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Culture-independent molecular analysis of the microbial communities in *burukutu* and *pito*, two Nigerian traditionally fermented alcoholic cereal beverages

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ABSTRACT

Burukutu and *pito* are two widely consumed, alcoholic fermented beverages, made from malted cereals, and popular in various regions of West Africa, including Nigeria, Ghana, Burkina Faso and The Republic of Benin. Traditionally produced at the cottage industries, they are primarily crafted from guinea corn, and the use of uncontrolled fermentation process yield a wide varieties of microorganisms present in the beverages. Therefore, to improve their quality and ensure safety, it is important to examine the microbial communities associated with these traditional beverages. In this study, *burukutu* and *pito* were investigated for their microbial community structure and composition, using culture-independent molecular techniques. Four samples of *burukutu* and *pito* were aseptically obtained from two production sites in Lagos, Nigeria. The V4 hypervariable region of the 16S rRNA gene, as well as the internal transcribed spacers 1 and 2 (i.e. ITS1 and ITS2) regions were targeted, to obtain detailed microbial community profiles. High-throughput illumina MiSeq sequencing platform revealed that the phyla Firmicutes and Ascomycota were dominant in both beverages, with an average relative abundance of 83 % and 73 %, respectively. *Lactobacillus*, *Saccharomyces* and *Candida* were the predominant genera identified, accounting for 78 %, 63 % and 12 %, respectively. Additionally, unreported and uncommon bacterial and fungal genera such as *Acinetobacter*, *Caulobacter*, *Sphingomonas*, *Comamonas*, *Diutina*, *Malassezia* and *Vishniacozyma* were detected in both beverages. Some potential food-borne bacterial pathogens like *Pseudomonas*, *Staphylococcus*, *Shigella*, *Escherichia* and *Bacillus*, were furthermore found present, but at a lower relative abundance. Consequently, *burukutu* and *pito* cannot be absolutely considered safe for human consumption, hence, improvements in their production methods are recommended, including staff training in-process monitoring, good hygienic and sanitary practices, as well as the development of a controlled fermentation system using defined starter cultures, to enhance the safety, quality, and overall acceptability of these traditional alcoholic beverages.

Keywords: Cereal fermentation, next generation sequencing, microbial community and structure, food safety, starter cultures

1.0 Introduction

Burukutu and *pito* are the two most popular, Nigerian indigenous fermented alcoholic cereal beverages, produced mainly from red and white varieties of guinea corn (*Sorghum bicolor* and *Sorghum vulgare*), including pearl millet and maize (*Pennisetum glaucum*/*Pennisetum maliaceum* and *Zea mays*) (Faparusi, 1970; Konfo *et al*., 2015). They are traditionally produced, via small-scale batch processes, typically 2 - 3 times a week (Gazuwa *et al*., 2021). Production and marketing are predominantly carried out by the women, who derive substantial income from these activities (Egwurochi *et al*., 2021). The processing stages involve malting, mashing,

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fermentation and maturation (Kolawole *et al*., 2007). *Burukutu* appears as a brown-coloured suspension, while *pito* is a cream-coloured supernatant liquor (Blandino *et al*., 2003). Both beverages possess a vinegar-like flavour, with a low pH (3.3 - 4.0), and are often consumed while fermentation is still ongoing (Odunfa and Oyewole, 1998). *Burukutu and pito* are rich in calories, B-group vitamins (i.e. thiamine, folic acid and riboflavin), including essential amino acids such as lysine, leucine and methionine (Lyumugabe *et al*., 2012).

These two alcoholic beverages are spontaneously fermented, which results in a wide variety of microorganisms, including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus* sp., *Streptococcus* sp., *Lactobacillus* sp., *Saccharomyces cerevisiae*, *Aspergillus* sp. and *Rhizopus* sp. (Anaukwu *et al*., 2015; Olaniyi *et al*., 2018; Atter *et al*., 2026). Previous studies that examined the microorganisms in *burukutu* and *pito*, primarily relied on phenotypic and biochemical characteristics for microbial identification (Raji and Igbokwe, 2005; Lynn *et al*., 2014; Minamor *et al*., 2017; Yusuf *et al*., 2020). Phenotypic features such as Gram staining, colony morphology, catalase, oxidase and sugar fermentation tests have been utilized for identifying the microbiota of these traditional beverages in Nigeria (Anaukwu *et al*., 2015). However, these conventional culture-dependent techniques are prone to various limitations, including biases, low taxonomic resolution, misidentification of species, and underestimation of species richness and diversity (Cocolin and Ercolini, 2015).

Culture-independent molecular methods and high-throughput sequencing platforms have recently been utilized, to study the microbial ecology of foods, as they offer higher sensitivity, and a more comprehensive identification of microbial species, compared to the conventional microbiological techniques (Nwaiwu *et al*., 2020). Therefore, this study aims to assess the microbial community structure and composition of *burukutu* and *pito*, obtained from two production sites in Lagos, Nigeria, using culture-independent molecular analysis and illumina sequencing platform.

2.0 Materials and Methods

2.1 Samples collection

A total of four samples of *burukutu* and *pito* were obtained from two production sites in Lagos, Nigeria. One litre each of *burukutu* and *pito* was aseptically collected from the following locations:

- Site 1: Maami market, TA Lagbaja cantonment (formerly Ikeja cantonment), Ikeja, Lagos State, Nigeria (Location coordinates: 3° 21' 8.08", 6° 33' 53.77" E). Samples were labelled as BL1 (*burukutu*) and PL1 (*pito*).
- Site 2: Maami market, Bonny camp, Victoria Island, Lagos State, Nigeria (Location coordinates: 3° 24' 24.03", 6° 26' 12.04"E). Samples from this site were labelled as BL2 (*burukutu*) and PL2 (*pito*).

All the alcoholic beverages were obtained in sterile bottles, labelled appropriately, and transported on ice to the Laboratory of the Department of Microbiology, University of Lagos, Nigeria. The samples were stored at -20 °C in a Westpoint chest freezer (WBP-5219.ERLS, Portugal), for one week, before DNA extraction and subsequent illumina MiSeq sequencing at GENEWIZ, Inc. (Suzhou, China). Sample collection for this study was limited to a small sample size and absence of replicates.

2.2 Culture-independent molecular analysis

2.2.1 DNA extraction, polymerase chain reaction (PCR) and sequencing

Total genomic DNA was extracted from all the four alcoholic beverages, using the FastDNA Spin Kit (MP Biomedicals, UK), according to the manufacturer's instructions. The DNA extracted was quantified, using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), and illumina MiSeq sequencing was performed at GENEWIZ, Inc. (Suzhou, China). For library preparation, 50 - 100 ng of DNA was used to generate amplicons, with a panel of primers designed by GENEWIZ (GENEWIZ Inc., South Plainfield, NJ, USA). The V4 hypervariable region of the bacterial 16S rRNA gene was amplified, using the 515F and 806R primers (Caporaso *et al*., 2010). For the fungal community analysis multiple

oligonucleotide primers targeting the relatively conserved sequences spanning the ITS regions were used. The ITS1 region was amplified using a forward primer (5′-ACCTGCGGARGGAT-3′) and reverse primer (5′-GAGATCCRTTGYTRAA-3′), while the ITS2 region was amplified using the forward primer (5′-GTGAATCATCGARTC-3′) and reverse primer (5′-TCCTCCGCTTATTGAT-3′) (Martin and Rygiewicz, 2005).

For the PCR set up, the reaction mixture included 100 ng template DNA, 2.5 µL of 10 µM each of the forward and reverse primers, 0.75 µL dimethyl sulfoxide, 25 µL of 2X NEBNext high fidelity master mixture, and nuclease-free water added to make 50 µL. For negative control master mixture, DNA template was not included. The PCR thermocycling consisted of, 3 min of initial denaturation at 98 °C, followed by 24 cycles of 98 °C for 30 s, 52 °C for 30 s, 72 °C for 45 s, and a final extension of 72 °C for 10 min.

2.2.2 Bioinformatic analysis of illumina MiSeq sequences

The Quantitative Insights into Microbial Ecology version 2 (QIIME2) software (Caporaso *et al*., 2010; Bolyen *et al*., 2019) was used to analyze the sequence data. The forward and reverse sequence reads were joined, and assigned to samples, based on barcode, and truncated by cutting off the barcode and primer sequences. Quality filtering on the joined sequences was performed, and those that did not fulfil the following criteria were discarded: sequence length <200 bp, no ambiguous bases, mean quality score >/= 20. The sequences were then compared with the reference database (RDP gold database), using UCHIME algorithm to detect chimeric sequences, which were later removed. The effective sequences were grouped into operational taxonomic units (OTUs), using the clustering program VSEARCH (1.9.6), against the Silva 119 database pre-clustered at 97 % sequence identity for 16S rRNA gene, and the UNITE ITS database (https://unite.ut.ee/) pre-clustered at 97 % sequence identity for ITS rRNA region. The Ribosomal Database Program (RDP) classifier was used to assign taxonomic category to all OTUs at confidence threshold of 0.8. Sequences were rarefied prior to calculation of alpha and beta diversities statistics. Alpha diversity indexes were calculated in QIIME, while beta diversity was calculated using weighted and unweighted UniFrac, and principal coordinate analysis (PCoA) performed.

3.0 Results

3.1 Bacterial composition and diversity

The 16S rRNA gene amplicon sequencing of the V4 hypervariable region in *burukutu* samples from both sites revealed a predominance of the phylum Firmicutes, accounting for over 80 % of the microbial community in each sample. In the sample from site 1 (BL1), Pseudomonadota (formerly Proteobacteria) constituted a substantial portion, comprising 14.88 % of the relative abundance, while Bacteroidetes represented 2.20 %. In contrast, the sample from site 2 (BL2) exhibited a higher dominance of Firmicutes, at approximately 96 %, with Pseudomonadota comprising 3.37 % of the relative abundance (Figure 1).

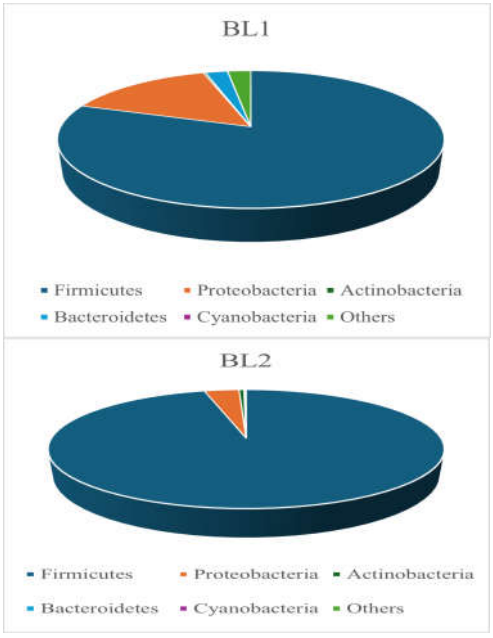

Figure 1: Bacterial taxonomic composition at phylum level of *burukutu* obtained from two production sites in Lagos, Nigeria.

A comparable phyla distribution was observed in the *pito* samples; however, the relative abundance of Firmicutes was slightly reduced, in comparison to the *burukutu* samples, where Pseudomonadota

were more prominent, accounting for 16 % in PL1 and 24 % in PL2, respectively (Figure 2).

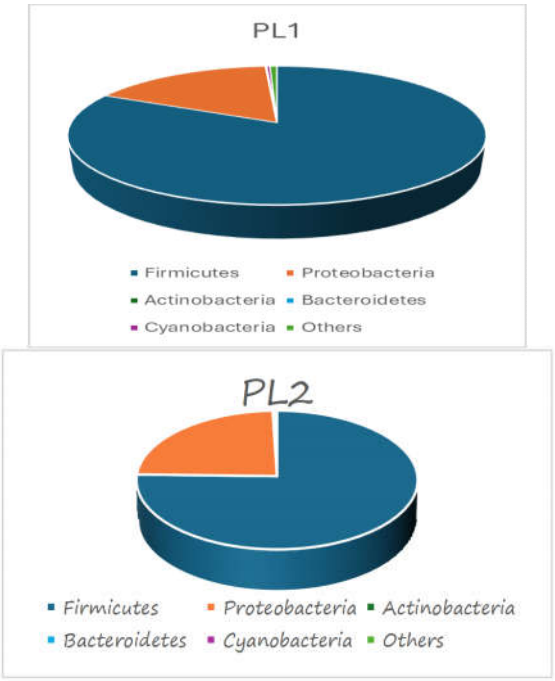

Figure 2: Bacterial taxonomic composition at phylum level of *pito* collected from two production sites in Lagos, Nigeria.

The genus *Lactobacillus* - now defined to include *Lactobacillus*, *Lactoplantibacillus* and *Limosilactobacillus* - was consistently detected in all the four *burukutu* and *pito* samples, representing over 70 % of the total bacterial community in each sample (Figure 3). Notably, both *pito* samples (PL1 and PL2) demonstrated the presence of *Acetobacter*, at relative abundances of 14.2 % and 6.6 %, respectively, whereas the both *burukutu* samples contained this genus at lower levels (<2 %). Additionally, Gram-negative bacterial genera such as *Pseudomonas*, *Acinetobacter*, *Caulobacter* and *Sphingomonas* were identified across the samples. Other lactic acid bacteria (LAB), including *Streptococcus*, *Leuconostoc*, *Weissella* and *Lactococcus*, were likewise detected (Figure 3).

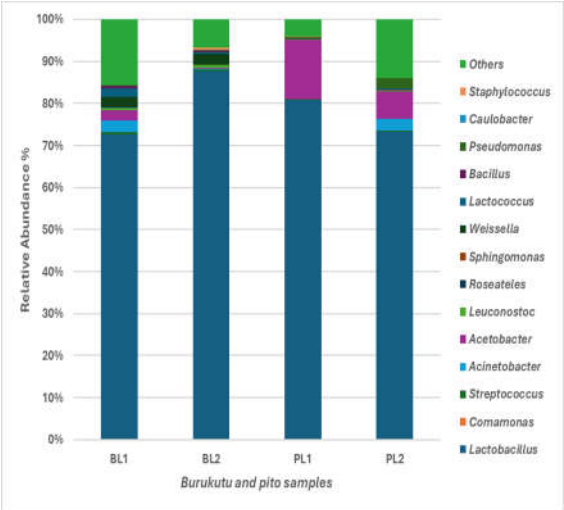

Figure 3: Bacterial taxonomic composition at genus level of *burukutu* and *pito* samples obtained from two production sites in Lagos, Nigeria.

Bacterial richness, evenness and diversity observed in *burukutu* samples were higher than those of *pito* samples. Phylogenetic diversity of BL1 and BL2 were 17.87 and 16.33, with Simpson indices of 4.8 and 3.4, and Shannon indices of 0.9 and 0.8, respectively (Table 1). PL1 showed the lowest richness and phylogenetic diversity, indicating a less complex and evolutionarily narrower microbial community. PL2 demonstrated moderate richness and relatively high evenness, suggesting a balanced bacterial community (Table 1).

Table 1: Alpha diversity of bacterial community in *burukutu* and *pito* samples collected from two production sites in Lagos, Nigeria.

Samples names	Observed species	Shannon index	Simpson index	Chao1	Phylogenetic diversity
BL1	277*	4.846	0.925	295.717	17.866
BL2	236	3.445	0.813	245.457	16.332
PL1	181	3.403	0.834	217.033	12.403
PL2	220	3.918	0.868	235.658	16.009

*Standard deviations are not reported, as samples collection did not include biological replicates.

3.2 Fungal composition and diversity

Amplicon sequence analysis of the ITS 1 and 2 regions of the fungal rRNA genes in *burukutu* samples from both sampling sites revealed the predominance of the phylum Ascomycota, comprising 65.39 % of the fungal community in BL1, and 77.12 % in BL2, respectively. In BL1, the phylum Basidiomycota was also conspicuously present, accounting for approximately 8 % relative abundance. In contrast, the *burukutu* sample from site 2 (BL2) exhibited lower relative abundances of Basidiomycota (1.85 %) and Mucoromycota (0.7 %) (Figure 4).

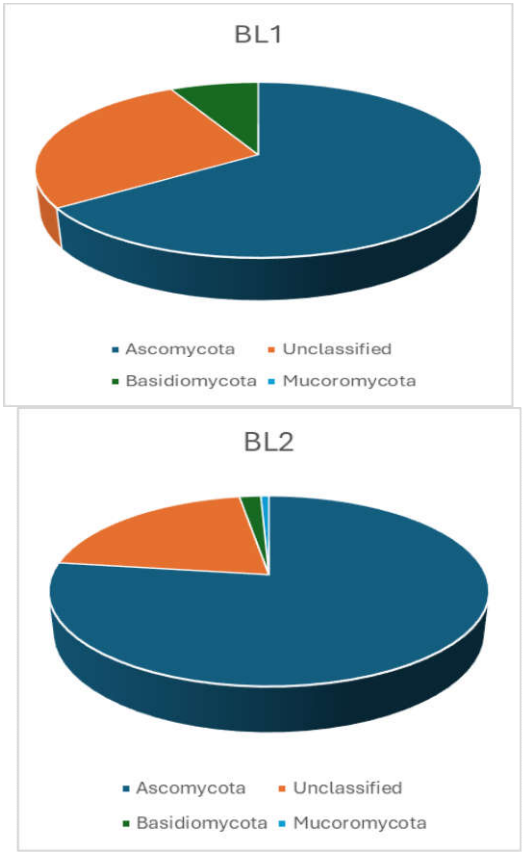

Figure 4: Fungal taxonomic composition at phylum level of *burukutu* collected from two production sites in Lagos, Nigeria.

Pito samples were observed to be highly dominated with fungi from the phylum Ascomycota, with relative abundance of over 70 % in both samples. Ascomycota displayed higher dominance in PL1, constituting approximately 82 % of the fungal community. In PL2, Ascomycota accounted for around 70 %, while Basidiomycota was in addition detected, at a relative abundance of 1.2 % (Figure 5).

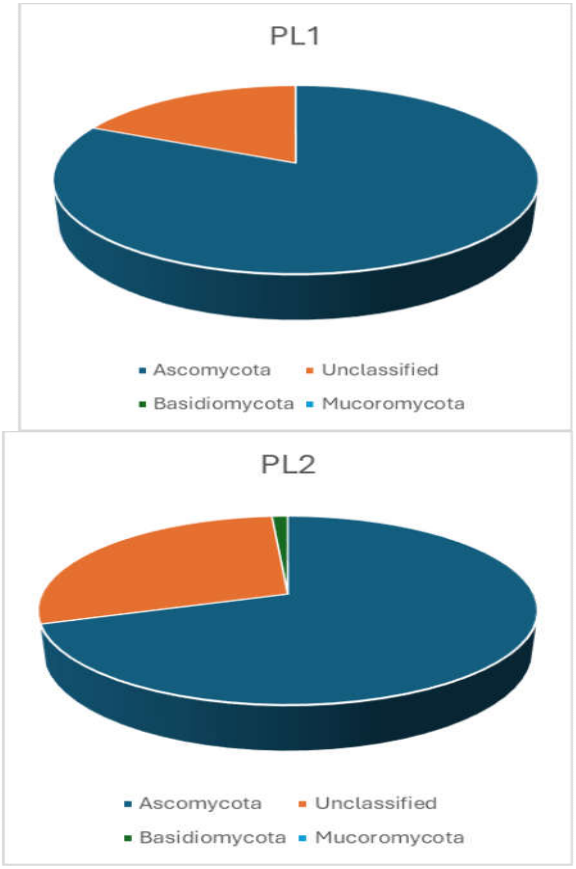

Figure 5: Fungal taxonomic composition at phylum level of *pito* obtained from two production sites in Lagos, Nigeria.

The genera *Saccharomyces* and *Candida* were detected in all the four *burukutu* and *pito* samples, with *Saccharomyces* exhibiting a dominance of over 60 % relative abundance, and *Candida* exceeding 10 % in each sample (Figure 6). Other fungal genera, including *Pichia*, *Aspergillus*, *Malassezia*, *Kluyveromyces*, *Trametes*, *Diutina*, *Vishniacozyma* and *Cladosporium*, were also present at lower relative abundances. Notably, samples collected from site 2 showed a higher proportion of *Aspergillus*, compared to those from site 1 (Figure 6).

Table 2: Alpha diversity of fungal community in *burukutu* and *pito* samples obtained from two production sites in Lagos, Nigeria.

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Samples names	ACE	Shannon index	Simpson index	Good's coverage
BL1	1032.05	5.776	0.949	0.997
BL2	929.798	5.325	0.95	0.997
PL1	946.893	2.947	0.704	0.996
PL2	713.835	1.185	0.625	0.997

Burukutu samples, BL1 and BL2 exhibited a higher fungal richness and diversity, compared to the *pito* samples. PL2 sample had the lowest fungal species richness and diversity. BL1 showed the highest ACE and Shannon values, indicating a complex and evenly distributed microbial community. In contrast, PL2 had reduced richness and diversity, along with a lower Simpson index, suggesting dominance by a limited number of taxa. Good's coverage values (~0.997) indicate sufficient sequencing depth across all the samples, confirming that observed differences reflect true community variation rather than sampling bias (Table 2).

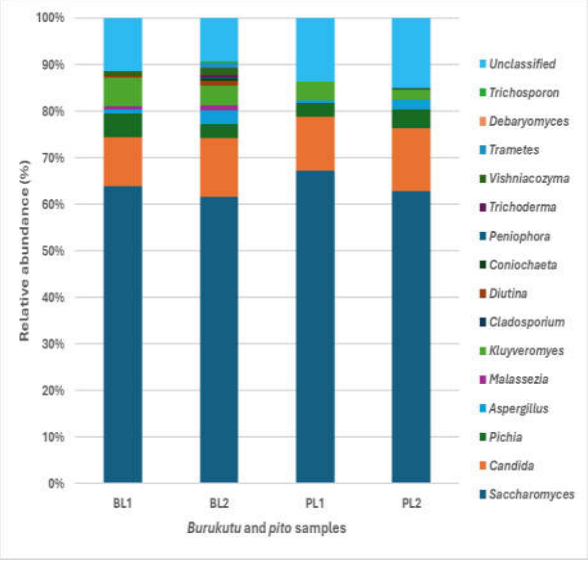

Figure 6: Fungal taxonomic composition at genus level of *burukutu* and *pito* samples collected from two production sites in Lagos, Nigeria.

4.0 Discussion

Next-generation sequencing platform based on illumina MiSeq was employed, to characterize the bacterial and fungal communities present in *burukutu* and *pito*. On the overall, both beverages exhibited a relatively complex microbial community, with notable differences observed between samples, obtained from the two production sites. *Burukutu* samples generally displayed greater phylogenetic diversity and richness, compared to *pito*. The observed differences among samples from the different locations may be attributed to environmental factors, such as water, soil, processing equipment, and the native microflora of the cereal grains (Blandino *et al*., 2003; Lyumugabe *et al*., 2012; Nwaiwu *et al*., 2020). Nevertheless, marked similarities were also detected, between the microbial communities of *burukutu* and *pito*, which is expected, given that both beverages are produced from the same cereal and fermentation process. Phylotypes such as *Lactobacillus*, *Comamonas*, *Sphingomonas*, *Acetobacter*, *Caulobacter*, *Pseudomonas* and *Bacillus* were detected across all samples of *burukutu* and *pito*. Consistent with previous microbiological studies on *burukutu* and *pito* (Egwurochi *et al*., 2021; Madilo *et al*., 2024), the dominance of *Saccharomyces* and *Lactobacillus* in both beverages is anticipated, as these genera represent the principal microorganisms in alcoholic and lactic acid fermentations, respectively, in various African cereal-based fermented foods and beverages, such as *kunun-zaki*, *mahewu*, *banku*, *hausa-koko* and *tchakpalo* (Baba-Moussa *et al*., 2012; Oguntoyinbo and Narbad, 2015; Fadahunsi

and Soremekun, 2017; Ire *et al*., 2020; Malomo and Popoola, 2020; Krabi *et al*., 2023; Atter *et al*., 2026).

Other LAB including *Streptococcus*, *Leuconostoc*, *Weissella* and *Lactococcus* were also identified, in agreement with previous reports by Kolawole *et al*. (2007), Eze *et al*. (2011), Olaniyi *et al*. (2018) and Yusuf *et al*. (2020). These LAB are responsible for both homolactic and heterolactic fermentation processes, which represent the second most important fermentation type in these beverages, after alcoholic fermentation. Their metabolic activities yield end products such as lactic, acetic and succinic acids, combined with ethanol, contributing to the distinctive organoleptic properties of *burukutu* and *pito* (Ire *et al*., 2020). Among the Pseudomonadota detected, were genera such as *Acinetobacter*, *Comamonas*, *Sphingomonas* and *Pseudomonas*, which are commonly found in soil, water, and other environmental sources. While this study was limited to genus-level identification, certain species within these genera have been implicated, in a variety of human infections, including urinary tract infections, bacteremia, endocarditis, and ocular infections (Ojeda-Varga *et al*., 1999; Lee *et al*., 2017).

Other fungal genera observed, like *Candida*, *Pichia* and *Aspergillus* have also been reported by Anaukwu *et al*. (2015) and Ire *et al*. (2020). The pathogenic potential of *Candida* species like *Candida parapsillosis*, *C. albicans* and *C. othopsillosis* raises safety concerns, as certain strains are known to be associated with humans, and are involved in nosocomial infections, neonatal and infant infections, and candidemia, with some exhibiting antifungal resistance (van Asbeck *et al*., 2009; Bonfietti *et al*., 2012; Kumar *et al*., 2016). The detection of *Aspergillus* in these products suggests a potential risk of mycotoxin contamination, as species such as *A. niger* and *A. flavus* have been reported to produce aflatoxins in fermented foods and beverages (Oguntoyinbo, 2012; Nwaiwu *et al*., 2020). The occurrence of *Aspergillus* in *burukutu* and *pito* has previously been documented (Kolawole *et al*., 2007; Eze *et al*., 2011). Additionally, the presence of *Acetobacter* in these alcoholic beverages indicates the likely production of acetic acid, a by-product of ethanol oxidation, which, if present in high amounts, can impart a vinegar-like flavour and undesirable off-tastes to the finished products (Kolawole *et al*., 2007; Fadahunsi *et al*., 2013; Fadahunsi and Olaniyi, 2015).

The use of high-throughput amplicon sequencing in this study, enabled the detection of rarely reported fungal pathogens in these commercial beverages, thereby revealing significant safety concerns associated with these products. Unreported fungal genera such as *Diutina*, *Malassezia* and *Vishniacozyma*, which may originate from either human or environmental sources, were likewise detected in these beverages. *Malassezia* (formerly known as *Pityrosporum*) are lipophilic yeasts that are part of the normal skin flora, but can become pathogenic, under certain conditions, causing diseases such as eczema, dandruff, dermatitis, folliculitis, and other skin disorders (Saunders *et al*., 2012; Saunte *et al*., 2020). *Vishniacozyma*, previously classified within the genus *Cryptococcus*, is a cosmopolitan yeast that has been isolated from soil and various environmental sources (Tsuji *et al*., 2019). *Diutina*, which was formerly included in the genus *Candida*, contains medically important species, such as *Diutina rugosa* and *D. catenulata* (Borman and Johnson, 2021).

The use of spontaneous and back-slopping methods of fermentation, contaminants from processing equipment, water and materials, processing environment and food processors or handlers are possible sources of bacterial and fungal pathogens in these beverages (Eze *et al*., 2011; Oguntoyinbo, 2012; Ire *et al*., 2020). Good manufacturing practices (GMPs) like storing the grains in a pest-free and dry environment, as well as safety inspection to screen out mouldy and infested grains are important in ensuring food safety, as the presence of mycotoxins in cereals, especially aflatoxins produced by fungal species, belonging to the genus *Aspergillus*, has been reported by Bala *et al*. (2017), Açar and Akbulut (2024) and Akter *et al*. (2025). Good hygienic practices (GHPs) implementation by farmers, suppliers, and processors are essential to prevent, eliminate, or mitigate microbiological hazards and ensure food safety, as inadequate personal hygiene among processors can lead to microbial contamination. In addition, good manufacturing

and hygiene practices, and the use of defined starter cultures with known functional properties that can impart desirable features during traditional fermentation as previously suggested by Oguntoyinbo (2012), will be required to reduce, or eliminate the risk of microbial contamination.

Conclusion

The use of next-generation sequencing platform provided a clearer understanding of the bacterial and fungal community structure and composition in *burukutu* and *pito*. These two alcoholic beverages were found to be highly rich, and diverse in both bacterial and fungal species. While this study was limited to genus-level identification, high-throughput sequencing facilitated the identification of previously unreported fungal genera in these beverages. Based on these findings, *burukutu* and *pito* cannot be completely considered safe, as they may harbour several potentially pathogenic bacteria and yeasts. To gain a deeper insight into the microbial communities present in these beverages, further investigations at the species level are recommended, along with studies on probiotic strains that could be used as starter cultures. Training local processors in GMPs, hygiene, and sanitary procedures, as well as adopting controlled fermentation system using identified starter cultures with probiotic and beneficial technological properties, would greatly enhance the safety, quality, consistency, and overall acceptability of these traditional alcoholic beverages.

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