Metagenomic Characterization of the Culturable Bacterial Community Structure of Tapuy, a Philippine Indigenous Rice Wine, Reveals Significant Presence of Potential Probiotic Bacteria

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ABSTRACT

Background. Tapuy is an indigenous fermented rice wine produced in the Northern areas of the Philippines. Fermented foods and drinks have gained interest due to their associated health benefits, attributable to probiotic bacteria in the food items. However, pathogenic bacteria may also be present in fermented food and pose health risks, signifying a need for standardization. Currently, there is limited knowledge on the bacterial content of tapuy.

Objectives. This study aimed to characterize the bacterial diversity and community structure of culturable bacteria in traditionally fermented tapuy samples, to perform standardization of tapuy fermentation, and to compare the bacterial diversity and community structure of culturable bacteria in laboratory fermented tapuy with that of the traditional samples.

Methods. Tapuy samples were obtained from four municipalities in Benguet, Philippines. Laboratory fermentation of tapuy was performed simultaneously with the fermentation in the sampling site using a standardized protocol. Samples were plated on de Man, Rogosa, and Sharpe (MRS) agar and NA, and the colonies were harvested for DNA extraction. DNA samples were sent for 16S rDNA metagenomic sequencing.

Results. Metagenomic analysis revealed the presence of many bacterial species that were previously unreported in tapuy. Traditional tapuy samples were composed primarily of members of the genera *Bacillus* and *Lactobacillus* in varying proportions. Potential probiotic bacteria were abundant in Kapangan (97.42%) and Sablan (99.89%) field samples. *B. wiedmannii* was present in all samples and was identified as a harmful species. Laboratory fermentation increased the abundance of potential probiotic bacteria in Itogon and La Trinidad samples (differences of 75.36% and 78.36%, respectively). It decreased the quantity of *B. wiedmannii* in La Trinidad (a difference of 97.1%). Laboratory fermented samples generally exhibited higher bacterial diversity and species richness compared to field samples.

Conclusions. Traditionally fermented tapuy samples contained a significant proportion of potential probiotic bacteria belonging to the genera *Bacillus* and *Lactobacillus*. Laboratory fermented samples were found to have higher bacterial diversity and richness compared to field samples. The significant presence of potential probiotic bacteria suggests that tapuy is a good candidate for development into functional food and a good source of likely probiotic species that could be explored for health applications. The presence of harmful bacteria suggests the need for possible standardization of fermentation practices.

Keywords: bacterial community structure, food microbiology, fermented foods and beverages, wine, tapuy, targeted metagenomics

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INTRODUCTION

Tapuy is an indigenous Philippine fermented rice wine produced mainly in northern Luzon, Philippines. It is typically made from the fermentation of cooked white or red glutinous rice by a native starter culture, called bubod. It is usually consumed during celebrations such as birthdays, weddings, holidays, and festivals.[1] As with other indigenous fermented products, fermentation of tapuy is commonly done at home and thus exhibits variations per area. Practices may differ between villages and utilize different indigenous materials.[2]

Fermented foods and drinks have been gaining interest in recent years due to research on their associated health benefits such as anti-aging, anti-carcinogenic, anti-obesity, and immunomodulating properties.[3] These health benefits have been attributed to probiotic bacteria in the food items, at least partially.

The World Health Organization defines probiotics as "live microorganisms, which when administered in adequate amounts confer health benefits to the host." These health benefits include, but are not limited to, improved intestinal health, enhanced immune response, reduced symptoms of lactose intolerance, reduced growth of pathogenic bacteria.[4] Some of the most well-studied and commonly occurring probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium*.[5] Knowledge of the potential probiotic bacterial species in a food item gives way to the development of probiotic strains for health applications and to the development of functional foods which confer additional health benefits aside from nutrition.[2] Furthermore, this increases interest in consuming the food item and promotes indigenous knowledge in indigenous food products.

In recent years, culture-independent methods such as metagenomic sequencing have been widely used to detect potential probiotic bacteria in fermented food products in a high throughput manner and to determine bacterial diversity. However, a culture of these bacteria is still necessary to screen them for probiotic characteristics and study them for further applications. Thus, a combination of culture-dependent and -independent methods may be advantageous because this will allow for the detection of potential probiotic bacteria that are culturable, and the detection can be done in a high throughput manner.

Metagenomic sequencing of food also helps to reveal the presence of potentially pathogenic bacteria. Previous studies have utilized metagenomic sequencing to identify contaminating pathogenic bacteria, which were not previously reported, in different fermented food items, resulting in efforts to standardize fermentation procedures.[6,7] Additionally, metagenomic sequencing has also been applied to fermented foods to identify microbial species that may produce desirable characteristics in the product.[8,9]

Currently, there are limited studies on the bacterial content of tapuy, and previous efforts to characterize these bacteria have utilized only culture-dependent methods, which are time-consuming and resource-intensive. Thus, the content of potential probiotic bacteria in tapuy may be severely underestimated, and the presence of potentially pathogenic bacteria in it is unknown. The present study then aims to characterize the community structure and diversity of culturable bacteria in tapuy from four municipalities in Benguet province, Philippines, using 16S rDNA metagenomic sequencing. The study also aims to perform standardization of tapuy fermentation and compare the bacterial community structure and bacterial diversity of laboratory fermented tapuy with traditional samples. The significance of this study is to establish tapuy as a source of potentially probiotic bacterial species and strains. The results of this study may also be used in the development of the probiotic bacteria for health applications, in the development of tapuy as a functional food, and the standardization of tapuy-making procedures.

MATERIALS AND METHODS

Ethical Clearance

Permission was sought from the National Commission on Indigenous Peoples Regional Office in Benguet to conduct the study and interview the locals regarding the tapuy-making procedure. The researchers were restricted from divulging enabling information regarding the fermentation recipe and process. Only the starting materials used in each municipality are reported in this study.

Sample collection

Traditionally fermented Tapuy rice wine and rice lees samples were purchased from local breweries in 4 municipalities in Benguet province, namely: Itogon, Kapangan, La Trinidad, Sablan. A map of the study site is shown in Figure 1. Upon collection, the samples were kept in their original containers and placed in ice (4°C) for transport to the laboratory. The samples were then processed within 4 hours of collection from the study sites.

Laboratory fermentation of tapuy

The starting materials used for tapuy-making in each municipality were obtained through interviews with the locals and followed during fermentation. In La Trinidad and Kapangan, Ballatinao black rice and Bongkitan white rice are used as the starting materials in differing ratios. In Itogon, Ballatinao black rice, Bongkitan white rice, and guava leaves are used as starting materials. In Sablan, Ballatinao rice and coarsely ground white corn are used as starting materials. The raw materials (Ballatinao black rice, Bongkitan white rice, and coarsely ground white corn) used for producing laboratory fermented tapuy were obtained from Baguio public market. Guava leaves were freshly picked from the backyard of the tapuy maker in Itogon.

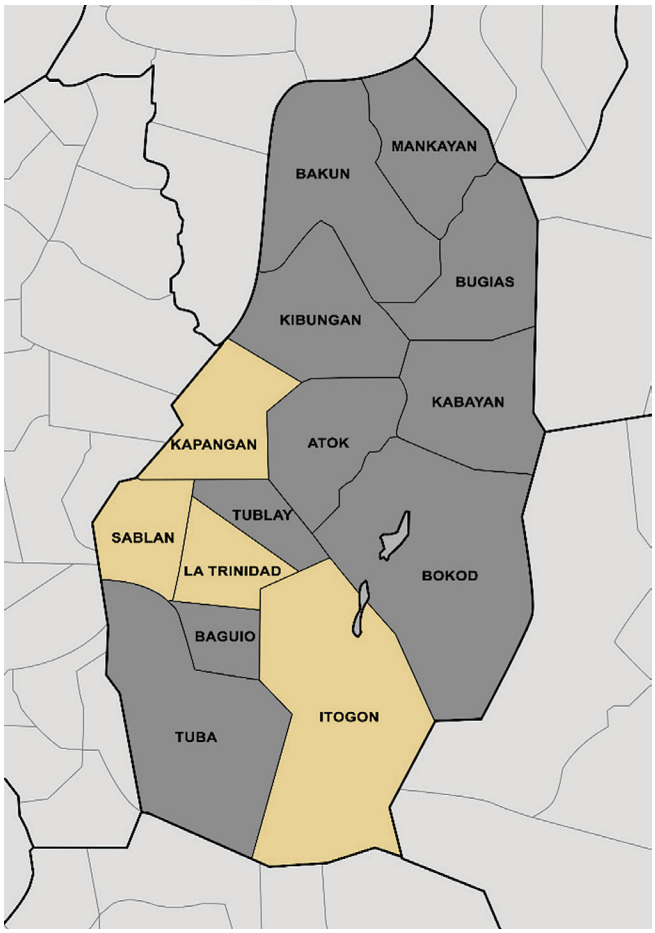

Figure 1. Map of Benguet, the Philippines, highlighting the municipalities sampled.

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Laboratory fermentation was performed simultaneously with the fermentation in the corresponding municipality. For all samples, the rice was washed once with tap water and once with distilled water. Distilled water was added to the rice in a ratio of 1 part rice: 2 parts water. The rice was cooked, transferred to a plastic container, and cooled to approximately 40°C before inoculation with a starter culture.

A 60 mL suspension containing *Mucor indicus, Rhizopus oryzae, Candida tropicalis*, and *Saccharomyces cerevisiae* was used as the starter culture. The starter culture was prepared as follows: 20 mL suspensions were made from 7-day cultures of *M. indicus* and *R. oryzae*. This was mixed with 10 mL suspensions made from 5-day cultures of *C. tropicalis* and *S. cerevisiae*. The suspension was transferred to a spray bottle and sprayed evenly on the cooled rice.

The container was covered with clean cheesecloth and stored at 20°C for three days to allow aerobic fermentation. After which, it was transferred into two clean 60-L glass jars in equal amounts, and the jars were sealed tightly. The jars were stored at 20°C for anaerobic fermentation for two months. Samples were then processed within 4 hours of the end of fermentation.

DNA extraction and sequencing

5 mL of wine and 5 mL of lees samples from each municipality were combined in a sterile 50 mL tube and vortexed continuously at high speed for 10 seconds. 100 µL of the homogenized sample was then used for serial dilution using saline solution. 10 µL of the dilution was spread plated onto de Man-Rogosa-Sharpe (MRS) agar and nutrient agar (NA). The plates were incubated under microaerophilic and aerobic conditions, respectively, for 24-48 hours at 37°C. All colonies were then harvested and used for DNA extraction. Bacterial DNA was isolated using DNeasy® PowerFood® Microbial Kit following manufacturer's instructions and sent to Macrogen, South Korea, for 16S rDNA sequencing using Illumina platform and subsequent metagenomic analysis. The primers used are as follows: 5' TCGTCGG-CAGCG TCAGATGTGTATAAGAGACAGCCTACGGGNG GCWGCAG for the forward primer and 5' GTCTCGT GGGCTCGGAGATGTGTATAAGAGACAGGACT ACHVG-GGTATCTAATCC for the reverse primer. The primers targeted the V3 and V4 regions of the 16S rRNA gene and yielded amplicons 460 bp in size.

Data analysis

Macrogen, South Korea, performed all data analyses. Assembly of reads was performed using Fast Length Adjustment of Short Reads (FLASH). Data pre-processing and clustering were performed using CD-HIT-OUT and rDnaTools programs. Taxonomic assignment, rarefaction analysis, and diversity analysis were performed using Quantitative Insights Into Microbial Ecology (QIIME). Phylogenetic trees were generated using the same program using unweighted pair groups with arithmetic mean (UPGMA) clustering algorithms.

RESULTS

Quality assessment of 16S rRNA gene sequences

Four hundred ninety-two thousand four hundred seventeen (492,417) high-quality sequences were obtained from the four fermented and four laboratories fermented tapuy samples. A total of 714,004 reads from the four samples were analyzed. Rarefaction analysis revealed that the number of sequences read was sufficient to capture all possible operational taxonomic units (OTUs) in the samples (Figure 2).

Table 1 shows the proportion of sequences identified up to the species level, above species level only, and unidentified even at the phylum level. Only the Itogon field sample contained unknown sequences at all levels (2.74% of sequences). For all other samples, all sequences were identified up to the species level. Further analysis was performed at the species level.

Bacterial community structure of traditionally fermented tapuy samples

A total of 19 different species were identified across all traditionally fermented tapuy samples belonging to Phyla Firmicutes and Proteobacteria (Table 2, Figure 3). Dominant bacteria are defined as those with relative abundance greater than 1.00%.[10]

In the Itogon sample, dominant species were identified to be *Acetobacter ascendens* and *Lactobacillus rhamnosus*. 2.74% of reads were unidentified. The Kapangan sample was dominated by *Bacillus subtilis*, *L. rhamnosus*, *Bacillus wiedmannii*, and *Lactobacillus fermentum*. Other *Lactobacillus* species were also detected in trace amounts, and these were: *Lactobacillus nasuensis, Lactobacillus paracasei,* and *Lactobacillus porcinae*. The La Trinidad sample was dominated by *B. wiedmannii*, followed by *B. subtilis*. Several *Lactobacillus* species were also detected, such as *Lactobacillus fermentum*, *Lactobacillus hilgardii*, and *Lactobacillus rhamnosus*. Dominating species in the Sablan sample were *B. subtilis* and *L. rhamnosus* in roughly equal abundance. *Lactobacillus paracasei* was also detected in small quantities.

Four species were found in all field-fermented samples: *B. subtilis, B. wiedmannii, L. paracasei,* and *L. rhamnosus* (Figure 4A). Samples from Kapangan, La Trinidad, and Sablan were composed entirely of species under Phylum Firmicutes, while the Itogon sample was composed of Firmicutes (18.52%) and Proteobacteria (78.74%).

Bacterial community structure of laboratory fermented tapuy samples

A total of 25 different species were identified in laboratory fermented tapuy samples falling under Phyla Firmicutes, Proteobacteria, and Bacteroidetes (Table 3, Figure 5).

The Itogon lab sample was dominated by *L. brevis*, *B. subtilis*, and *Carnobacterium alterfunditum*, the last unique to this sample. The presence of Gram-negative *Bacteroides xylanolyticus* and *A. ascendens* were also noted. Dominant species in the Kapangan sample were identified as *B. subtilis*, *L. paracasei*, and *B. carboniphilus*. Interestingly, *Enterococcus faecalis* was detected in the sample. La Trinidad lab sample was dominated by *B. subtilis*, *L. dextrinicus*, and *C. neuense*. *B. xylanolyticus* was also detected as in the Itogon sample. Sablan's lab sample was dominated by *B. wiedmannii* and *L. curieae*.

Four species are common to all fermented laboratory samples: *B. carboniphilus, B. subtilis, B. wiedmannii*, and *L. curieae* (Figure 4B). Kapangan and Sablan samples were composed entirely of Firmicutes. In contrast, the La Trinidad sample was composed of Firmicutes (98.69%) and Bacteroidetes (1.31%), and the Itogon sample was

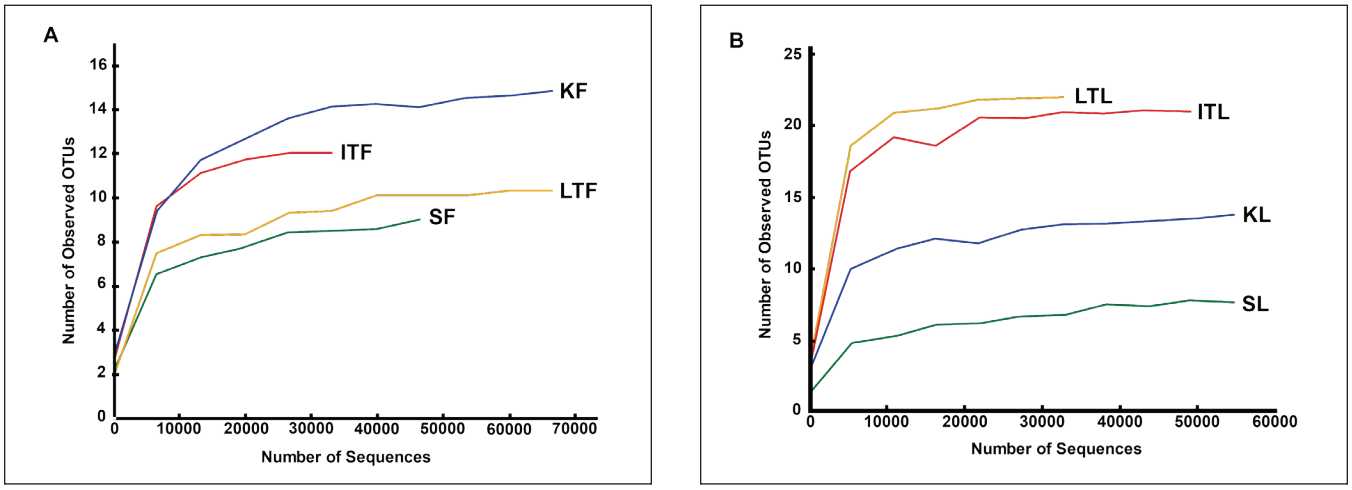

Figure 2. Rarefaction curve for **(A)** traditionally fermented and **(B)** laboratory fermented Tapuy samples.

ITF = Itogon field; KF = Kapangan field; LTF = La Trinidad field; SF = Sablan field; ITL = Itogon lab; KL = Kapangan lab; LTL = La Trinidad lab; SL = Sablan lab.

Table 1. The proportion of sequences in each sample identified up to the species level, above species level only, and unidentified even at the phylum level

Level of identification	Field samples				Lab samples			
	Itogon	Kapangan	La Trinidad	Sablan	Itogon	Kapangan	La Trinidad	Sablan
At the species level	97.26	100	100	100	100	100	100	100
Above species level	0	0	0	0	0	0	0	0
Unidentified at any taxonomic level	2.74	0	0	0	0	0	0	0

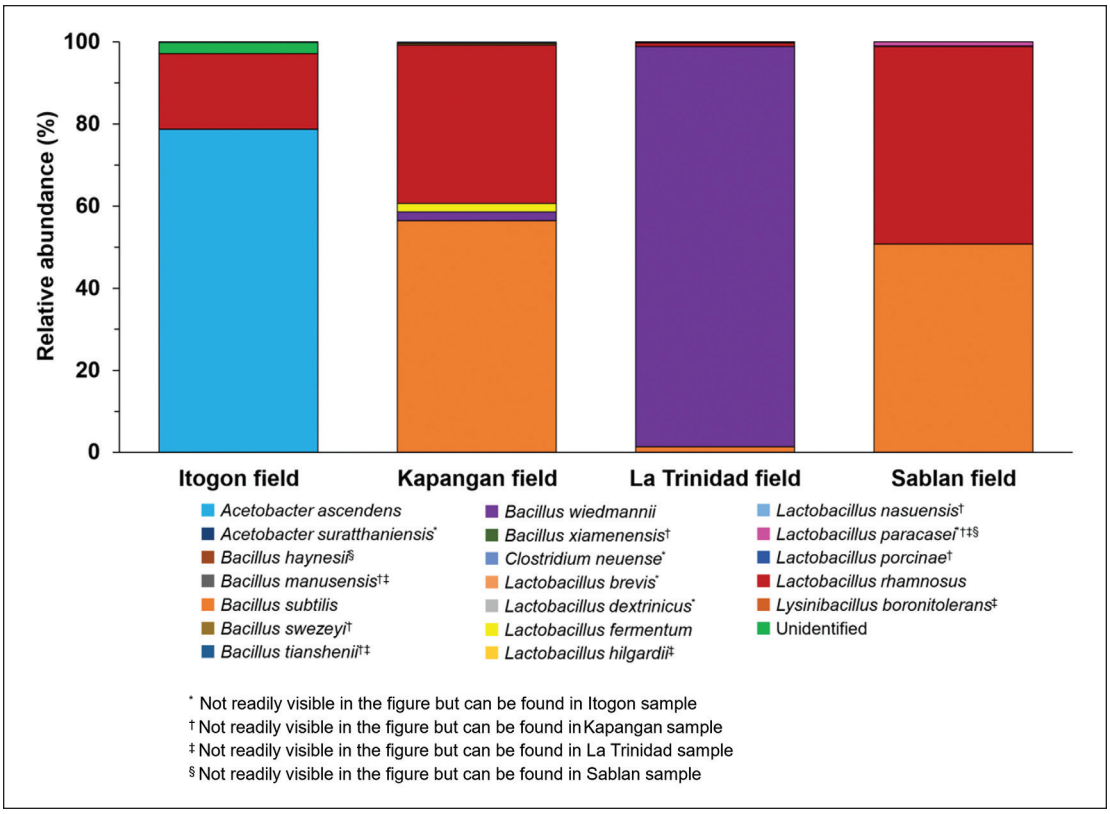

Figure 3. Bacterial community composition of traditionally fermented tapuy samples from different municipalities in Benguet presented as the relative abundance (%) of each bacterial species.

Table 2. Bacterial community composition of laboratory fermented tapuy samples following the starting materials used by different municipalities in Benguet

Species	Relative abundance (%)			
	Itogon	Kapangan	La Trinidad	Sablan
Acetobacter ascendens	78.71%	–	–	–
Acetobacter suratthaniensis	0.03%	–	–	–
Bacillus haynesii	–	–	–	0.02%
Bacillus manusensis	–	0.01%	0.03%	–
Bacillus subtilis	0.02%	56.50%	1.36%	50.80%
Bacillus swezeyi	–	0.43%	–	0.07%
Bacillus tianshenii	–	0.01%	0.13%	–
Bacillus wiedmannii	0.01%	2.13%	97.58%	0.00415%
Bacillus xiamenensis	–	0.01%	–	0.01%
Clostridium neuense	0.04%	–	–	–
Lactobacillus brevis	0.03%	–	–	–
Lactobacillus dextrinicus	0.01%	–	–	–
Lactobacillus fermentum	–	2.04%	0.00189%	–
Lactobacillus hilgardii	–	–	0.03%	–
Lactobacillus nasuensis	–	0.00352%	–	–
Lactobacillus paracasei	0.01%	0.24%	0.00378%	0.96%
Lactobacillus porcinae	–	0.00352%	–	–
Lactobacillus rhamnosus	18.41%	38.63%	0.85%	48.13%
Lysinibacillus boronitolerans	–	–	0.02%	–
Unidentified	2.74%	–	–	–

The relative abundance of each bacterial species is represented as a percentage of reads. A dash '-' indicates none detected

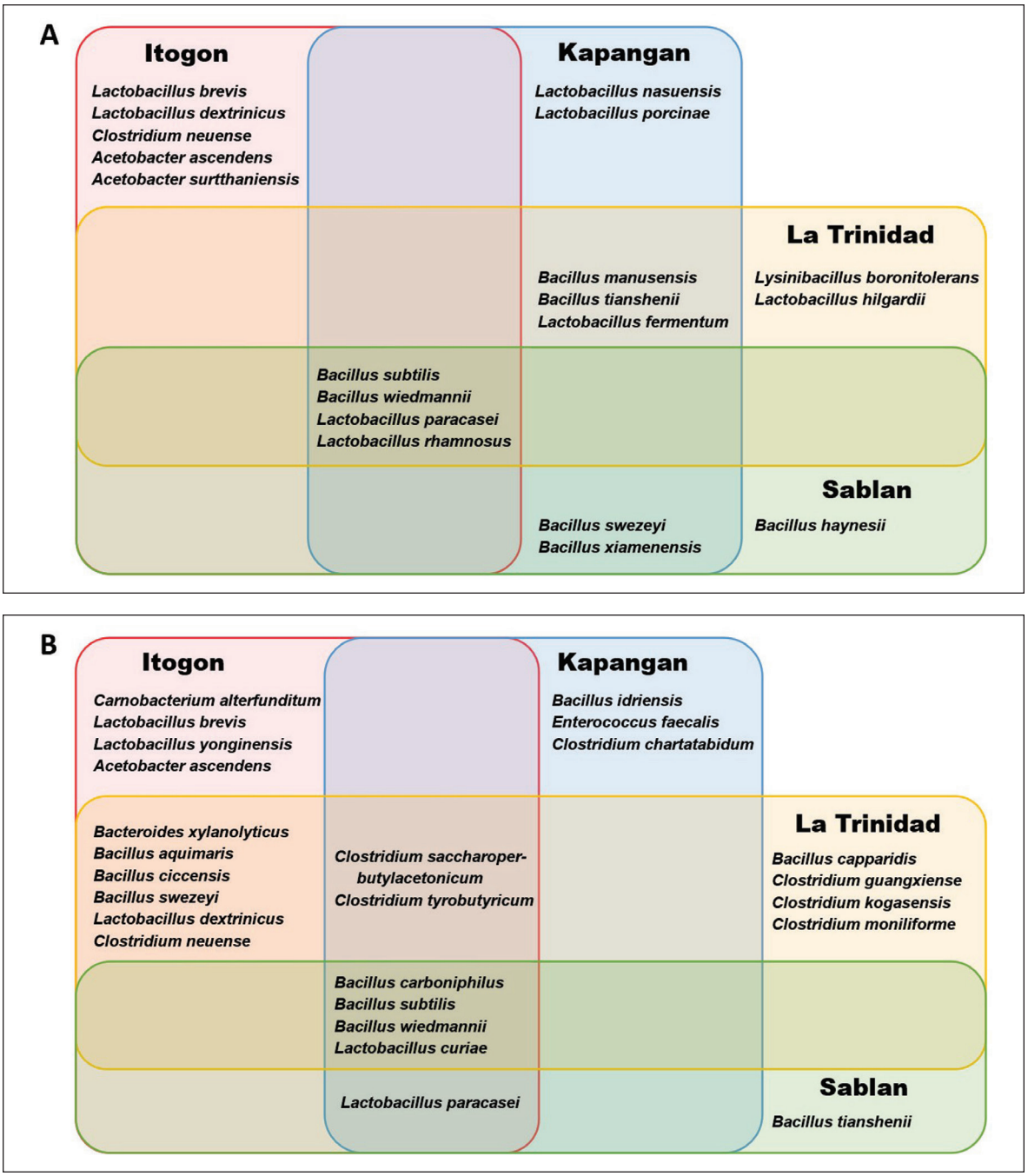

Figure 4. A four-way Venn diagram shows the bacterial species' overlapping among **(A)** traditionally fermented and **(B)** laboratory fermented Tapuy samples.

composed of Firmicutes (99.47%), Proteobacteria (0.42%), and Bacteroidetes (0.11%).

Bacterial diversity and clustering of samples

Generally, lab samples were characterized by higher species richness (Table 3), with the highest difference being eight species between the Itogon lab and field samples. Although the Kapangan and Sablan field samples exhibited higher species richness than their lab counterparts, only one species differed for both areas. Lab samples were also characterized by increased bacterial diversity (Table 4). An increased presence of members of the genera *Clostridium* was observed in lab samples. The smallest difference in abundance was 0.59% between Itogon samples, and the largest difference was 13.52% between La Trinidad samples. Laboratory fermented samples of Itogon and La Trinidad exhibited a higher proportion of *Lactobacillus* (with a difference of 49.98% and 17.65%, respectively) and *B. subtilis* (difference

Table 3. Bacterial community composition of laboratory fermented tapuy samples following the starting materials used by different municipalities in Benguet

Species	Relative abundance (%)			
	Itogon	Kapangan	La Trinidad	Sablan
Acetobacter ascendens	0.42%	–	–	–
Bacillus aquimaris	0.01%	–	0.26%	–
Bacillus capparidis	–	–	0.15%	–
Bacillus carboniphilus	0.37%	4.59%	2.61%	0.00148%
Bacillus ciccensis	0.10%	–	1.05%	–
Bacillus idriensis	–	0.02%	–	–
Bacillus subtilis	25.39%	58.25%	62.07%	0.07%
Bacillus swezeyi	0.02%	–	0.03%	–
Bacillus tianshenii	–	–	–	0.33%
Bacillus wiedmannii	0.01%	0.44%	0.48%	98.40%
Bacteroides xylanolyticus	0.11%	–	1.31%	–
Carnobacterium alterfunditum	4.51%	–	–	–
Clostridium chartatabidum	–	0.01%	–	–
Clostridium guangxiense	–	–	0.01%	–
Clostridium kogasensis	–	–	0.03%	–
Clostridium moniliforme	–	–	0.03%	–
Clostridium neuense	0.54%	–	12.58%	–
Clostridium saccharoperbutylacetonicum	0.05%	1.93%	0.32%	–
Clostridium tyrobutyricum	0.03%	0.00169%	0.56%	–
Enterococcus faecalis	–	0.65%	–	–
Lactobacillus brevis	68.14%	–	–	–
Lactobacillus curieae	0.06%	0.00169%	3.20%	1.19%
Lactobacillus dextrinicus	0.20%	–	15.33%	–
Lactobacillus paracasei	0.03%	34.11%	–	0.00296%
Lactobacillus yonginensis	0.01%	–	–	–

The relative abundance of each bacterial species is represented as a percentage of reads. A dash '-' indicates none detected

Table 4. Shannon diversity index of the field and laboratory fermented tapuy samples

Sample	Field	Lab
Itogon	0.912	1.258
Kapangan	1.294	1.406
La Trinidad	0.200	1.862
Sablan	1.086	0.136

of 25.37% and 60.71%, respectively), compared to their field counterparts. On the other hand, lab samples of Kapangan and Sablan exhibited a lower abundance of *Lactobacillus* (difference of 6.8% and 47.89%, respectively) compared to their field counterparts. Sablan lab sample also showed a higher quantity of *B. wiedmannii* than in the field sample, with the difference being 98.39%.

Phylogenetic analysis of the field samples revealed that Kapangan and Sablan were the most closely related bacterial community structure, followed by La Trinidad. Itogon was found to be the most distant (Figure 6A). This clustering appeared to follow the geographic locations of the sample sites (Figure 1). However, a different pattern was obtained when analyzing the lab samples (Figure 6B). La Trinidad and Kapangan were the most closely related, followed by Itogon and then Sablan as the most distant. The overall distances between the lab samples were also found to be smaller than those in the field fermented samples suggesting that the laboratory samples are more closely related to each other and have less variation.

DISCUSSION

Previous studies on the bacterial content of Tapuy reported the presence of *Pediococcus pentosaceus*[11,12], *Lactobacillus viridescens*[11,12] (now *Weissella viridescens*[13]), *Lactobacillus brevis*[11,12], *Lactobacillus plantarum*[12,14], *Lactobacillus lactis* subsp. *cremoris*[14], and *Leuconostoc* sp.[1]. Of these, only *L. brevis* was detected in the present study and trace amounts. Moreover, many of the bacteria reported here have not been seen in previous studies. This is due to the difference in culture media and method of identification used. Previous studies utilized glucose yeast peptone (GYP) agar for isolation and phenotypic identification.

Tapuy field samples were found to contain bacterial species that may be beneficial (*Lactobacillus* spp. and *B. subtilis*), species that may be harmful (*B. wiedmannii*), and species whose effects on health are still unclear (*Acetobacter* spp. and *Clostridium neuense*). It is also noted that 2.74% of the sequenced DNA in the Itogon field sample was unidentified. These may be derived from bacteria that have not yet been sampled and sequenced and may be novel species.

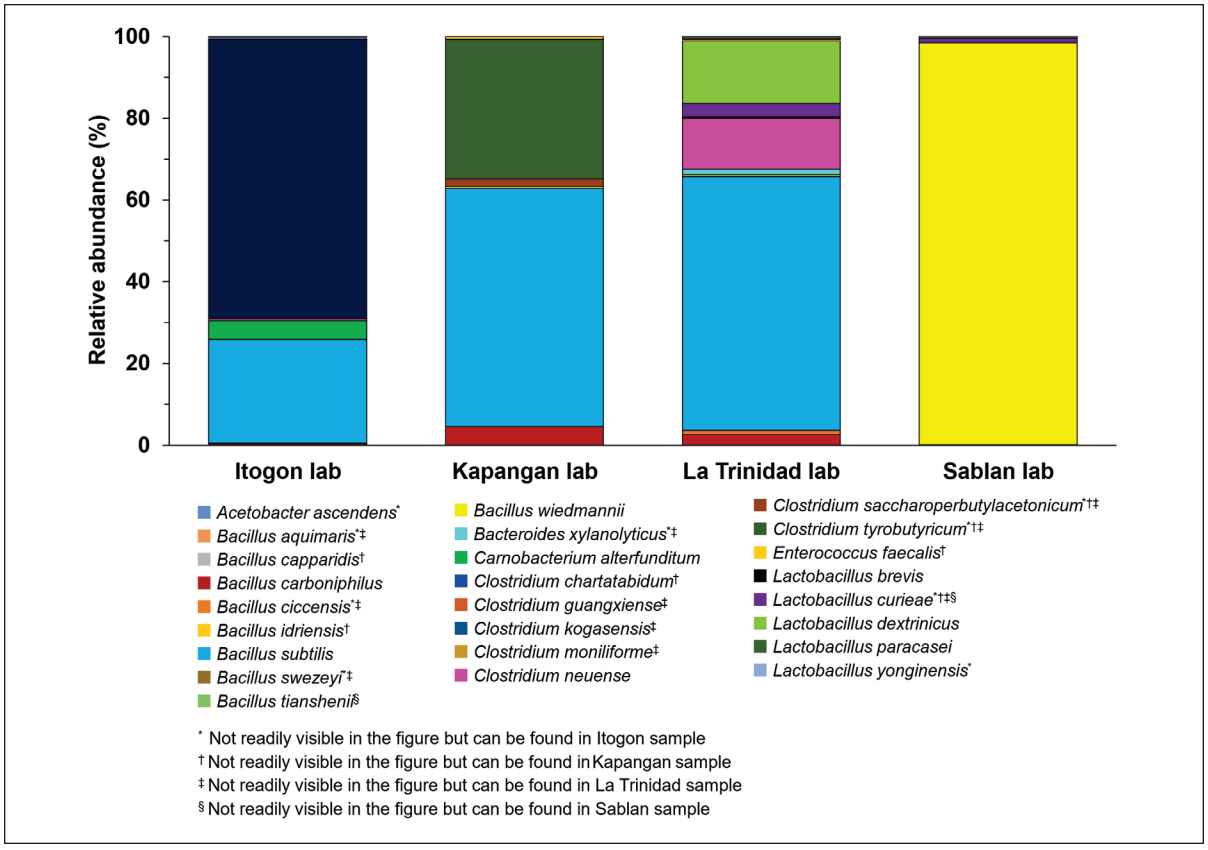

Figure 5. Bacterial community composition of laboratory fermented tapuy samples following the starting materials used by different municipalities in Benguet presented as each bacterial species' relative abundance (%).

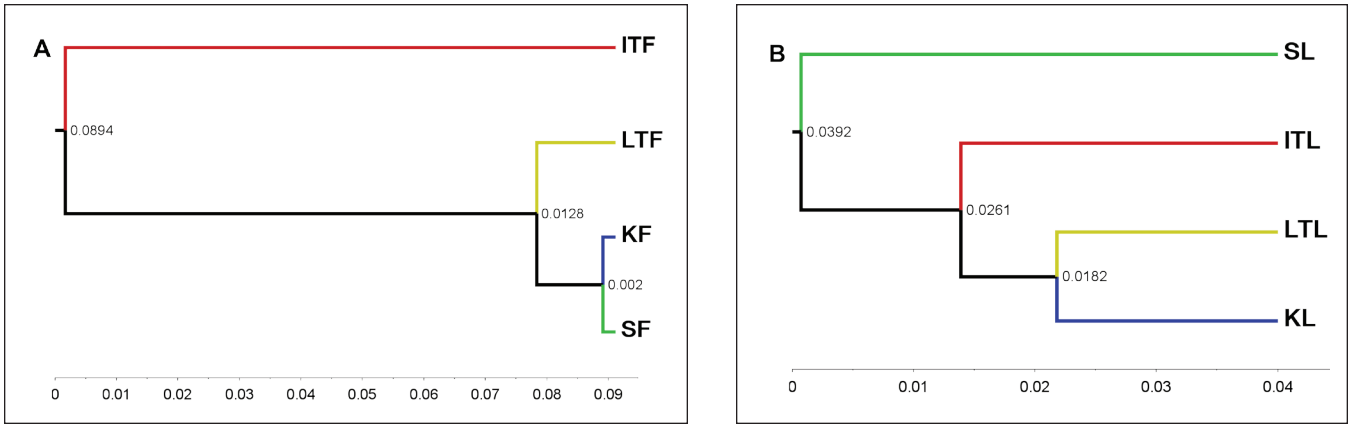

Figure 6. Phylogenetic tree showing the relationship of the bacterial community structures of **(A)** traditionally fermented and **(B)** laboratory fermented Tapuy samples generated using UPGMA hierarchical clustering algorithm. Values on the graph denote the distance between groups.

IT1 = Itogon field; KF = Kapangan field; LTF = La Trinidad field; SF = Sablan field; ITL = Itogon lab; KL = Kapangan lab; LTL = La Trinidad lab; SL = Sablan lab.

The genus *Lactobacillus* is a widely regarded taxon of probiotic bacteria. Lactobacilli are generally recognized as safe (GRAS) and are part of the normal microflora. They are also commonly found in dairy products and fermented food. Many species have been found to exhibit probiotic activity, which has been reviewed extensively in previous publications.[15] Several species of *Lactobacillus* have also been isolated from a variety of Philippine fermented food.[2,16]

Members of the genus *Bacillus* also formed a considerable proportion of the samples. The genus *Bacillus* is home to numerous medically and economically important species, both pathogenic and non-pathogenic. Efforts to characterize the phylogenetic relationships in this genus often divide the species into several groups or clades. There does not appear to be a consensus in the naming of these groups. However, they typically include a *B. subtilis* group/clade and *B. cereus* group/clade, among others. The former often contains non-pathogenic bacteria that have also been explored for probiotic potential (e.g., *Bacillus indicus, Bacillus coagulans*), while the latter usually includes other pathogenic species (e.g., *B. anthracis*).[5,17]

B. subtilis, which was found in all samples, have been well-studied for probiotic activity.[18-22] *B. subtilis* is a Gram-positive bacterium ubiquitous in the environment. It can be isolated from soil, water, and plant matter and can also be found in the normal gut microflora of animals. It has also been found in fermented soybean foods and patis, a Philippine fermented fish sauce.[16,23]

On the other hand, *B. wiedmannii*, also quite prevalent in the samples, is closely related to the *B. cereus* group, posing a threat to the food safety of the product.[24] It is a recently described species and was first isolated from raw milk. It was found to produce hemolysin BL and non-hemolytic enterotoxins.[24] In a separate study, it was isolated from shrimp in a hydrothermal environment and was reported to exhibit hemolytic and cytotoxic activity and putative virulence genes in its genome.[25]

A. ascendens, which was found to be the dominant organism in the Itogon field sample, is commonly found in fermented liquids and beverages such as vinegar and kombucha.[26] While this species is relatively understudied, other *Acetobacter* species have been explored for probiotic properties.[27]

Clostridium neuense, which was prevalent in the La Trinidad sample, was initially isolated from lake sediment.[28] Phylogenetic analysis revealed that it belongs to the Cluster I group of clostridia, which contains pathogenic (e.g., *Clostridium tetani, Clostridium perfringes, Clostridium botulinum*) and non-pathogenic members. Non-pathogenic species are usually involved in the degradation of polymeric carbohydrates and the production of solvents, organic acids, and hydrogen.[29] *C. neuense* is most closely related to *Clostridium acetobutylicum* which is widely used in the production of acetone and butanol.[28] Its presence in fermented food and effects on health have not been previously documented.

E. faecalis is also a gut commensal and abundant in nature in soil and vegetation.[30] Its presence in the Kapangan lab sample is alarming as it is commonly considered a sign of fecal contamination and is an opportunistic pathogen, particularly in nosocomial infections.[30,31] *E. faecalis* can also act as a reservoir for virulence and antibiotic resistance genes, spreading to other bacteria through horizontal gene transfer.[31] However, the genus *Enterococcus,* in general, is being used in the food industry more frequently and is thus becoming more tolerated as part of the normal microbiota.[31]

Two bacterial species were common in all field and lab samples: *B. subtilis* and *B. wiedmannii*. This may be due to the Ballatinao black rice, which was the starting material that was common to all areas. They may have been present in the rice, and because they are both spore-formers, they were not killed during the cooking process.

In the present study, lab samples of tapuy were found to have higher bacterial diversity and richness than field samples. This may be due to the absence of inhibitory fungi in the lab samples and the controlled fermentation temperature used in the laboratory fermentation. Open-air fermentation and less hygienic handling of tapuy in the field may contribute to fungal species that produce antibacterial compounds, thereby limiting the growth of certain bacterial species. Sterilization and sanitization of materials used in laboratory fermentation would remove these fungi and allow the growth of bacterial species that would otherwise be restricted in the field, resulting in higher bacterial diversity and richness in the lab samples. However, further studies exploring the fungal composition of field and lab tapuy samples would be necessary to confirm this.

The higher bacterial diversity and richness in the lab samples compared to the field samples may also be due to differences in the fermentation temperature. Field fermentation is done under ambient temperatures and is therefore subjected to day-to-day fluctuations in fermentation temperature. These fluctuations cause stress to the bacteria, and only those capable of adapting to the temperature changes can thrive in the field samples. Previous studies exploring the effect of temperature on fermentation have reported that higher temperatures yielded food products that exhibited lower bacterial diversity than food products that were fermented under lower temperatures.[32,33] The reason for this is that increased temperature results in increased activity and growth of yeast and lactic acid bacteria, decreasing the sample's pH.[34] The acidic environment inhibits the growth of other bacterial species resulting in a lower bacterial diversity.

Meanwhile, laboratory fermentation made use of a constant fermentation temperature. Therefore, there is less stress on the bacteria. Those inhibited in the field samples were allowed to proliferate in the lab samples, resulting in higher bacterial diversity and richness in the lab samples.

The bacterial composition of fermented foods is highly reliant on the starting materials used in fermentation. The starting materials provide the substrates such as carbohydrates and amino acids which the bacteria may use

for growth. Thus, those bacteria that can utilize the available substrates will be selected for, and those that cannot use the substrates are selected against. Because the different municipalities use different starting materials (such as white corn in Sablan and guava leaves in Itogon), other substrates are available in the samples resulting in varying bacterial compositions. Additionally, the starting materials may contribute inorganic compounds that differentially affect the growth of bacteria. For example, guava leaves may contain antimicrobial compounds that limit the growth of certain bacterial species.[35] Different rice varieties also contribute varying amounts of anthocyanins and phenolic compounds, which also have antimicrobial properties.[36,37]

This was observed in the study as the clustering of the lab samples reflected the differences in the starting materials used (Figure 6B). La Trinidad and Kapangan samples used similar starting materials, which differed only in the ratio of Ballatinao black rice to Bongkitan white rice and were the most closely related in bacterial community structure. Itogon samples also used Ballatinao black rice and Bongkitan white rice with guava leaves and were found to be the next most closely related. Sablan samples used Ballatinao black rice and coarsely ground white corn instead of Bongkitan white rice and were the most distant.

In contrast, the clustering of the field samples suggests that other environmental factors greatly influence the bacterial community structure of tapuy (Figure 6A). Kapangan and Sablan field samples, although differing in starting materials, were found to be most closely related in bacterial composition. Environmental variations may have arisen from the open-air fermentation and the handling by tapuy-makers as these introduce contaminants from the environment, which affect the bacterial composition. Although the specific details and differences of each tapuy-making practice cannot be disclosed, what can be said of the procedure is that all field samples underwent cold fermentation in Benguet and all used commercially available bubod from the public market. These findings are consistent with Zhao *et al.,* wherein the same fermented product from different areas shows considerable variation in bacterial structure.[10]

The study of the functional properties of fermented foods and beverages to develop these food items into functional foods is an emerging field of research in the country. The existing studies in this field focus on fermented food items that contain known probiotic strains.[2] In the present study, tapuy from the different municipalities had a significant proportion of potential probiotic bacterial species such as *L. rhamnosus* and *B. subtilis*. This makes tapuy a good candidate for development into functional food. In addition to this, tapuy is usually consumed fresh, not long after fermentation is finished, which means that viable potential probiotic bacteria are consumed. Other properties of tapuy also lend to its potential as a functional food. Recently, it was reported that tapuy made from Ballatinao black rice, such as in the study, exhibited high antioxidant activity and total phenolic content.[36] Prebiotics such as sugars and dietary fibers, commonly found in grains, may also be present in tapuy.[38]

As previously mentioned, tapuy field samples were also found to contain potentially pathogenic bacteria such as *B. wiedmannii*. This suggests a need to standardize the fermentation procedure to decrease the abundance of pathogens and minimize their health risk. In the study, fermentation was standardized using a defined starter culture and controlled fermentation environment to reduce contaminants. For Itogon and La Trinidad, standardization resulted in an increased abundance of beneficial bacteria such as *Lactobacillus* and *B. subtilis* and a decreased abundance of harmful bacteria such as *B. wiedmannii*. This suggests that the starting materials used in these two areas (Ballatinao black rice, Bongkitan white rice, and guava leaves) produce tapuy of good quality and are a consistent source of potential probiotic bacteria. Thus, they may be used in future endeavors to standardize the tapuy-making process.

CONCLUSION

This was the first study to describe the bacterial content and community structure of tapuy from four municipalities in the Benguet province using a high-throughput method and to compare this with the bacterial content and community structure of laboratory fermented tapuy samples which used a standardized fermentation procedure.

Traditional tapuy samples were composed primarily of members of the genera *Bacillus* and *Lactobacillus* in varying proportions. Potential probiotic bacteria were abundant in Kapangan (97.42%) and Sablan (99.89%) field samples. Two species, *B. subtilis* and *B. wiedmannii,* were present in all samples. These may have been sourced from the Ballatinao black rice used by all municipalities as a starting material. Laboratory fermented samples differed in community structure as a result of the different starting materials used. Meanwhile, in the field samples, the difference in starting materials could not fully account for the variations seen in community structure which suggests that environmental factors in each locality are a contributory factor.

The study also identified potential probiotic bacterial species in tapuy, many of which were previously unreported. Thus, tapuy may be a good source of potential probiotic bacteria that may be explored for health applications. Furthermore, due to the high content of potential probiotic bacteria, tapuy may be a good candidate for development into a functional food product. However, the presence of potentially pathogenic bacteria such as *B. wiedmannii* suggests the need for standardization of the tapuy-making procedure. The initial attempt to standardize tapuy fermentation performed in the study revealed that the laboratory fermented Itogon and La Trinidad samples yielded higher probiotic bacteria abundantly than their field counterparts. Thus, the starting materials used by these two practices may be beneficial in subsequent standardization efforts.

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Disclaimer

The views expressed in this article are those of the authors alone and are not the official position of the institution or funding agency.

Statement of Authorship

Both authors contributed in the study implementation, data analysis and interpretation, writing and editing of the manuscript and approved the final version to be published.

Author Disclosure

Both authors declared no conflicts of interest.

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