Agilent). 1 ul of the library and 10 uls of D5000 sample buffer (Catalog: 5067-5589) was vortexed using IKA vortexer at 2000 rpm for 1 min. The collected sample at the bottom was loaded on the Agilent 4150 TapeStation system, Agilent.

4.2.1.5. Sequencing Methodology: 25 ng of DNA was used to amplify *16S rRNA* hyper variable region *V3-V4*. The reaction includes KAPA HiFi HotStart Ready Mix and 100 nm final concentration of modified 341F and 785R primers. The PCR involved an initial denaturation of 95°C for 5 min followed by 25 cycles of 95°C for 30s, 55°C for 45s and 72°C for 30s and a final extension at 72°C for 7 min. Ampure beads were used to purify the amplicons with additional 8 cycles of PCR with Illumina barcoded receptors to prepare the sequencing libraries.

4.2.1.6. Sequence Data QC: The sequence data was generated using Illumina MiSeq. Data quality was checked using FastQC (Andrews, 2017) and MultiQC (MultiQC, 2017) software. The data was checked for base call quality distribution, % bases above Q20, Q30, %GC, and sequencing adapter contamination. All the samples passed the QC threshold (Q20>95%).

4.2.1.7. Data Analysis: The reads were trimmed (20bp) from 5' end to remove the degenerate primers. The trimmed reads were processed to remove adapter sequences and low quality bases using Trimgalore (Babraham Bioinformatics, 2017). The QC passed reads were imported into MOTHUR (Schloss et al. 2009) and the pairs were aligned with each other to form contigs. Any contig with ambiguous base calls were rejected and 300bp and 532bp contigs were retained and checked for identical sequences and duplicates were merged. The gaps and the overhang at the ends were removed and processed for chimera removal and nonspecific amplification of other regions were corrected by aligning the contigs to a known database of *16s rRNA.* UCHIME algorithm (Edgar et al. 2011) was used to flag contigs with chimeric regions postulating it to a known reference of all the chimeric sequences. The filtered contigs were processed and classified into taxonomical outlines based on the GREENGENES v.13.8-99 database (DeSantis et al. 2006). The contigs were then clustered into OTUs (Operational Taxonomic Unit) and OTU abundance was estimated using PICRUSt (Langille et al. 2013) to predict gene family abundance. The 16s RNA copy numbers were normalized by PICRUSt's precalculated file and the metagenomes were predicted using predict_metagenomes.py script. The predicted pathways were merged into higher categories and OTU contributions for the particular functions were estimated by metagenome contributions.py script.

4.2.1.8. Statistical analysis and Bio- accession No: The statistical analysis of alpha diversity measurements were performed using Phyloseq R package and the raw metagenomic reads were submitted to Sequence Read archive (SRA), NCBI to obtain Bio-Sample and SRA accession numbers. The Bio- Sample ID SAMN18079902 and SRA accession no. PRJNA705276 are now publicly available in the NCBI Database.

4.2.2. Methodology of *ITS* fungal metagenome analysis

4.2.2.1. DNA Extraction, Quantification and QC check: 500 ul of fermented beverage sample was used for DNA extraction using DNeasy PowerSoil Kit (Catalog: 12888-100, Qiagen) and the DNA concentration was determined by using Qubit™ dsDNA BR Assay Kit (Catalog: Q32853, Thermo Fisher Scientific). The readings were taken in a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and the sample was QC passed after taking it for the amplification using *ITS2* specific primers by taking 20 ng of input DNA which showed an intact amplification at approximately 450 bp.

4.2.2.2. Library Preparation: 20 ng of DNA was used to amplify *ITS2* regions of *18S* rDNA gene using KAPA HiFi HotStart ReadyMix PCR Kit (Catalog: 0370, KAPA BIOSYSTEMS) following Kumar and Shukla et al. 2005. The PCR involved an initial denaturation of 95°C for 5 min followed by 25 cycles of 95°C for 30s, 55°C for 45s and 72°C for 30s and a final extension at 72°C for 7 min and holding at 4^0C. The amplification was confirmed by loading 3 uls of the PCR product on 2% agarose gel and the presence of a band at ~430 bp. Upon the confirmation on the amplification, the PCR products were cleaned up, using 0.9X AMPure XP beads (Catalog: A63881, Beckman Coulter) to remove unused primers and eluted in 10 uls of 0.1X TE buffer. Further unique P7 (AGATCGGAAGAGCACACGTCTGAACTCCAGTCA) and P5 (AGATCGGAAGAG CGTCGTGTAGGGAAAGAGTGT) barcoding was performed for the individual samples by additional 8 cycles of PCR by taking 4 uLs of the purified PCR product. The final PCR products were again purified using 0.9X AMPure XP beads and the final library was eluted in 15 uLs of 0.1X TE buffer.

4.2.2.3. Library Quantification and validation: The library concentration was determined in a Qubit.3 Fluorometer (Catalog: Q33216, Life technologies using The Qubit™ dsDNA BR (Broad Range) Assay Kit (Catalog: Q32853, ThermoFisher Scientific). The library quality assessment was done using Agilent D5000 ScreenTape System (Catalog: 5067-5588, Agilent) in a 4150 TapeStation System (Catalog: G2992AA, Agilent).

4.2.2.4. Sequencing Methodology: 25 ng of DNA was used to amplify Fungal *ITS2* hypervariable regions using 100 nm final concentration of *ITS3* mix and *ITS4* NGS primers that have partial Illumina sequencing adapters as overhangs and KAPA HiFi HotStart Ready Mix (Tedersoo et al. 2015). The PCR involved an initial denaturation of 95°C for 5 min followed by 30 cycles of 95°C for 30s, 55°C for 45s and 72°C for 30s and a final elongation at 72°C for 7 min followed by purification of amplicons using Ampure beads to remove unused primers. Libraries were quantitated using the Qubit dsDNA High Sensitivity assay kit. Sequencing was performed using Illumina Miseq with 2x300 PE V3 sequencing kit.

4.2.2.5. Sequence Data QC: The sequence data was generated using Illumina MiSeq. Data quality was checked using FastQC (Babraham, 2017) and MultiQC (Ewels et al. 2016) software. The data was checked for base call quality distribution, % bases above Q20, Q30, %GC, and sequencing adapter contamination.

4.2.2.6. Data Analysis: The reads were trimmed (20bp) from 5’ end to remove the degenerate primers. The trimmed reads were processed to remove adapter sequences and low quality bases using Trimgalore (Babraham, 2017). The QC passed reads were imported into USEARCH v11 (Edgar, 2010) and the pairs were aligned with each other to

form contigs allowing for 10 mismatches while aligning and simultaneously screening for errors to reject any contig with ambiguous base calls. The high quality contigs were checked for identical sequences and duplicates were merged to get unique sequences as representative sequence for OTU (Operational Taxonomical Unit) clustering. The unique sequences are then clustered at 97% sequence similarity into OTUs while simultaneously detecting and removing chimeric contigs. OTUs with only 1 representative sequence are considered as singletons and discarded from further analysis. This OTU clustering and chimera removal was carried out by the UNOISE algorithm (Edgar 2016). Next, the OTUs were populated by mapping back the filter passed contigs onto the representative sequence of OTUs and the abundance calculated. The OTUs were then classified using UNITE ITS fungal database version 8.0 (Kõljalg et al. 2013).

4.2.2.7. Statistical analysis: The statistical computing for alpha diversity measurements were performed using Phyloseq R package for microbiome data analysis.

4.2.2.8. Data availability: Raw metagenomic reads were submitted to Sequence Read Archive (SRA), NCBI to obtain the Bio-Sample and SRA accession numbers. The Bio-sample accession number obtained is SAMN18079738 and the accession to cite the SRA data is PRJNA705285 respectively.

4.3. Results

4.3.1. Sequence Data Quality Check: The sequence data showed that both the samples passed the QC threshold (Q20>95%).

Table 4.1. Summary of Raw sequence data and quality

Sample-ID	Number of reads	Read Length	GC%	% Bases > Q20
Zusem	226466	301	52	99.65
Katsing	331772	301	50.5	99.29

4.3.2. Metagenomic profile of *Zusem* and *Katsing*

4.3.2.1. *16S* microbial profile of *Zusem*: The rarefaction curve of *Zusem* indicated a high read sample value (Figure 4.1) and the Taxonomic Diversity Analysis of filtered *16S rRNA* gene amplicons showed that the value of observed richness was 397, Chao1 and ACE estimated richness was 502.175 and 491.143. Shannon, Simpson, Inverse Simpson, and Fisher's alpha diversity values showed 2.122, 0.108, 5.228, and 52.91 respectively (Figure 4.3). The data revealed dominance of gram positive against gram negative bacteria with the top 3 phylla identified as Firmicutes, Cyanobacteria and Proteobacteria (Table 4.2, Figure 4.5), *Weissella, Pediococcus* and *Lactobacillus* as the top 3 genus (Table 4.3, Figure 4.6) while at the species level *acidilactici, oryzae* and *manihotivorans* formed a major part of the microbial community (Table 4.4, Figure 4.7). The KEGG metabolic pathways (Figure 4.8) and gene categories abundance predictions, COGs (Figure 4.9) revealed that in KEGG pathway, "metabolism" dominated the pathway followed by

"genetic information processing" and "unclassified group". The COGs analysis showed the "general function prediction only" as the highest represented category, followed by "function unknown" and "replication, recombination and repair". A Krona chart illustrating the complete bacterial community of *Zusem* was also constructed (Figure 4.11).

4.3.2.2. Microbial diversity indices of *Katsing*. The alpha diversity indices estimated for *Katsing* revealed rich species diversity as depicted by the rarefaction curve (Figure 4.2). The Taxonomic alpha diversity analysis showed that observed richness was 202, while Chao1 and ACE estimated richness value was 202 and 202 ± 4.514. Shannon, Simpson, inverse Simpson, and Fisher's alpha diversity showed 2.287, 0.726, 3.652 and 23.632, respectively (Figure 4.4).

4.3.2.3. ITS fungal community of *Katsing*. The complete fungal community is depicted by Krona chart using Krona tool (Figure 20). The fungal community was found to be comprising of a total number of 4 phyla with maximum representation of species from the phylum Ascomycota (73.4%). This was followed by Neocallimastigomycota (17%), Unassigned (8.13%) and Basidiomycota (1.48%) (Table 4.5, Figure 4.10A). At genus level 27 Outgroup taxonomic Units (OTUs) were observed, among them the genera *Pichia* (50.2 %) were found in abundance, followed by unassigned groups (42.3%) and *Penicillium* (5.72 %). Other genera found in less abundance are represented in decreasing order of their percentage abundance in the table below (Table 4.6, Figure 4.10B). At the species level, "Unassigned" group occupied the highest level (Table 4.7, Figure 4.10C).

Table 4.2. Table representing the top 10 Phyla abundance in *Zusem*

Phyllum	Abundance %
Firmicutes	57897
Cyanobacteria	27199
Proteobacteria	9029
Actinobacteria	563
Bacteroidetes	347
TM7	66
Verrucomicrobia	56
Planctomycetes	42
Chloroflexi	40
Acidobacteria	32

Table 4.3. Table representing the top 10 Genus abundance in *Zusem*

Genus	Abundance%
Weissella	25422
Pediococcus	23591
Lactobacillus	6162
Leuconostoc	284
Streptomyces	171
Bacillus	113
Rhodococcus	108
Pseudomonas	70

Lactococcus	70
Stenotrophomonas	66

Table 4.4. Table representing the top 10 Species abundance in *Zusem*

Species	Abundance%
acidilactici	23090
oryzae	19674
manihotivorans	4323
ghanensis	2694
plantarum	1151
cibaria	1120
beninensis	258
mesenteroides	257
geniculata	59
marisflavi	45

Table 4.5. OTUs abundance percentage of *Katsing* at Phylum level

Phylum	Abundance %
Ascomycota	73.4
Neocallimastigomycota	17
(Unassigned)	8.13
Basidiomycota	1.48

Table 4.6. OTUs abundance percentage of *Katsing* at Genus level

Genus	Abundance %
Pichia	50.2
Unassigned	42.3
Penicillium	5.72
Malassezia	0.778
Aspergillus	0.288
Talaromyces	0.136
Anaeromyces	0.0854
Gjaerumia	0.0813
Zopfiella	0.0575
Candida	0.0411
Debaryomyces	0.0402
Toxicocladosporium	0.0361
Curvularia	0.0296
Zygoascus	0.023
Cladorrhinum	0.0214
Sarocladium	0.0214
Thermomyces	0.0205
Anthracocystis	0.0197
Trichosporon	0.0189
Sterigmatomyces	0.0172
Periconia	0.0164

Xenoacremonium	0.0156
Occultifur	0.0148
Psathyrella	0.0131
Orpinomyces	0.00903
Nigrospora	0.00739

Table 4.7. OTUs abundance percentage of *Katsing* at Species level

Species	Abundance%
Unassigned	99.8
Gjaerumia_minor SH1565762.08FU	0.0813
Candida parapsilosis SH1570474.08FU	0.0238
Zygoascus meyerae SH1508099.08FU	0.023
Cladorrhinum bulbillosum SH1551592.08FU	0.0214
Sterigmatomyces halophilus SH1546026.08FU	0.0172
Malassezia globosa SH1565713.08FU	0.0164
Xenoacremonium recifei SH1561515.08FU	0.0156
Psathyrella efflorescens SH1643610.08FU	0.0131

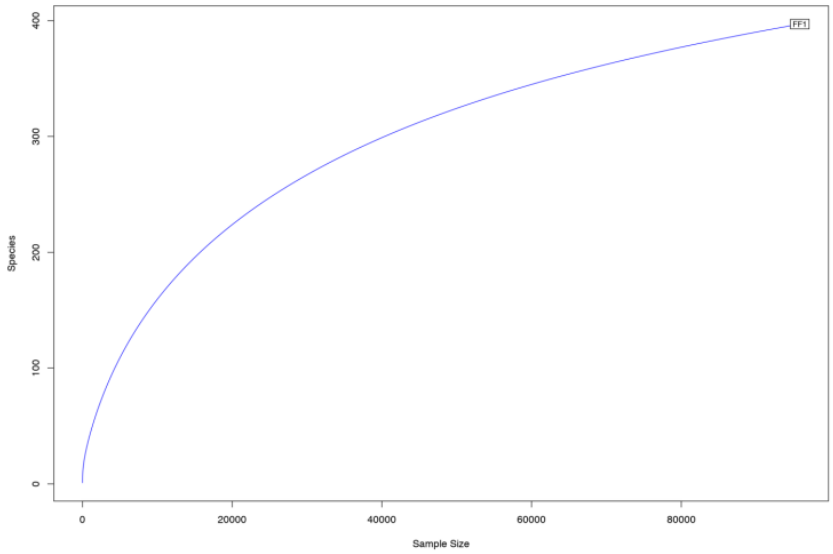

Figure 4.1: Rarefaction curve representing the measure of diversity that was captured by a given number of reads in *Zusem* (fermented bamboo shoot)

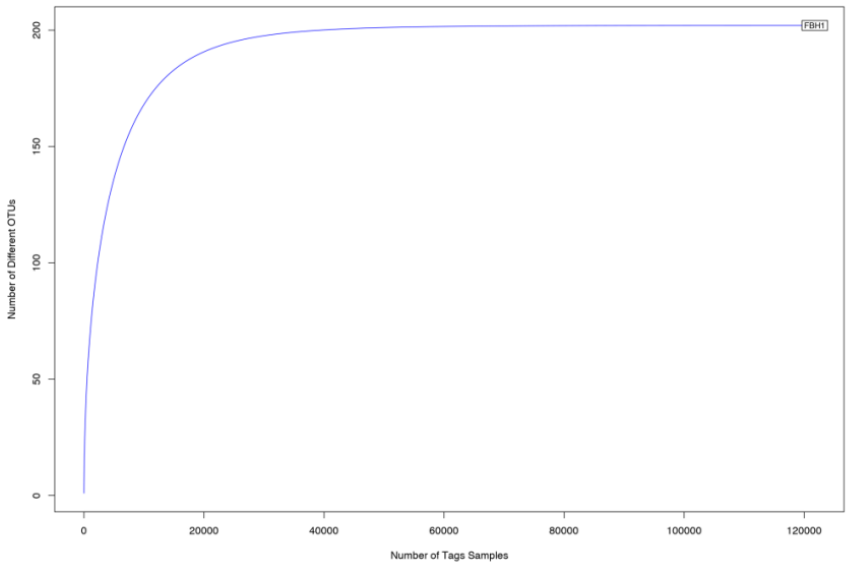

Figure 4.2: Rarefaction curve representing the measure of diversity that was captured by a given number of reads in *Katsing* (fermented rice beer)

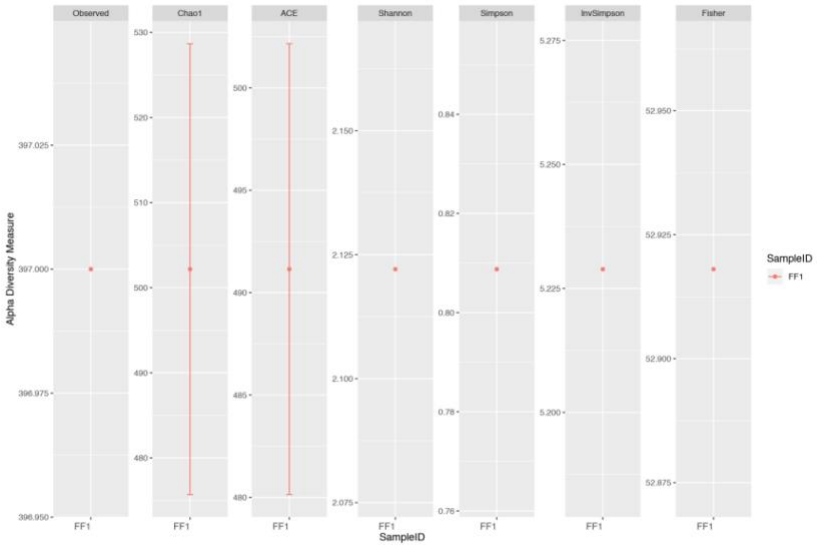

Figure 4.3: Alpha diversity measurements of FF1 (*Zusem)* (Chao1 and ACE represent the richness of the sample and Shannon, Simpson, InvSimpson and Fisher represent both richness and relative abundance)

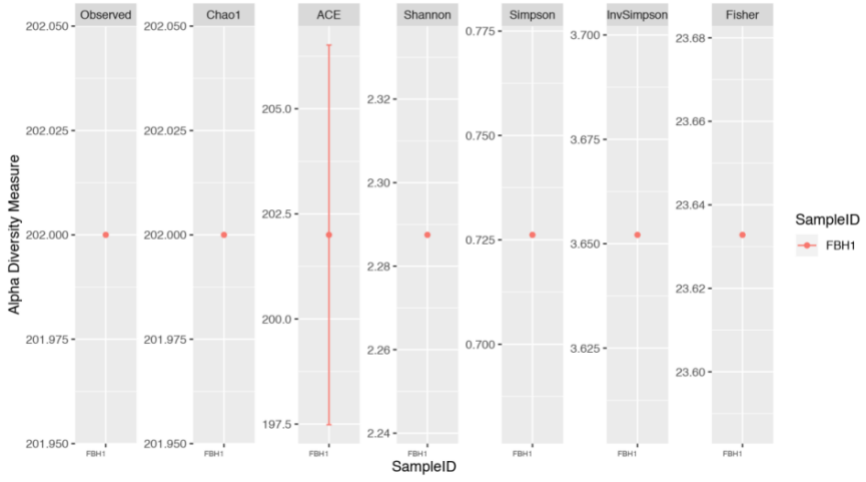

Figure 4.4: Alpha diversity measurements of FBH1 (*Katsing)* (Chao1 and ACE represent the richness of the sample and Shannon, Simpson, InvSimpson and Fisher represent both richness and relative abundance)

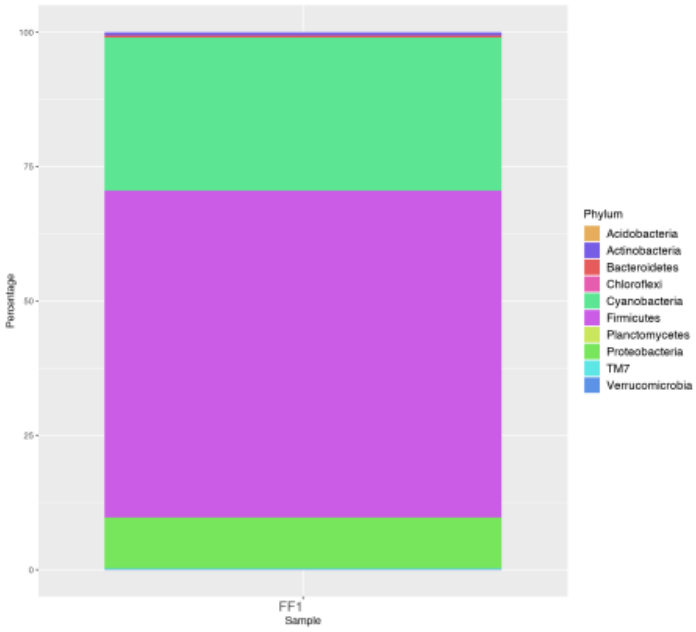

Figure 4.5: Figure representing the top 10 Phyla abundance distribution in *Zusem*

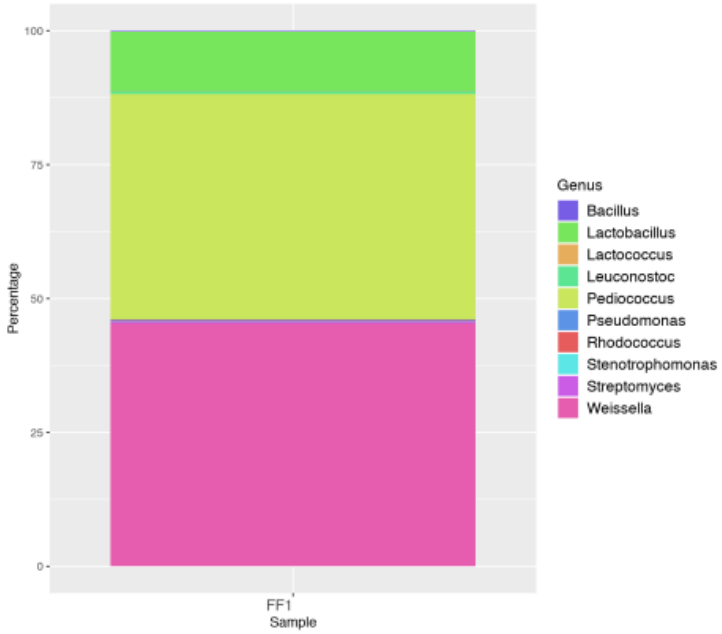

Figure 4.6: Figure representing the top 10 Genus abundance distribution in *Zusem*

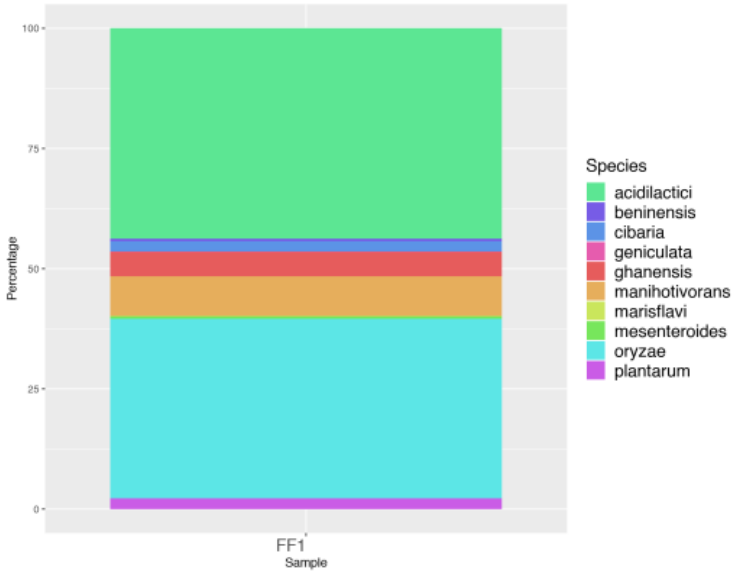

Figure 4.7: Figure representing the top 10 Species abundance distribution in *Zusem*

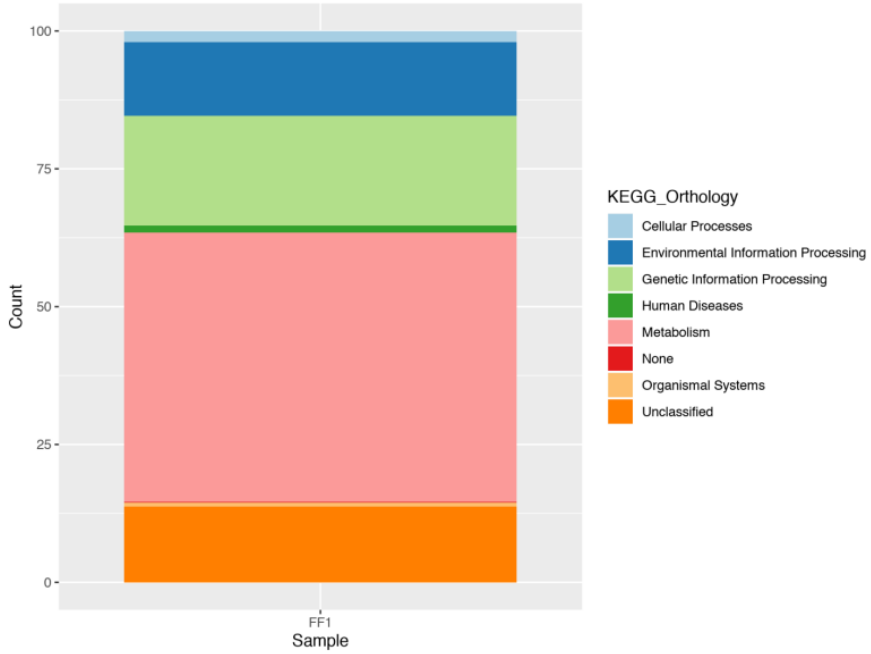

Figure 4.8: KEGG orthology predicted using PICRUSt

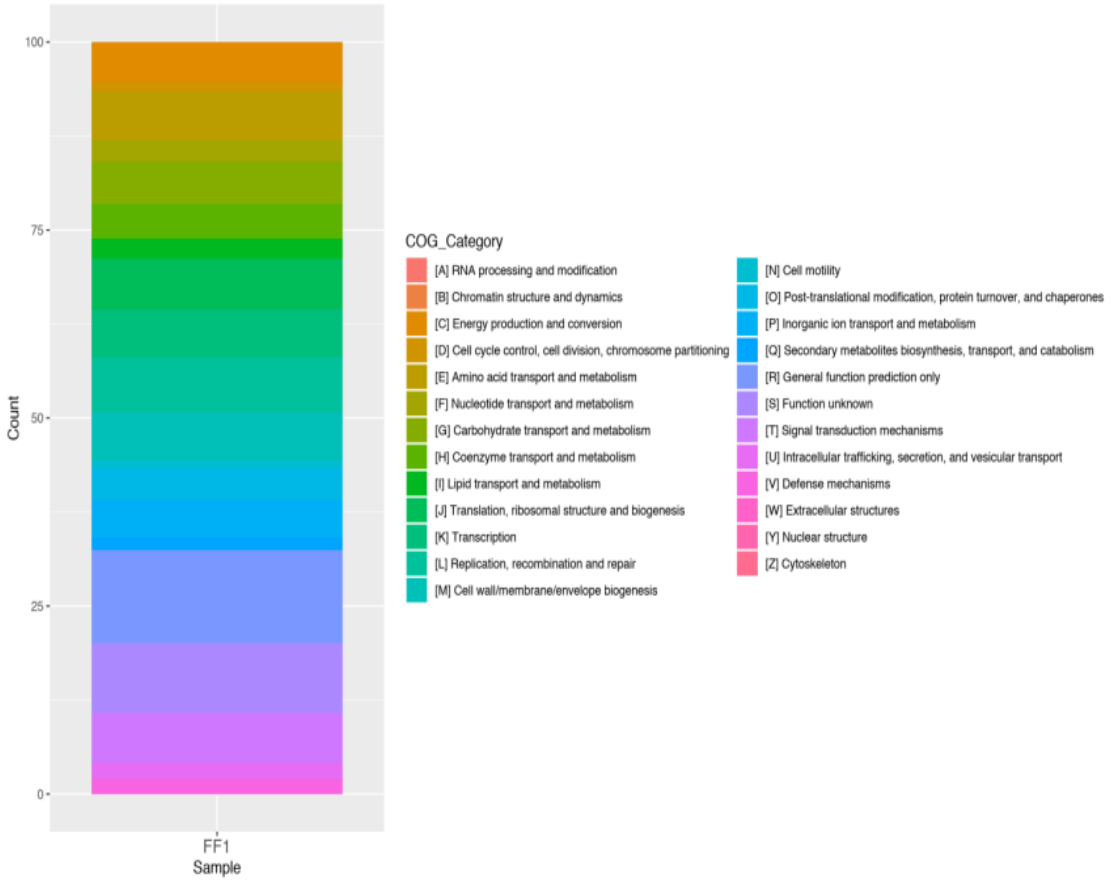

Figure 4.9: COG Category predicted using PICRUSt

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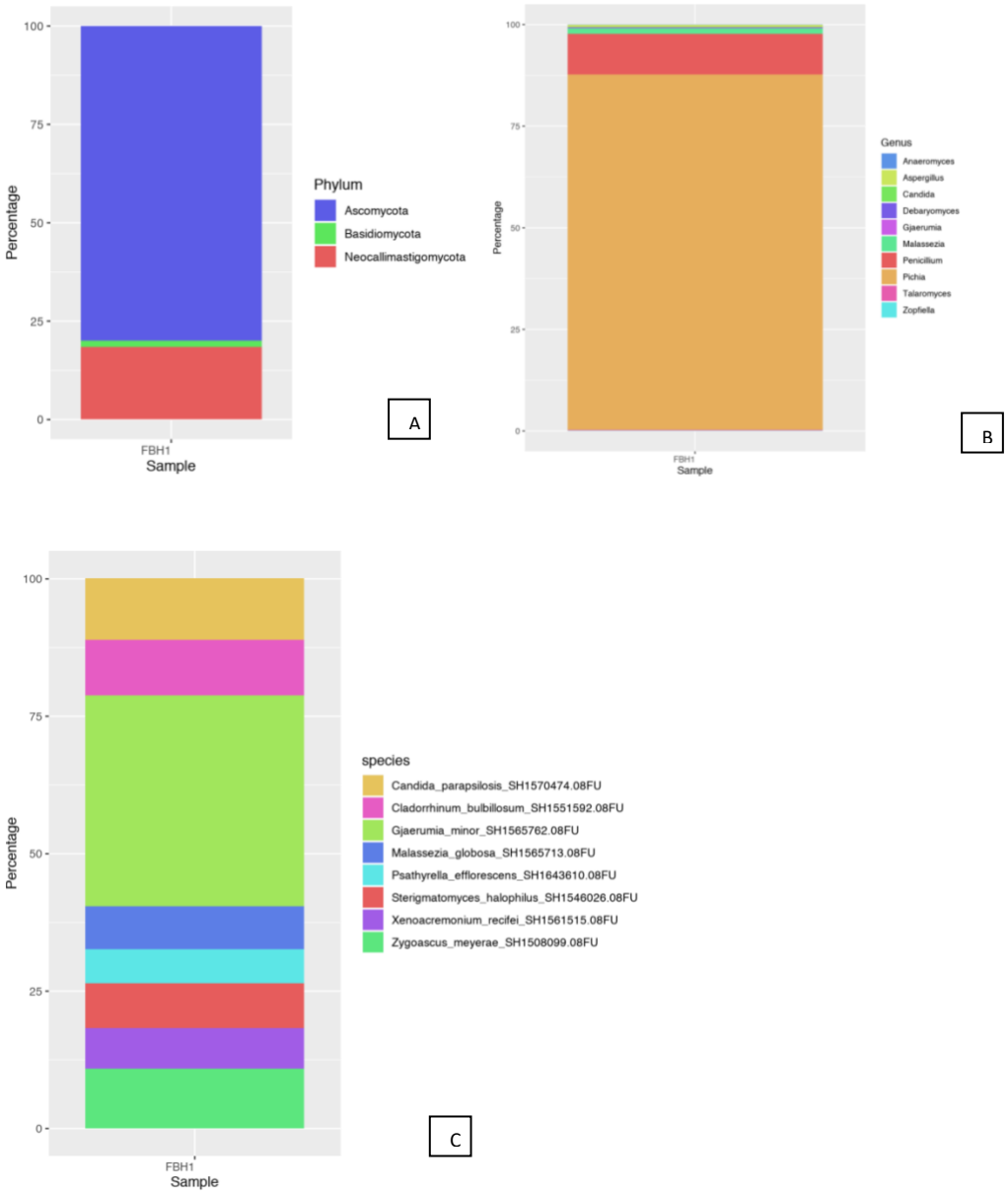

Figure 4.10(A-C): Figure representing the Phyla, Genus and Species abundance distribution in *Katsing*. A- Phyla abundance distribution, B-Top 10 Genus abundance distribution, C- Species abundance distribution

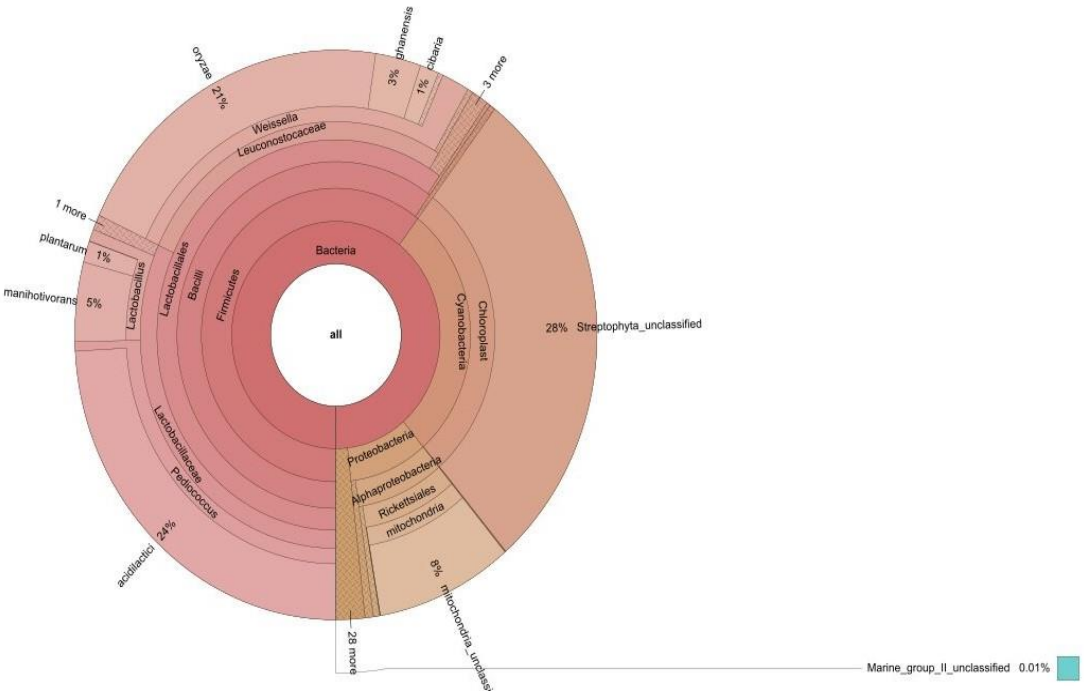

Figure 4.11: A Krona chart illustrating the complete bacterial community of *Zusem*

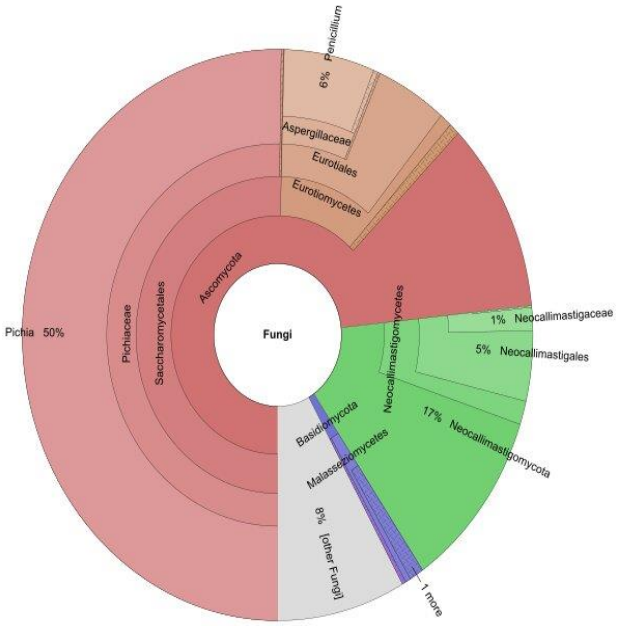

Figure 4.12: A Krona chart depicting the fungal community of *Katsing*

4.4. Discussion

4.4.1. *Zusem*: Fermented bamboo shoots commonly referred to as the green gold of the twenty first century (Behera and Balaji 2021) houses profuse no. of microbial flora where LAB is reported to be the dominant organism present. Lactic Acid Bacteria (LAB) are predominant microorganisms of many fermented food products imparting a characteristic flavor, taste and aroma. Our results indicate the dominance of gram positive LAB with Firmicutes, Cyanobacteria and Proteobacteria representing the top 3 phylla, *Weissella, Pediococcus* and *Lactobacillus* as the top 3 genus and *acidilactici, oryzae* and *manihotivorans* as the top 3 species level. Among the top 10 genera, 7 genus belonged to phyla Firmicutes, while 2 genus belonged to Proteobacteria and 1 to Actinobacteria. Data showed dominance of gram positive bacteria LAB, the most abundant genus *Weisella* belonging to family Leuconostocaceae. A study on the probiotic properties of *Weisella* strain showed that *W. cibaria* and *W.confusca* conferred probiotic potential (Lee et al. 2011). *Pediococcus* and *Lactobacillus,* ranked below, *Weisella,* both of which are LAB belonging to family Lactobacillaceae. *Pediococcus* and *Lactobacillus* are common microorganisms employed in the dairy industry and *Lactobacillus* is the most common probiotic as it forms biofilms in the gut microbiota. *Leuconostoc* another genus detected in *Zusem* is responsible for the fermentation of cabbage into sauerkraut along with *Pediococcus* and *Lactobacillus.* At the species level, *acidilati* ranked as the most dominant microorganism. *Pediococcus acidilactici* is a homo-fermentive gram positive bacteria known for its potential probiotic role found commonly in fermented vegetables, meat and dairy products (Barros et al. 2001). An interesting case with regard to species *oryzae* belonging to genus *Xanthomonas* is reported in the sample. Many species of *oryzae* are

pathogenic to plant and are typically host or tissue specific with unique colonization strategies (Shi-Qi An et al. 2020). *Xanthomonas oryzae* belongs to Proteobacteria where *Oryza sativa* is the major host known. Our findings on the abundance of *oryzae* in *Zusem* may attribute to the fact that both the host plant belongs to the same family i.e Poaceae. The explanation of imparting a characteristic sour taste by LAB is probably combination of raw substrates used and the environmental condition of fermentation which shifts the competitive advantage towards the LAB. However the underlying mechanisms of metabolism, host pathogen cycle, assessment of food safety remains to be investigated as microbial diversity is subjected to change with successive stages of fermentation. The KEGG metabolic pathways predicted "metabolism" as the most active pathway while COGs analysis predicted the "general function prediction only" as the highest represented category. KEGG pathway predicting "metabolism" as the highest representative pathway encompasses carbohydrate, energy, lipid, nucleotide and amino acid metabolism suggesting active microbial metabolism during fermentation. However both metabolomics features require larger sample size to make further generalized absolute conclusion.

4.4.2. *Katsing*: Traditional fermented beverage harbors unique microbial populations owing to its ethnic taste. In Japan, *Aspergillus niger* (Koji mold) is used for the production of *sake* for over a millennium (Katsukiho 2015). Similarly in Korea, Wild Yeast Strain *Pichia anomala Y197-13* has been well characterized for brewing *Makgeolli* which is the national rice beer of Korea (Kim et al. 2013). The list of microorganisms according to 2011 International Dairy Federation (IDF) Inventory included 62 genera of microbes with the fungal genera listed into 24 eukaryotic genera present as natural fermenters or in inoculants (Bourdichon et al. 2012). The fungal community in *Katsing* was dominated by

the genus *Pichia* (50.2 %) and it is frequently found in fermented drinks and foods where it plays a role in spontaneous fermentation (Masoud et al. 2004; Sujaya et al. 2004), and belongs to the non *Saccharomyces* wine yeasts (Rojas et al. 2003). Studies on similar rice beer products from Assam and Tripura, 2 neighboring states of Nagaland revealed the presence of *Lactobacillus casei, Pediococcus pentosaceus, Lactobacillus pentosus, Lactobacillus plantarum* (Arup et al. 2019) and *Pichia kudriavzevii* (Ghosh et al. 2019). Relatively few genera present in *Katsing* such as *Malassezia*, *Candida* and *Trichosporon* are natural inhabitants of the skin microflora of humans, *Thermocyces* (hemicellulose degraders) and *Sterigmatomyces* belongs to fungi imperfecti and few genera can be grouped as belonging to opportunistic pathogens of plants and humans. *Katsing* harbors unique microbial populations owing to its ethnic taste, however, further research is required to classify and assign potential metabolic roles of microbes, isolate their productive strains and enhance their role in promoting human health.

4.5. Conclusion

The post-genomic age of microbiology facilitates the knowledge on sequence of many microorganisms used for food fermentation or microorganisms isolated from food fermentations and this offers a new knowledge-based approach, from metabolic engineering to produce antimicrobials or nutritionals, to the molecular mining of unknown activities which could potentially benefit food production. Metagenomics has enabled the emergence of a new era coupled with transcriptomics, proteomics and metabolomics to make known what only seemed obscure a few decades ago. New integrated findings targeting microbial genomes, active participation of the microbes in fermentation, transmission pathways, microbe- host association, time -dependent studies of fermented foods are requisite for radical studies on fermented foods. Furthermore, detection of microbiological contaminations for cross contamination studies and protection of food safety and quality are needed to authenticate fermented foods. Hence, metagenomic analysis triumphs over the complementary traditional culture techniques, however our study is not without limitation. We analyzed only a single time point, which is subjected to microbial succession, likely to be dynamic with each stage of fermentation. Thus to assess the viability of the taxa detected via our DNA base analysis, further investigation is required to improve and widen our limited knowledge.

The real challenge in the present era, however, as applied to food systems is the harnessing of this huge wealth of information to improve culture performance and activities to design novel antimicrobials targeting the essential functions of food pathogenic and spoilage bacteria and aim to improve the safety, quality and composition of our food supply. Hopefully, further scientific research will continue to illuminate the

ways of our ancestral food culture connected to the emerging discipline of fermentation and its products thereof.

Summary

Fermentation solders the ancient old interest in preserving foods prepared in unique and varied ways and highlights the traditional gastronomy and cultural cuisines across the globe. Traditionally the Nagas lived a hunter- gatherer lifestyle but the transition mainly influenced by religion, education and migration has led to sedentary phase with changes in beliefs and palatable flavour profile. The ethnic food culture of Nagas maybe categorized as a blend of *sattvic* symbolizing strength, health, and happiness, which includes fruits, vegetables, legumes, cereals, sweets etc and *rājasic* food symbolizing activity, passion, and restlessness, which include hot, sour, spicy, alliaceous plants including onions, garlic and salty foods. Most of the ethnic food products of Nagaland are vegetable based products and are either fermented, sundried or smoked using unique techniques of preservation. The culinary culture also sequels acquired taste to supplement the organoleptic and palatability of the ethnic food products. Food is reflected as a part of our rich culture and the range of fermented food products produced and consumed serves as a gateway to understand our ethnic and unique food habits prepared at its best. Distinct cuisines separate a certain tribe from others while most food overlaps between the various tribes. A typical traditional Naga meal is cooked rice, meat or vegetable cooked with a fermented food product supplemented with chutney in a traditional wooden plate. A cup of red tea or water at the end of the meal wraps up the repast. The traditional and culinary knowledge are passed down to younger generations mostly by mothers to their daughters by cooking together with them. As a result, traditional gastronomy links the study of perception and practices of a particular food that distinguishes a certain tribe and

highlights culinary tradition that goes beyond the boundaries of a single community to counter the oblivion caused by modernity that affects a rural community.

Fermentation is exclusively a beneficial application to food and beverage production and the traditional knowledge possessed by the tribal community on the diversity of plant and animal products employed for the art of fermentation supported by the abundance of rich natural resource has culminated in culinary skills blended with cultural legacy that have created unique flavor profiles of traditional food items in Nagaland. The nutritional values of some fermented foods of Nagaland namely *Ashikumna* (ASK, fermented pork fats of Sumi Naga), *Kese* (KES, fermented bamboo shoot of Angami Naga), dry *Bastenga* (DBA, common dried fermented bamboo shoot), *Zusem* (ZU, fermented bamboo shoot of Ao Naga) and *Jangpangngatsu* (JAP, fermented crab of Ao Naga) using various standard analytical techniques were analyzed. Among the fermented bamboo shoots of Nagaland, *Kese* (KES) showed the highest total phenolic (mg/100mg FW) and flavonoid (mg/100mg of DW) content (1.959 ± 0.9, 8.035 ± 1.175) which corresponds to its high antioxidant activity. Our results also highlighted the acidic nature of the fermented foods which may aid in preventing the growth of harmful microbes. *Katsing*, an alcoholic rice beer showed alcoholic content as 5 % which is considered a safe level for consumption according to safety norms laid by WHO. All the biochemical parameters studied revealed a higher value of *Katsing* sample bought from market against its positive control. It was also observed that, even though the same method was adopted for preparation of positive control, differences in terms of alcoholic content and bio- chemical properties appeared. The *p*H of fermented crab (JAP) revealed its neutral nature (7.1) with low protein content (0.148 ± 0.09 mg/100mg of FW) and high

flavonoid content (14.461 ± 5.079 mg/100mg of DW). Fermented pork fats (ASK) showed negligible difference against its negative control in all the biochemical parameters studied, with slightly higher values inclined towards fermented product except for total flavonoid content. It can be ascertained that fermentation of pork fats reduces its *p*H but undergoes moderate biochemical and physiochemical changes. Standard and all the extracts showed a dose dependent inhibition on the DPPH radicals. The IC50 value of KES - *Kese*, ZUPC - *Zusem* positive control, ZU - *Zusem* , FBS - fresh bamboo shoot, JAP- *Jangpangngatsu*, FPF - fresh pork fats , DBA - dry *Bastenga* ,KAPC – *Katsing* Positive control, KAT- *Katsing* and ASK – *Ashikumna* were found to be 26.02, 1.7, 2.1, 2.5, 2.2, 9.02, 1.6 and 1.5 mg/ml respectively. KES exhibited the strongest DPPH radical scavenging activity and the extracts radical scavenging activity were effective in the order KES> ZUPC> DBA> ZU> FBS> JAP> FPF> KAPC> KAT> ASK. Hence, our study revealed that fermented foods of Nagaland are a potential source of nutrition or natural antioxidants owing to their biochemical attributes such as phenol and flavonoid contents which are known as good antioxidants. These foods can be further exploited for their health-giving attributes as they are highly valued for their promising prebiotic and probiotic prospects. The nutritional status of fermented foods can also be improved by the rational choice of food-fermenting microbes based on the understanding of their interaction with diet and human gastrointestinal microbiota.

Metagenomic application in our study elucidated the microbial diversity, structure, functional annotation and mapping into metabolic pathways and helped understand the dynamic balance between microbes and the food substrate by DNA based extraction. It explored the usefulness of culture – independent method in detecting the bacterial

community in *Zusem* and fungal repertoire of *Katsing*. *Zusem* is an indigeneous fermented bamboo shoot while *Katsing* is a traditional fermented rice beverage, both of which are unique to the Ao Nagas of Nagaland. Lactic Acid Bacteria (LAB) are predominant microorganisms of many fermented food products imparting a characteristic flavor, taste and aroma. *16S* analysis of *Zusem* indicate the dominance of gram positive LAB with 7 genus belonging to phyla Firmicutes, 2 genus to Proteobacteria and 1 to Actinobacteria in *Zusem*. The most abundant genus was *Weisella* belonging to family Leuconostocaceae followed by *Pediococcus* and *Lactobacillus*. Firmicutes, Cyanobacteria and Proteobacteria represented the top 3 phyla while the species level was dominated by *acidilactici, oryzae* and *manihotivorans* as the top 3 species. KEGG pathway predicting "metabolism" as the highest representative pathway includes carbohydrate, energy, lipid, nucleotide and amino acid metabolism suggesting active microbial metabolism during fermentation. COGs also predicted the "gene performing function" as the most abundant category. *ITS* fungal profile of *Katsing* revealed the microbial profile to be dominated by the genus *Pichia* (50.2 %) along with 24 other genera and the species level dominated by "unassigned" category (99.8%) followed by *Gjaerumia minor* and *Candida parapsilosis* with 6 other species. Ascomycota (73.4%) represented the highest phylum category followed by Neocallimastigomycota (17%). However both metabolomics features require larger sample size to assess the viability of the taxa detected and hopefully, further research will continue to illuminate the ways of our ancestral food culture connected to the emerging discipline of fermentation and its products thereof.

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Appendix I

Primers and adapter sequences used in the study

NAME	SEQUENCE	BASES	REGION
ITS3mixF	CAHCGATGAAGAACGYRG	18	*ITS*
ITS4R	TTCCTSCGCTTATTGATATGC	19	
P7 adapter	AGATCGGAAGAGCACACGTCTGAACT CCAGTCA	33	
P5 adapter	AGATCGGAAGAGCACACGTCTGAACT CCAGTCA	33	
V3V4F	CCTACGGGNGGCWGCAG	17	*16S rRNA*
V3V4R	GACTACHVGGGTATCTAATC	19	gene
P7 adapter	AGATCGGAAGAGCACACGTCTGAACT CCAGTCA	33	
P5 adapter	AGATCGGAAGAGCGTCGTGTAGGGAA AGAGTGT	33	

Appendix II

Bio-Sample and Sequence Read Archive (SRA) accession numbers

Name of the sample	**Bio-sample ID**	**SRA accession numbers**
Katsing	SAMN18079738	PRJNA705285
Zusem	SAMN18079902	PRJNA705276

List of Publications

Lydia Yeptho, T. Ajungla, Asangla Kichu and Maibam Romeo Singh. Ethnic food habits of the Sumi tribe, Nagaland, India. Current Science. Vol 119, No. 4, 25th August 2020. Doi: 10. 18520/cs/v119/i4/708-712. ISSN: 0011-3891.

Lydia Yeptho, T. Ajungla and Keviphruonuo Kuotsu. Ethnic study on *bastenga*, a fermented bamboo shoot product of Nagaland, India. Current Science. Vol 120, No. 4, 25th February 2021.

Lydia Yeptho and T. Ajungla. Ethnobiology of the traditional alcoholic rice beers of Nagaland, India, In: Bioresources and Sustainable Livelihood of Rural India, edited by Chitta Ranjan Deb and Asosii Paul, Mittal Publication, New Delhi. ISBN-10-9390692571.

T. Ajungla, Lydia Yeptho, Asangla Kichu and Gloria Nyenthang. Some ethnic fermented foods and beverages of Nagaland, In: Ethnic fermented foods and beverages of India: Science, History and Culture, J.P. Tamang (ed.), Springer Nature, 2020. 459-477.

Nyenthang, G., Kichu, A., Ajungla, T. and Yeptho, L., 2019. The first encounter of *Fistulina hepatica* (Schaeff.) With. belonging to the family Fistulinaceae in Nagaland, India. Current Science, 2019, 9, 1433-1434.

Kichu, A., Nyenthang, G., Ajungla, T. and Yeptho, L., 2019. Colonial and morphological characteristics of soil fungi from jhum land. Indian Journal of Agricultural Research. Doi: 10.18805/IJARe.A-5265.

List of Seminar/ Symposium, Conferences Attended and Presented Papers

Standard Operating Procedures (SOPs) for the Isolation and Characterization of Bacteria and Fungi from Diverse Habitats during July 2nd and 3rd 2018, organised by The North East Microbial Repository Centre (MRC), Institute of Bioresources and Sustainable Development (IBSD), Imphal, Manipur, India.

Hands on training on "genomics and gene expression analysis". Organised by Department of Biotechnology, Govt. Of India sponsored Advance Level Institutional Biotech Hub, Department of Botany, Nagaland University, Lumami 798627, Nagaland. July 18th -23rd, 2018.

'Skill and Entrepreneurial Development of the Tribal Youth' with the theme 'Value-additions to rich Bio-resources with special reference to Medicinal and Aromatic plants' at Nagaland University, Lumami between July 25th – 28th, 2018. Jointly organised by Biotech Park, Lucknow and Institutional Biotech Hub, Department of Botany, Nagaland University under the aegis of the National Academy of Sciences, India.

National conference of Stakeholders on conservation, cultivation, resource development and sustainable utilization of Medicinal plants of North- Eastern India held on March 6-7, 2019 at Nagaland University, Lumami jointly organised by Dept. of Botany (UGC- SAP DRS-III, FIST), Nagaland University Lumami and Society for Conservation and Resource Development of Medicinal plants (SMP) New Delhi.

The 9th Conference on Taxonomy and Systematics in Thailand (TST9) October 2-4, 2019. Chiang Mai University, Thailand.

'Research Ethics, Paper Writing & IPR' Organised & Sponsored by UGC-SAP(DRS-III), Department of Botany & Department of Biotechnology, Govt. of India sponsored. Advanced Level Institutional Biotech Hub, Nagaland University, Lumami. November 14-15, 2019

"Bioresources and Sustainable Livelihood of Rural India" organised by Department of Botany, Nagaland University, Lumami 798627, Nagaland, India on September 28 and 29, 2020 sponsored by "Ministry of Environment, Forest and Climate Change" supported by NMHS Programme and UGC- SAP (DRS- III) Programme, Department of Botany, Nagaland University.

Poster presentation on "**Traditional knowledge on health benefits and preparation of ethnic fermented rice and food beverages of Nagaland, India**" was presented on National conference of Stakeholders on conservation, cultivation, resource development and sustainable utilization of Medicinal plants of North- Eastern India held on March 6-7, 2019 at Nagaland University, Lumami. (Awarded 1st position)

Oral presentation on "**Ethnic food diversity of some common fermented foods and beverages of the Indigenous tribes of Nagaland, India"** presented at the 9th Conference on Taxonomy and Systematics in Thailand (TST9) October 2-4, 2019. Chiang Mai University, Thailand.

Oral presentation on **"Ethnobiology of the Traditional Alcoholic Rice beers of Nagaland, India"** was presented at the "Bioresources and Sustainable Livelihood of

Rural India" organised by Department of Botany, Nagaland University, Lumami 798627, Nagaland, India on September 28 and 29, 2020 sponsored by "Ministry of Environment, Forest and Climate Change" supported by NMHS Programme and UGC- SAP (DRS- III) Programme, Department of Botany, Nagaland University.