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Research Article

High-throughput microbiome profiling of fermented grain drinks: insights across manufacturers and batches

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Abstract

Microbial communities underpin the safety, consistency, and functional properties of fermented foods; however, traditional culture-based methods can result in poorly characterized communities across manufacturers and batches. Molecular approaches enable rapid, cultivation-independent profiling of microbial community composition and function. To gain deeper insights into microbial communities, high-throughput metabarcoding was applied to characterize bacterial and fungal communities in fermented plant-based drink concentrates from seven European manufacturers. Samples were collected at two time points representing independent batches. Bacterial and fungal communities were profiled using 16S rRNA gene and internal transcribed spacer 2 (ITS2) amplicon sequencing, respectively. Taxonomic profiles were complemented by functional assessment to flag beneficial taxa associated with fermentation and taxa with potential spoilage or safety relevance. Bacterial communities were dominated by lactic acid bacteria, including *Lactobacillus* spp. (e.g., *L. acidophilus*), *Leuconostoc* spp., and *Lactococcus* spp., alongside probiotic genera such as *Bifidobacterium* spp. Low-abundance sequences assigned to taxa with potential safety relevance, including *Clostridium perfringens*, were detected. Fungal communities were dominated by the fermentative yeast *Saccharomyces* spp., alongside environmental taxa such as *Fusarium*, some of which may have mycotoxin-producing potential. Community composition varied by manufacturer and batch for both bacteria and fungi; however, manufacturer effects were substantially stronger for bacterial communities, explaining 74.8% of bacterial variance compared to 35.4% for fungi. These findings indicate that metabarcoding with functional assessment can serve as an initial flagging system for routine microbial monitoring and enables tracking of community composition and consistency across batches and markets, thereby improving quality insights in fermented food production.

Key words: Fermented foods, functional analysis, metabarcoding, microbial diversity, microbial monitoring, microbial screening system, production batch variability

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Introduction

Fermentation is one of the oldest forms of biotechnology, used for centuries in food preservation and transformation. Often regarded as a pioneer of biotechnological innovation (Galimberti et al. 2021), fermentation continues to play a vital role in developing next-generation food ingredients and products (Augustin et al. 2024). Fermented foods are defined as "foods made through desired microbial growth and enzymatic conversions of food components," distinguishing deliberate fermentations from spoilage processes (Marco et al. 2021).

From a biochemical perspective, fermentation is typically defined as an ATP generating metabolic process with organic compounds acting as both electron donors and acceptors. In food systems, however, the term is used more broadly and may include mixed microbial systems with aerobic or alternative metabolic pathways, as in koji, vinegar, and kombucha (Marco et al. 2021). Across fermentation systems, microbial growth drives enzymatic and metabolic transformations of food substrates, including the breakdown of proteins into peptides and amino acids and the formation of diverse metabolites that alter the nutritional and sensory properties of foods (Sharma et al. 2020). Fermented foods are globally distributed and culturally diverse, including East Asian staples such as kimchi and cheonggukjang; Eastern European kefir and kvass; Japanese and Indonesian tempeh; Central European sauerkraut; East African togwa and injera; and globally traded beverages such as kombucha (Shiferaw Terefe and Augustin 2020).

Fermented foods offer numerous benefits, including improved preservation, enhanced nutritional value, removal of undesirable compounds, increased antioxidant capacity, improved organoleptic qualities, and contributions to food security and sustainability (Bourdichon et al. 2012; Okoye and Oni 2017; Sharma et al. 2020; Augustin et al. 2024). Growing consumer interest in natural and health-promoting products has renewed attention to fermentation-based foods (Shiferaw Terefe and Augustin 2020). Many commercially available fermented foods are labeled as containing probiotics or prebiotics (Marco et al. 2021; Whelan and Jones 2022). Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit, whereas prebiotics are non-digestible substrates that selectively promote beneficial gut microbes (Parvez et al. 2006; Hill et al. 2014). While these foods are generally perceived as health-promoting (Sanlier et al. 2019; Yuan et al. 2024), clinically documented benefits exist for specific products and conditions (e.g., fermented dairy effects on cardiometabolic risk and lactose intolerance), and evidence for broad health claims remains limited (Companys et al. 2021; Marco et al. 2021; Rul et al. 2022).

Despite these benefits, fermented foods require careful microbial management to ensure safety, quality, and consistency. Microbial composition and relative abundance strongly influence product characteristics (Sharma et al. 2020), and the emergence of novel products necessitates evaluation of the safety and functional potential of both established and new microbial strains (Augustin et al. 2024; Niyigaba et al. 2025). Current industry standards are limited, and manufacturers typically screen only a subset of regulated or high-profile organisms using culture-based methods targeting starter cultures or common pathogens (Laulund et al. 2017). Voluntary verification of culture viability and abundance is performed via plating and colony enumeration (Sanders 2000; Patro et al. 2016). While valuable for targeted quality control, these methods provide a

narrow view of microbial communities and may overlook slower-growing or minor taxa, limiting insight into batch-to-batch consistency and emerging risks (Lok 2015; Cao et al. 2017; Jackson et al. 2019; Kovacs et al. 2025).

High-throughput sequencing (HTS) offers a complementary, system-level perspective. Amplicon metabarcoding and shotgun metagenomics can profile the breadth and structure of microbial communities without cultivation, revealing both dominant and minor taxa and enabling cross-sectional and longitudinal comparisons (Shiferaw Terefe and Augustin 2020; Zago et al. 2021). For fermented foods, these tools can (i) characterize microbial communities beyond starter cultures, (ii) detect unexpected or potentially hazardous constituents not captured by targeted tests, and (iii) evaluate batch-to-batch variability, a key quality assurance metric. Limitations remain, including restricted taxonomic resolution for some bacterial genera (often to genus level), PCR bias, and the inability to distinguish among viable, metabolically active, or dead cells (De Filippis et al. 2017; Muhamad Rizal et al. 2020). Nevertheless, metabarcoding and metagenomics provide a robust first-pass approach to guide subsequent culture-based or functional assays and inform process control.

In this study, an HTS metabarcoding framework was applied to assess the microbiological value and quality consistency of fermented plant-based beverages from seven different manufacturers in Lithuania, Europe. These beverages, primarily grain-based, are gaining popularity due to their perceived nutritional properties, including antioxidant, anti-hypertensive, and antidiabetic effects, and their suitability as vegan-friendly probiotic delivery vehicles (Melini et al. 2019; Andrade and de Castro 2023). Samples were collected at two distinct manufacturing time points and analyzed using next-generation sequencing targeting the V3–V4 region of the bacterial 16S ribosomal RNA (16S rRNA) and fungal internal transcribed spacer 2 (ITS2) gene to (i) profile bacterial and fungal communities, (ii) compare composition across manufacturers, (iii) evaluate batch-to-batch consistency, and (iv) classify taxa functionally. This approach complements conventional microbiological testing by providing a comprehensive view of product microbiomes. When combined with functional assessment, taxonomic profiling can flag both beneficial and potentially harmful taxa, creating a sensitive system for monitoring, tracking consistency, and supporting quality management.

Materials and methods

Sample background and collection

Fermented beverages from seven local manufacturers (anonymized as M1–M7) were obtained as commercially available products in Klaipėda, Lithuania. For each product, two time points representing separate production batches were chosen. The products were primarily acidic, plant- or grain-derived fermented concentrates. Product characteristics by manufacturer are summarized as follows: M1 produces fermented probiotic concentrates from water and grains (buckwheat, oats, and rice) with lactic acid and *Bifidobacterium* cultures. The M2 product line includes fermented oat drinks with oatmeal flakes and starter cultures, marketed as acidic, concentrated, unpasteurized, vegan, GMO-free, and lactose-free. M3 offers natural fermented beverage products, including kombucha

and traditional fermented drinks based on regional Lithuanian recipes, using plant-derived substrates and microbial fermentation. M4 sells fermented grain and seed beverages (e.g., oats with turmeric) as unpasteurized, vegan drinks enriched with beneficial microbes. M5 produces water-based concentrates with honey, buckwheat, oats, or rice and biologically active microbes. M6 markets flavored fermented concentrates of water, grain flours, or flakes with high levels of lactic acid and *Bifidobacterium* cultures. M7 offers fermented probiotic drinks and concentrates made from organic oats, which are fermented together with organic juices or honey and contain no added sugar, gluten, or lactose.

Detailed compositional parameters (pH, ethanol, and residual sugar) were not provided by manufacturers and were not measured.

DNA extraction

A 45 mL volume of the homogenized beverage was centrifuged at 4,000 rpm for 20 min. Approximately 0.3 g of the resulting pellet was transferred into a PowerBead Tube supplied with the DNeasy PowerSoil Pro DNA Extraction Kit (Qiagen). DNA was extracted and purified according to the manufacturer's instructions. For each beverage, three biological replicates were processed independently. DNA extractions were performed at Sequench Lithuania laboratory facilities (Klaipėda, Lithuania). Extracted DNA samples were sent to Sequench Ltd. (Nelson, Aotearoa, New Zealand) for downstream processing.

High-throughput sequencing

Amplicon PCR and library preparation were performed in separate clean laboratory rooms to avoid cross-contamination. Each room was UV-sterilized for a minimum of 30 min before use. PCR was conducted in a DNA/RNA UV-cleaner box with a UV cleaner–recirculator (BioSan, Latvia). Aerosol barrier tips (Accumax, Interlab, NZ) were used throughout.

DNA samples were amplified for the V3–V4 region of the bacterial 16S ribosomal RNA (16S rRNA) gene and the internal transcribed spacer 2 (ITS2) region, representing bacterial and fungal diversity, respectively. Primers for 16S rRNA (~464 bp) were NexR-16SFWD: 5'-CCTACGGGNGGCWGCAG-3' and NexR-16SREV: 5'-GACTACHVGGGTATCTAATCC-3' (Klindworth et al. 2013). ITS2 primers (~330 bp) were ITS3: 5'-GCATCGATGAAGAACGCAGC-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3' (White 1990). Illumina overhang adaptors were added as described in Kozich et al. (2013).

PCR reactions for 16S rRNA used a T100 Thermal Cycler (Bio-Rad, USA) in a 30 µL total volume: 15 µL MyFi Mix (Bioline, UK), 0.75 µL of each 10 µM primer, 11 µL DNase-/RNase-free water, and 3 µL template DNA. ITS2 reactions used the same total volume with 1.5 µL of each 10 µM primer and 9 µL water. Thermocycling for 16S rRNA was 95 °C for 1 min; 35 cycles of 95 °C for 15 s, 50 °C for 15 s, 72 °C for 15 s; and a final extension at 72 °C for 7 min. ITS2 thermocycling was 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s; and a final extension at 72 °C for 10 min. Negative controls (no template controls, NTCs) were included in all PCR assays.

Amplicons were purified and normalized using the SequalPrep Normalization Kit (Thermo Fisher Scientific, USA) to ~1 ng/µL. Samples were indexed with

xGen™ Amplicon UDI Primers (IDT, USA) with Illumina Nextera-compatible adapters and sequenced on an Illumina NextSeq 2000 platform with a 30% PhiX spike. Raw reads were deposited in NCBI SRA (PRJNA1347471).

Bioinformatics

In this study, the focus was on unique eDNA sequences, or amplicon sequence variants (ASVs). The denoising approach used to generate ASVs provides high taxonomic resolution without relying on the arbitrary similarity thresholds associated with clustering into molecular operational taxonomic units (OTUs), and because ASVs are inferred as true biological sequences, they improve reproducibility, reusability, and comparability across studies (Callahan et al. 2016). This approach is particularly advantageous for characterizing the complex and dynamic microbial communities present in fermented food products and has been shown to provide more accurate taxonomic representation and improved potential for pathogen detection (Hussain et al. 2021; Palmnäs-Bédard et al. 2023). Bioinformatic pipelines for fungal and 16S rRNA analysis followed the methodology outlined below. Primer sequences were removed using cutadapt (version 5.0; Martin 2011), applying the default error rate of 0.1 for primer mismatches. Sequences were then processed using the DADA2 package (Callahan et al. 2016) in R (R Core Team 2024). In DADA2, 16S rRNA reads were truncated to 226 bp (forward) and 220 bp (reverse), whereas ITS2 sequences were not truncated due to length variation at this locus (Callahan 2022). Truncation values for 16S rRNA were selected based on inspection of read quality profiles to retain high-quality bases while ensuring sufficient overlap for merging, following standard DADA2 guidelines (Callahan et al. 2016; Callahan 2022). Reads were filtered using a maximum expected errors (maxEE) threshold of 2; any reads exceeding this threshold were discarded. A parametric error matrix was created, and sequence variants were dereplicated for both forward and reverse reads. Singletons were removed, and paired-end reads were merged without mismatches, requiring a minimum overlap of 10 bp. Chimeric sequences were filtered out using removeBimeraDenovo with the consensus option.

For taxonomic classification, 16S rRNA ASVs were compared with the SILVA database (version 138.2; Quast et al. 2013), whereas ITS2 amplicons were compared against the UNITE database (version 10.0; Abarenkov et al. 2024). Taxonomic assignment was performed using the DADA2 assignTaxonomy script and the RDP classifier (Wang et al. 2007) with default settings.

To eliminate potential contamination, the maximum sequence count across DNA extraction, PCR, and sequencing controls was applied as a removal threshold (Bell et al. 2019). This was implemented using ps_decon() from the R package 'biohelper' (version 0.0.10.0; Laroche 2023) with the max_v method. ASVs detected in samples and controls with counts below this threshold were excluded, whereas ASVs exceeding the threshold had the threshold value subtracted.

Statistical analysis

All statistical analyses were conducted in R (version 4.4.1). Rarefaction curves evaluated sequencing depth for biodiversity comparisons, and read depth was standardized across all samples using the R package ‘phyloseq’ (version 1.46.0; McMurdie and Holmes 2013). Samples were rarefied to equal sequence numbers

(67,900 for 16S rRNA and 1,250 for ITS2) to remove bias from sequencing depth. Rarefaction depths were selected based on inspection of rarefaction curves and sample sequencing depth distributions, choosing values that retained the maximum number of samples while ensuring curves approached a plateau. For the 16S rRNA dataset, the minimum read count across samples was used, whereas for the ITS2 dataset, the second-lowest read count (excluding zero-read samples) was selected. Rare ASVs, represented by fewer than 50 reads across the dataset, were excluded. These sequences likely reflected environmental noise or legacy signals from handling rather than the inherent product microbiome. Unclassified data at the Kingdom level (NA) were removed. Manufacturers were anonymized and assigned identifiers M1–M7 for confidentiality.

Taxonomic community composition was visualized using 'microeco' (version 1.13.0; Liu et al. 2021) and 'phyloseq' (version 1.46.0; McMurdie and Holmes 2013).

Alpha diversity was assessed using the Shapiro–Wilk test for normality and Levene's test for homogeneity of variance. The data did not follow a normal distribution (Suppl. material 1: table S1), so a non-parametric Kruskal–Wallis test compared observed ASVs between manufacturers, followed by Dunn's test with Bonferroni correction for significant pairwise comparisons. All tests were conducted using cal_diff() from 'microeco'.

Beta diversity was evaluated via Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarity. Clustering by manufacturer and production batches (time_rep) was visualized. Differences between groups were tested using PERMANOVA with cal_manova() from 'microeco,' assessing main effects and covariate interactions (e.g., Manufacturer × time_rep). Homogeneity of multivariate dispersion was evaluated using cal_betadisper().

Functional analysis

For functional inference supporting microbial categorization, fungal ITS2 sequences were assigned ecological functions using FUNGuild (version 1.1; Nguyen et al. 2016). Bacterial 16S rRNA gene sequences were annotated using the FAPROTAX database (Louca et al. 2016), which has previously been shown to be suitable for microbial communities involved in fermentation (Yang et al. 2022a). Both datasets were subsequently glommed to the assigned functional group level using tax_glom() from the 'phyloseq' package. For bacterial taxa, those lacking functional annotation were assigned as "Unassigned." Taxa associated with fermentation, gut-related functions, or nitrogen fixation were labeled "Beneficial." Taxa with functions linked to human pathogenicity were labeled "Potentially Harmful," whereas those associated with spoilage were classified as "Undesirable." All other taxa were considered "Neutral." Taxa that exhibited both beneficial traits (e.g., fermentation, nitrogen fixation) and functions linked to pathogenicity or spoilage were flagged as "Verify." These taxa were manually checked against the literature and assigned to one of the five categories (Beneficial, Potentially Harmful, Neutral, Undesirable, or Unassigned) based on pathogenic risk, potential probiotic effects, or the absence of known effects. For example, *Clostridium perfringens* is widely distributed in the environment and commonly found in human intestines; certain types (e.g., type A with the *cpe* enterotoxin gene) are associated with food poisoning (FDA 2012). Based on this classification and literature review, *C. perfringens* was reported as

"Potentially Harmful." However, because this HTS approach cannot distinguish pathogenic from non-pathogenic strains or isotypes, risk cannot be confirmed without strain-level verification and assessment of variability checks.

To ensure contextual relevance, all taxa initially labeled as "Beneficial" were further verified. Taxa classified as "Beneficial" were required to have documented participation in traditional anaerobic fermented grain beverages (yeast and lactic acid bacterial metabolism, e.g., kvass) (Ziarno and Cichońska 2021), in aerobic grain or grain-malt fermentations dominated by acetic acid bacteria (e.g., kombucha-type processes) (Wang et al. 2022), or documented use as probiotics in other fermented food products (Tamang et al. 2016; Ziarno and Cichońska 2021). Several taxa annotated with fermentation-related pathways (e.g., *Turicibacter* sp., *Klebsiella variicola*, *Bacteroides graminisolvens*, and *Paraclostridium bifermentans*) were reclassified, as these organisms are primarily associated with gut or environmental niches and are not established contributors to beverage fermentation. For final visualizations, only taxa resolved to species level were retained. Exceptions were made for well-characterized lactic acid bacteria genera (*Lacticaseibacillus* and *Limosilactobacillus*), which were summarized at the genus level due to their documented beneficial roles in fermented grain beverages (Basinskienė et al. 2016; Yang et al. 2022b; Lee et al. 2024) and the limited species-level resolution of standard 16S rRNA amplicon sequencing for closely related lactic acid bacteria (Laref and Belkheir 2022). The corrected table was then used for downstream visualizations (Suppl. material 2: appendix S1 – bacterial ASVs with functions and microbial categorical classifications).

For fungal taxa, classification criteria were as follows: taxa lacking guild or trophic information were classified as "Unassigned"; taxa exhibiting multiple ecological roles were classified as "Mixed"; those identified as opportunistic human pathogens or likely opportunistic human pathogens by FUNGuild, with the exception of *Pichia* due to its frequent use as a non-conventional yeast in fermented drinks (Staniszewski et al. 2025), were classified as "Potentially Harmful"; symbiotrophic taxa were labeled "Beneficial"; and saprotrophic taxa were categorized as "Neutral." In addition, taxa associated with plants (e.g., plant saprotrophs, wood saprotrophs) were verified as environmental fungi and, if no known human health risk existed, were assigned a status of "Neutral." Taxa with evidence suggesting potential opportunistic human pathogenicity were assigned "Mixed." As with bacterial species, all fungal taxa classified as "Beneficial" or "Potentially Harmful" were cross-referenced with the literature. Visualization of fungal taxa was conducted at the genus level due to limited resolution at the species level.

Overall, several caveats with this approach are acknowledged, including that the associated risk is influenced by factors such as the specific strains involved, their pathogenicity or toxicity levels, their impact on human health, and the variable cell abundances within the samples.

Results

Sequence data and microbial biodiversity overview

After all bioinformatics processing and decontamination steps, a total of 6,352,987 reads (mean: 151,261.6 reads; 1,332 ASVs) for 16S rRNA and 1,295,070 reads (mean: 39,244.6 reads; 133 ASVs) for ITS2 were obtained

across 42 samples (seven different manufacturers, two sampling time points, and three replicates). Nine ITS2 samples were removed after denoising due to zero or extremely low fungal read abundance: two from M2, two from M6, and five from M7. A rarefaction analysis plot confirmed adequate sequencing coverage across all samples, as the rarefaction curves plateaued—indicating that the majority of the target taxonomic diversity was captured (Suppl. material 1: fig. S1). Following rarefaction, 65 ASVs were removed from the 16S rRNA dataset, as they were no longer present in any sample after random subsampling. Similarly, eight ASVs and one sample were removed from the ITS2 dataset.

After rarefaction and the removal of rare ASVs (<50 reads), the 16S rRNA marker dataset comprised 375 ASVs, whereas the ITS2 dataset included 65 ASVs. Taxonomic assignment revealed the following classifications across the 16S rRNA and ITS2 datasets, respectively: 10 and two phyla; 13 and eight classes; 40 and 12 orders; 57 and 16 families; 84 and 18 genera; and 80 and 19 species.

The 10 most abundant bacterial genera detected across all samples using 16S rRNA sequencing included lactic acid bacteria (LAB) such as *Lacticaseibacillus*, *Limosilactobacillus*, *Lactobacillus*, *Streptococcus*, and *Sporolactobacillus*; probiotic-associated genera such as *Bifidobacterium*; acetic acid bacteria such as *Acetobacter*; and other genera, including *Anoxybacillus*, *Weizmannia*, and *Clostridium* (Table 1).

Table 1. Top 10 bacterial genera detected in fermented beverages.

Genus	Description/relevance	Species detected	References
Lacticaseibacillus	Genetically close lactic acid bacteria common in fermented products and cereal-based beverages (e.g., *L. rhamnosus*, *L. paracasei*, *L. casei*)	*L.* spp.	Colautti et al. 2024; Ziarno and Cichońska 2021
Limosilactobacillus	Lactic acid bacterial genus involved in cereal fermentation and probiotic beverage development (e.g., *L. fermentum*, *L. reuteri*)	*L.* spp.	Lee et al. 2023; Ziarno and Cichońska 2021
Lactobacillus	Lactic acid bacterial genus central to cereal-based fermentations such as boza and togwa	***L. acetotolerans***, ***L. acidophilus***, ***L. amylovorus***, ***L. delbrueckii***, ***L. equicursoris***, *L. fructivorans*, ***L. gallinarum***, *L. homohiochii*, ***L. kitasatonis***, *L.* spp.	Dan et al. 2019; Ziarno and Cichońska 2021
Streptococcus	Lactic acid bacteria; some species relevant to fermented foods	*S. gallolyticus*, *S. lactarius*, *S. lutetiensis*, *S. oralis*, ***S. salivarius***, ***S. thermophilus***, ***S. vestibularis***, *S.* spp.	Yu et al. 2021; Ziarno and Cichońska 2021
Sporolactobacillus	Spore-forming lactic acid bacteria occasionally found in cereal fermentations	***S. inulinus***, ***S. laveus***, *S.* spp.	Guo et al. 2024
Bifidobacterium	Gut-associated genus widely used in probiotics	***B. animalis***, ***B. breve***, *B. globosum*, ***B. pseudolongum***, *B. psychraerophilum*, ***B. thermophilum***, *B.* spp.	Bourdichon et al. 2012
Clostridium	Diverse genus with pathogenic and probiotic species (e.g., *C. butyricum*); probiotics rare in fermented beverages	*C. acetobutylicum*, *C. baratii*, *C. beijerinckii*, *C. butyricum*, *C. chromiireducens*, *C. intestinale*, *C. isatidis*, *C. kolgasensis*, *C. pasteurianum*, *C. paraputrificum*, *C. perfringens*, *C. saccharoperbutylacetonicum*, *C. tarantellae*, *C. tertium*, *C. tyrobutyricum*, *C.* spp.	Cassir et al. 2016; Grenda et al. 2022; Guo et al. 2020
Acetobacter	Acetic acid bacteria contributing to souring and flavor in beverages	***A. aceti***, *A. ascendens*, *A. fabarum*, *A. indonesiensis*, *A. lambici*, ***A. lovaniensis***, *A. orientalis*, ***A. pasteurianus***, *A. persici*, *A. senegalensis*, *A. syzygii*, *A. tropicalis*, *A.* spp.	Ashaolu et al. 2025; Han et al. 2024; Pothakos et al. 2016
Anoxybacillus	Thermophilic spore-forming genus from soil and hot springs; detection in fermented grain beverages likely reflects environmental presence, contamination, or dormant spores	*A. flavithermus*, *A. rupiensis*, *A.* spp.	Tasara et al. 2017
Weizmannia	Spore-forming soil and plant bacteria; some species such as *W. coagulans* are probiotics, though not well-documented in fermented grain beverages	*W.* spp.	Maresca et al. 2024; Sreenadh et al. 2022; Zhang et al. 2023

Table notes: Species in bold indicate representative taxa that have been associated with other acidic, plant- or grain-derived fermented beverages, as reported in the literature.

For fungal data, the ten most abundant genera detected using ITS2 sequencing included core fermentative yeasts such as *Saccharomyces* and minor or secondary fermentative yeasts such as *Pichia*. Other prominent taxa included common environmental yeasts (*Dioszegia*, *Microbotryozyma*, and *Vishniacozyma*); wood-decay fungi (*Heterobasidion* and *Peniophora*); saprotrophic fungi (*Mycena*); skin-associated fungi (*Malassezia*); and soil-borne molds (*Fusarium*), which are frequently found in cereal crops (wheat, sorghum, rice, maize, and barley), with some species capable of producing harmful mycotoxins (Table 2).

Table 2. Top 10 fungal genera detected in fermented beverages.

Genus	Description/relevance	Species detected	References
Fusarium	Frequently found in cereal crops (wheat, sorghum, rice, maize, and barley); some species capable of producing mycotoxins under suitable conditions	*F. langsethiae*, *F. poae*, *F.* spp.	Lattanzio et al. 2013; Masenya et al. 2024
Pichia	Non-*Saccharomyces* yeasts occasionally found in fermented beverages (e.g., *Pichia kluyveri*); may act as minor, secondary, or spoilage yeasts with variable fermentative activity	*P. ethanolica*, *P. membranifaciens*, *P.* spp.	Chu et al. 2023; da Silva Vale et al. 2023; Tomicic et al. 2017
Malassezia	Skin-associated fungi; detected in SCOBY cultures of kombucha	*M. restricta*, *M. globosa*	Kindo et al. 2004
Dioszegia	Environmental yeast	*D. hungarica*	Renker et al. 2004
Saccharomyces	Core fermentative yeast (e.g., *S. cerevisiae*)	***S.* spp.**	Bourdichon et al. 2012; da Silva Vale et al. 2023
Heterobasidion	Wood-decay fungus	*H. annosum*, *H.* spp.	Garbelotto and Gonthier 2013; Piri and Vainio 2024
Microbotryozyma	Environmental yeast	*M.* spp.	Tian et al. 2025
Vishniacozyma	Environmental yeast	*V. victoriae*	Liu et al. 2025
Peniophora	Wood-associated fungus	*P.* spp.	Zou et al. 2022
Mycena	Saprotrophic fungi	*M. mirata*, *M. zephirus*	Ke et al. 2020

Table notes: Species in bold indicate representative taxa that have been associated with other acidic, plant- or grain-derived fermented beverages, as reported in the literature.

Microbial compositional data

Analysis of the compositional data across manufacturers and production batches revealed distinct patterns in microbial community structure. In the 16S rRNA dataset, comparisons between manufacturers highlighted dynamic shifts in dominant genera. For example, manufacturers M3 (batch 1), M4, M5 (batch 1), and M6 exhibited relatively high proportions of *Lactobacillus*, comprising approximately 41–85.5% of the total detected genus-level composition (Fig. 1A). In contrast, M2's bacterial community, particularly batch 2, was largely dominated by *Clostridium* (72.1%). Manufacturer M1 showed high abundances of other LAB, including *Lacticaseibacillus* (batch 1: 39.1%) and *Sporolactobacillus* (batch 2: 34.3%), whereas M7 displayed over 50% relative abundance of *Weizmannia* across both batches (58% and 52.2%, respectively) (Fig. 1A). Probiotic taxa such as *Bifidobacterium* were detected sporadically across batches in M1, M2, and M4. While M3 batch 2 reads were largely dominated by *Acetobacter* (56.1%), primarily *Acetobacter pasteurianus* (Suppl. material 1: fig. S2). With the exception of M2 batch 2, M3 batch 2, and M7, both batches, the majority of genera (≥50%) were LAB.

Fungal community composition showed lower taxonomic resolution in some cases—for example, M6 batch 2 could only be assigned at the phylum level, preventing genus-level visualization. Compared to bacterial communities, fungal samples displayed greater diversity within the "Other" category, particularly

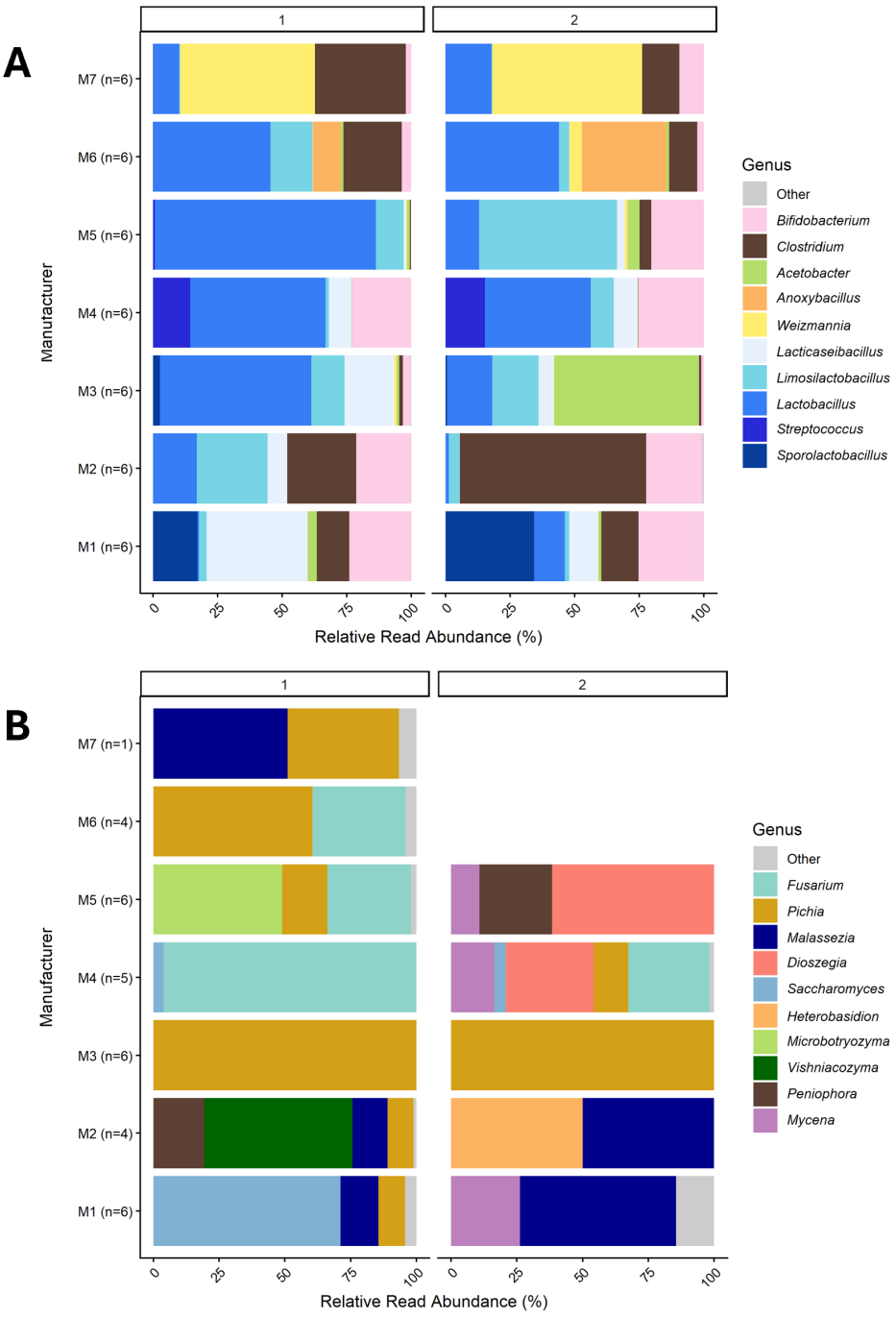

Figure 1. Relative abundance of the top ten genera grouped by manufacturer and faceted by production batches (time replicates 1 and 2) for (**A**) bacteria 16S ribosomal RNA (16S rRNA) and (**B**) internal transcribed spacer 2 (ITS2) metabarcoding analysis. Note that for manufacturer M7, all fungal samples from the second production batch were removed during denoising in the bioinformatics pipeline, and for M6, samples from the second production batch could only be classified to the phylum level (Ascomycota).

M1 batch 2 (Fig. 1B). *Pichia*, a non-*Saccharomyces* yeast, was present in at least one batch across all manufacturers, with notably high levels in M3 (100%), M6 batch 1 (60.4%), and M7 batch 1 (42.4%), particularly of *Pichia ethanolica* (Suppl. material 1: fig. S3). *Malassezia* was a dominant genus in M7 (51.0%), M2 batch 2 (50%), and M1 batch 2 (59.3%). Notably, *Fusarium* constituted 96.1% of the fungal community in M4 in batch 1 (31.0% in batch 2), and *Saccharomyces*

accounted for ~71.1% in batch 1 for manufacturer M1 (Fig. 1B). Other dominant environmental yeasts included *Heterobasidion* and *Vishniacozyma* in M2 (batch 2: 50%; batch 1: 56.5%) and *Microbotryozyma* and *Dioszegia* in M5 (batch 1: 49%; batch 2: 61.7%).

When comparing production batches (time replicates), temporal changes in dominant bacterial genera were observed across most manufacturers (Fig. 1A). While core genera remained relatively consistent, shifts in dominance were evident in M2, M3, and M5 (Fig. 1A). For example, in M3, *Lactobacillus* accounted for 58.6% of reads in batch 1 but dropped to 17.5% in batch 2, with *Acetobacter* becoming dominant (from 0.78% in batch 1 to 56.1% in batch 2). Similarly, *Lactobacillus* dominated M5's batch 1 (85.5%) but decreased to 13% in batch 2, with a corresponding increase in another LAB genus, *Limosilactobacillus* (Fig. 1A). In M4 and M7, dominant bacterial taxa remained stable, whereas M2 batch 2 showed increased *Clostridium*, and M6 batch 2 showed increased *Anoxybacillus*.

Shifts in fungal genera mirrored bacterial trends, with all manufacturers showing differences in dominant genera between batches (Fig. 1B). In M2, batch 2 showed increased *Heterobasidion* and *Malassezia*, with a decrease in *Pichia* and Ascomycota. In M4, *Fusarium* decreased substantially between batches, whereas *Dioszegia*, *Pichia*, and *Mycena* increased. In M5, environmental yeasts shifted from *Microbotryozyma* to *Dioszegia*, with additional contributions from *Mycena* and *Peniophora* (Fig. 1B). Overall, yeasts associated with fermentation, such as *Saccharomyces*, decreased in some manufacturers (e.g., M1), whereas contaminant genera including *Malassezia* and *Mycena* became more prominent.

Comparison between manufacturer and production time points

When examining the number of unique molecular species (ASVs) detected within each manufacturer, the results indicate a significant difference in the number of observed bacterial ASVs for the 16S rRNA dataset (Kruskal–Wallis rank sum test, p = 7.14E-06) (Suppl. material 1: table S2; Fig. 2A). In contrast, the number of observed fungal taxa in the ITS2 region did not differ significantly between manufacturers (Kruskal–Wallis rank sum test, p = 0.396) (Suppl. material 1: table S2; Fig. 2B).

For 16S rRNA, pairwise comparisons revealed significant differences between M3 and two other manufacturers (M7 p = 3.08E-04; M2 p = 1.90E-05). Additionally, M6 differed significantly from M7 (p = 1.88E-02) and M2 (p = 2.04E-03) (Suppl. material 1: table S3). These results suggest that the richness of bacterial communities may be influenced by manufacturer-specific factors, whereas fungal richness appears relatively consistent across manufacturers. However, as shown in Fig. 1, differences in community composition between manufacturers, as well as production batches, are evident (Fig. 3).

To further explore community composition between manufacturers, beta diversity analysis was performed to assess how bacterial and fungal communities differ across manufacturers and batches. The results revealed distinct microbial community differences by manufacturer, with PERMANOVA indicating significant effects of both manufacturer (bacteria p = 0.001; fungi p = 0.001) and production batch (time_rep) (bacteria p = 0.001; fungi p = 0.023), as well as a significant interaction between these factors (bacteria p = 0.001; fungi p = 0.024) (Table 3).

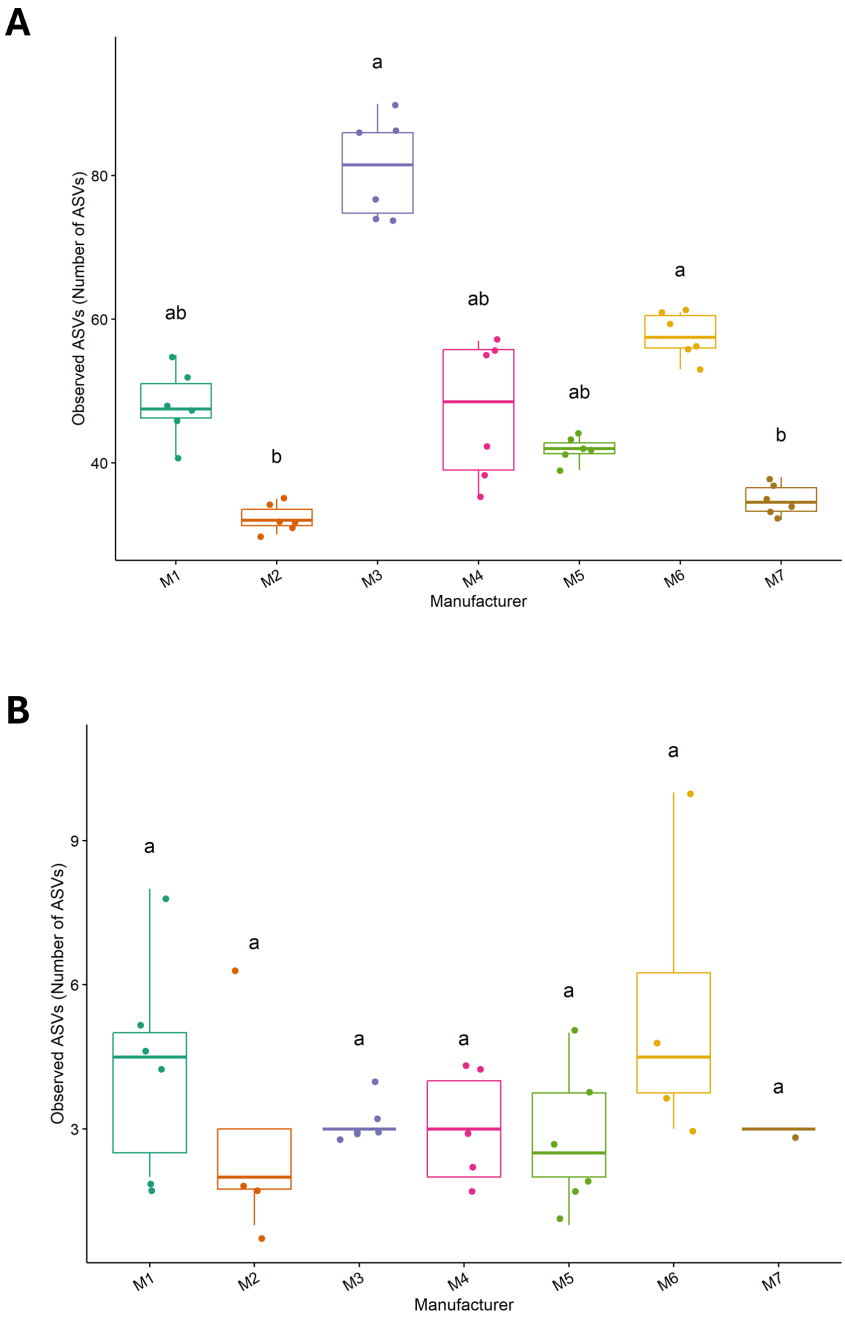

Figure 2. Alpha diversity, observed amplicon sequence variant (ASVs), plots for (**A**) bacteria 16S ribosomal RNA (16S rRNA) gene and (**B**) internal transcribed spacer 2 (ITS2) metabarcoding analysis. Letters above the boxplots indicate groupings based on post hoc Dunn's Kruskal–Wallis multiple comparisons test with Bonferroni adjustment. Groups sharing at least one letter are not significantly different from each other ($p > 0.05$), whereas those with no letters in common differ significantly ($p < 0.05$).

Beta dispersion analysis revealed significant differences in within-group variability between manufacturers and batches (time_rep) ($p = 0.001$), indicating that the significant PERMANOVA results may be influenced by both differences in inferred community composition and heterogeneity among manufacturers and batches for both bacterial and fungal datasets (Table 3).

The manufacturer and production batch accounted for 98.02% of the total variance in the 16S rRNA dataset (manufacturer: 74.8%, batch (time_rep):

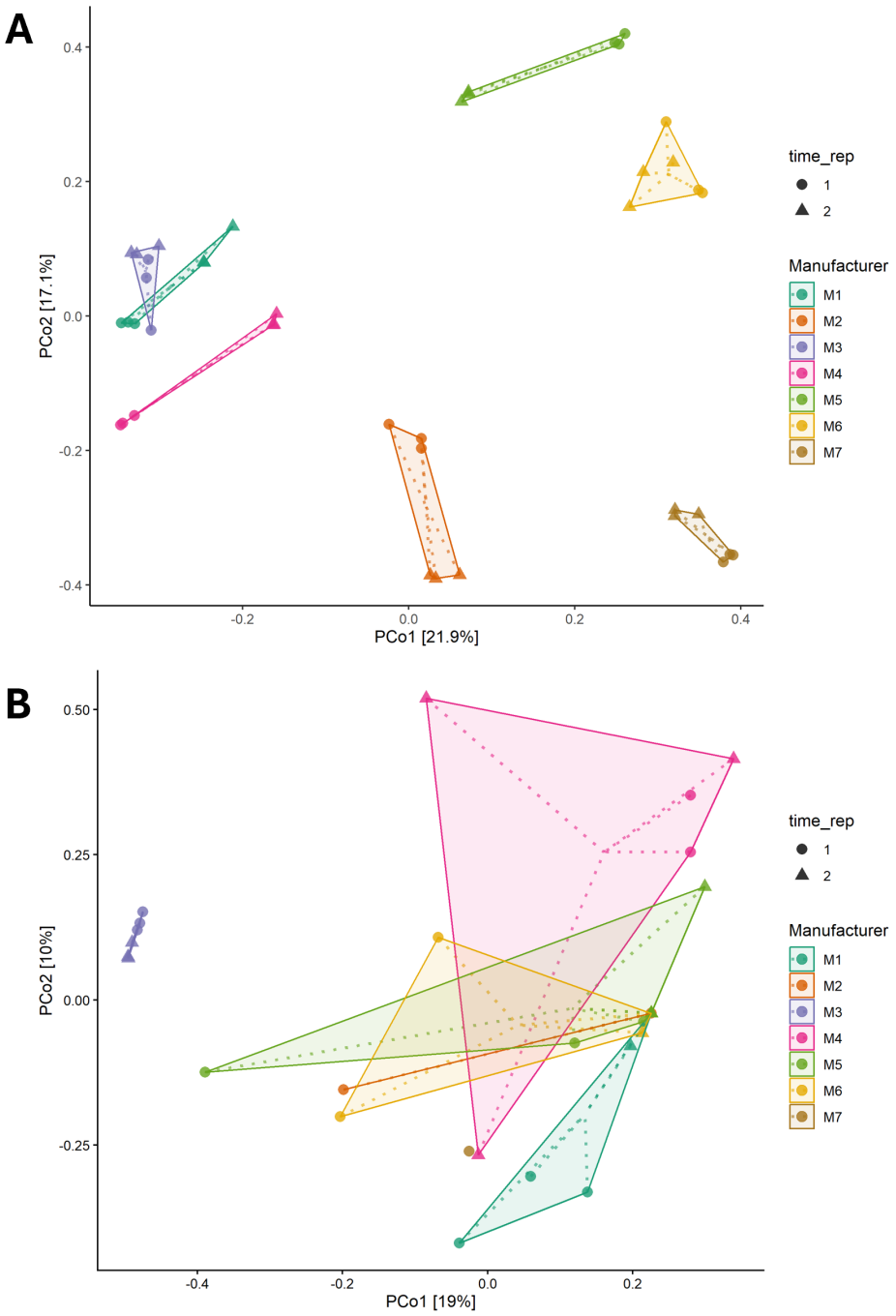

Figure 3. Beta diversity PCoA (Principal Coordinates Analysis) plots (**A**) bacteria 16S ribosomal RNA (16S rRNA) gene and (**B**) internal transcribed spacer 2 (ITS2) metabarcoding analysis. Distance was measured using Bray–Curtis dissimilarity. Data are grouped by both manufacturer and production batch (time_rep).

2.52%, and their interaction: 20.7%) (Table 3). For the ITS2 dataset, manufacturer and production batch accounted for only 55.53% of the total variance (manufacturer: 35.4%, time_rep: 4.33%, and their interaction: 15.8%) (Table 3). This suggests that most of the variance within the 16S rRNA gene dataset is related to the manufacturer, which is clearly visible on the PCoA plot (Fig. 3A). In contrast, distinct clustering by manufacturer in the fungal ITS2 dataset was less pronounced on the PCoA plot (Fig. 3B), with only one manufacturer, M3, showing a distinct separation from the others.

Table 3. Permutational multivariate analysis of variance for 16S ribosomal RNA (16S rRNA) gene and internal transcribed spacer 2 (ITS2) datasets.

	df	SumofSqs	R2	F	Pr (>F)	beta dispersion
16S rRNA						
Manufacturer	6	10.7	0.748	176.1	**0.001**	0.001 ***
Time_rep	1	0.359	0.0252	35.5	**0.001**	0.355
Manufacturer:time_rep	6	2.96	0.207	48.8	**0.001**	
Residual	28	0.283	0.0198	NA	NA	
Total	41	14.3	1	NA	NA	
ITS2						
Manufacturer	6	4.83	0.354	2.52	**0.001**	0.001 ***
Time_rep	1	0.591	0.0433	1.85	**0.023**	0.129
Manufacturer:time_rep	5	2.16	0.158	1.35	**0.024**	
Residual	19	6.07	0.445	AN	NA	
Total	29	13.66	1	NA	NA	

Table notes: Permutation: 999 permutations were used, and for significance, * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, and *** denotes $p \leq 0.001$.

Functional analysis and categorical assignment of microbes

One key aim, alongside characterising the microbial community composition in fermented products, is to understand the functional roles these microbes may play in the fermentation process and any potential risks they may pose to human health, as well as to identify potentially beneficial microbes that could positively impact consumers. Therefore, functional analysis was conducted, and microbes were categorized according to the criteria outlined in the Methods.

Overall, for bacteria, many appeared to play a role in fermentation, with the top 10 most abundant functions related to processes such as nitrogen fixation, mammalian and human gut association, fermentation, and both anaerobic and aerobic chemoheterotrophy (Suppl. material 1: fig. S5). Functional classification at the species level indicated that most resolved ASVs were beneficial across manufacturers and production batches, particularly for M3, M2, M4, M5, and M7 batch 2 (Fig. 4A). Importantly, most manufacturers had "Potentially Harmful" bacteria below 1%, and this was consistent across batches, with the exception of both production batches from M6 and production batch 1 from M3 (Suppl. material 1: table S6). These samples warrant further investigation to verify safety and associated risk, including methods that can resolve strain-level variability and pathogenic potential. Products from M1 and M6 contained higher levels of undesirable taxa, likely including *Clostridium tyrobutyricum* and *Anoxybacillus flavithermus* (Suppl. material 1: fig. S2), which may contribute to spoilage or off-flavor formation. This supports broader product quality assessment, particularly for batch 2 from M6, where 84.3% of taxa were classified as "Potentially Harmful" or "Undesirable" (Fig. 4A; Suppl. material 1: table S6). Flagging was conducted at the species level; however, many ASVs were not resolved to the species level, further limiting risk interpretation.

Fungal functional groups were predominantly associated with plant saprotrophs, including agaricoid forms (gilled mushrooms), as well as animal and plant pathogens, likely associated with the fermentation substrate raw

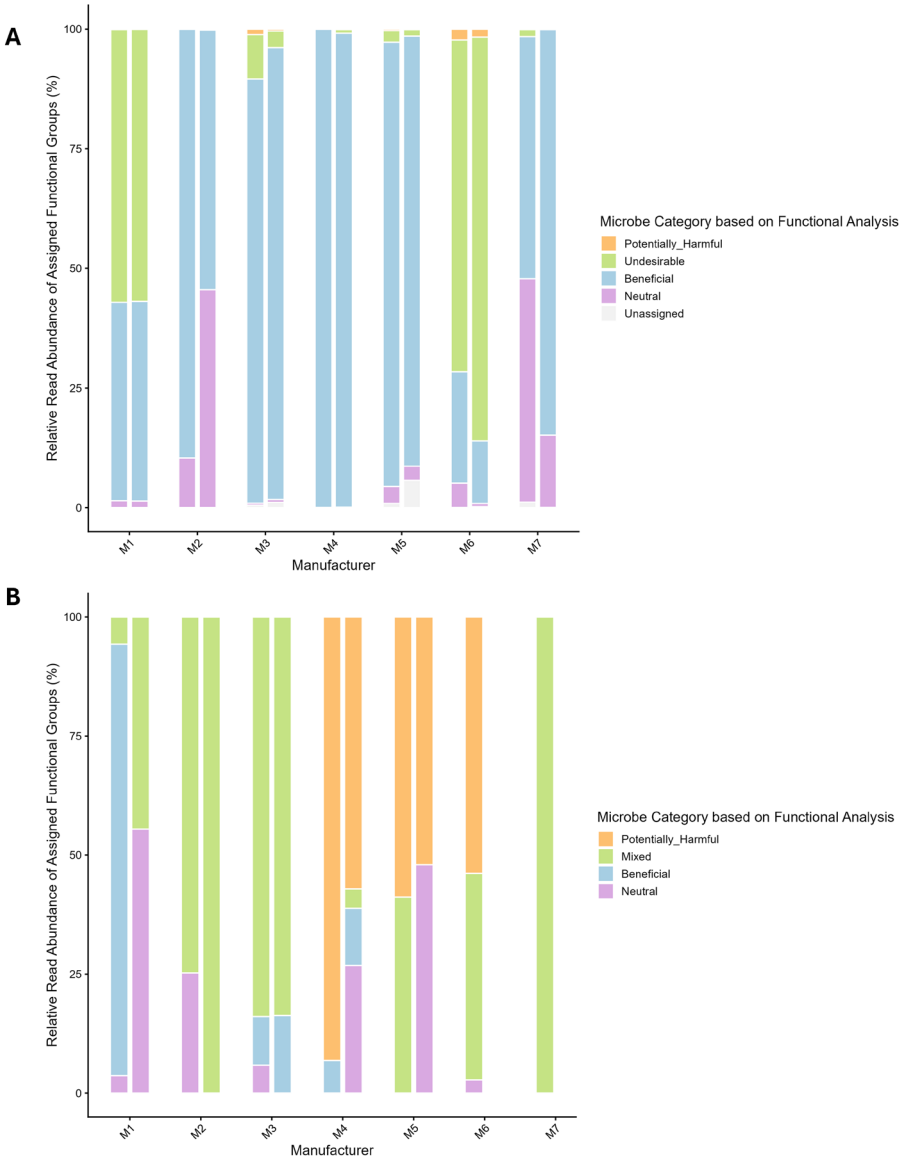

Figure 4. Categorization of microbes based on analysis of (**A**) bacteria 16S ribosomal RNA (16S rRNA) gene and (**B**) internal transcribed spacer 2 (ITS2) datasets. Data are grouped by manufacturer, with each manufacturer showing results for each batch (time_rep). Taxa were assigned to categories based on functions related to potential fermentation pathways, human health, and pathogenicity. These categorizations were also verified against the literature, as described in the Methods. Relative abundances represent sequence reads assigned to functional groups following taxonomic and functional annotation. Note that for manufacturer M7, all fungal samples from the second production batch were removed during denoising in the bioinformatics pipeline. Bacterial categorization was performed at the species level, whereas fungal categorization was performed at the genus level; consequently, second-batch fungal samples for manufacturer M6 were removed because classification could only be resolved to the phylum level.

material (Suppl. material 2: appendix S2 – fungal ASVs with functions and microbial categorical classifications). Many taxa were identified as yeasts, microfungi, or facultative yeasts, with some associated with wood decay and other wood-saprotrophic roles. Consequently, the contribution of fungi to beneficial microbial functions is less clear. Most fungal taxa with assigned functions were

categorized as “Mixed,” indicating combined symbiotrophic and pathogenic potential (Fig. 4B). Manufacturers M2, M3, and M7 were dominated by taxa with mixed ecological roles (Fig. 4B; Suppl. material 1: table S7). Notably, M4, M5, and M6 exhibited higher proportions of genera classified as "Potentially Harmful", including taxa with opportunistic or pathogenic lifestyles, such as *Fusarium* (in M4 and M6) and *Issatchenkia* (in M5) (Suppl. material 1: fig. S3). However, these observations should be interpreted with caution, as additional approaches would be required to resolve strain-level variation and assess potential risks. Functional annotation based on amplicon data alone cannot provide this level of resolution.

Discussion

This study demonstrates that high-throughput metabarcoding can provide a comprehensive view of microbial composition in fermented grain beverages, complementing conventional culture-based methods. Clear manufacturer-specific bacterial community signatures and measurable batch effects were observed, with manufacturer identity explaining most of the variance in bacterial profiles, along with contributions from production batch and their interaction. In contrast, fungal communities clustered less distinctly by manufacturer, reflecting both their differing ecological roles in grain fermentations and the lower richness observed in the ITS2 data. These patterns underscore the ability of metabarcoding to detect both consistent and variable components of complex fermented microbial ecosystems.

Production batch consistency

Across products, bacteria known for their probiotic functions, such as *Bifidobacterium* spp., were frequently detected and often dominant, consistent with their expected presence in fermented grain beverages (Hill et al. 2014; Marco et al. 2021). Other LAB were also prevalent, demonstrating the dominance of fermentative taxa. However, community structure varied widely by producer and, in some cases, between batches from the same producer.

It was somewhat unexpected to observe substantial temporal shifts between batches for both bacteria and fungi (e.g., lactic acid and acetic acid bacteria turnover; changes in yeast dominance), considering the comparatively short span of the study (3–4 weeks between sampled batches). However, this inter-batch variation is consistent with previous metabarcoding studies of spontaneously fermented beers, which report significant differences between production batches and vintages, often linked to variation in raw materials and processing conditions (Ohwofasa et al. 2025). This indicates that lot-to-lot variability is a realistic concern.

PERMANOVA supported significant effects of manufacturer, batch, and their interaction for bacterial communities, with smaller but significant effects for fungi. This indicates that microbial composition is influenced by both production-specific and temporal factors. However, the results also indicate that, under controlled production practices, reproducibility of microbial composition is achievable even in artisanal fermentation systems. For instance, the bacterial community profiles of manufacturer M4 showed a consistent dominance of

LAB across both production batches, demonstrating reproducibility. In contrast, only M3 exhibited a consistently dominant yeast group. In both cases, observed variability may also reflect the limited taxonomic resolution achievable with microbial metabarcoding, which limits reliable comparison below the genus level, particularly for yeasts (Rué et al. 2023).

For tracking general microbial communities within manufacturers, these findings argue for (i) baseline microbial specifications (expected starter taxa and exclusion lists); (ii) routine community profiling at defined production stages (e.g., post-fermentation and pre-packaging) to quantify compositional drift; and (iii) triggers for corrective action when communities deviate from validated ranges. In addition, incorporating increased taxonomic resolution—through improved reference databases, long-read sequencing, or complementary methods—would enhance the ability to monitor consistency, align microbial content with product claims, and reduce the risk of unrecognized spoilage or safety issues (Hutkins et al. 2016; Ouwehand 2017).

Functional potential inferred from community composition and raw-material links

While DNA-based metabarcoding does not directly indicate viability or metabolic activity, community composition can provide insight into functional potential. Across products, the frequently detected LAB and *Bifidobacterium* spp. demonstrated that taxa with fermentative and potentially probiotic-associated functions are present. Functional assignments inferred from known ecological roles also identified taxa commonly associated with organic acid production, highlighting their contribution to fermentation outcomes. These observations demonstrate the capacity of high-throughput sequencing to identify taxa of interest for downstream functional and ecological studies, even without viability information, although viability would be required to fully determine probiotic benefits (Fenster et al. 2019; Ullah et al. 2019).

Several taxa flagged as having potentially undesirable properties were also detected. On the bacterial side, some products showed high relative abundance of *Clostridium* spp., a genus encompassing benign species as well as toxin producers (Cassir et al. 2016). Among fungi, *Fusarium* reached notable relative abundance in one manufacturer's dataset. The latter is noteworthy given the well-documented association of cereal substrates with *Fusarium* mycotoxins (e.g., deoxynivalenol and T-2/HT-2) (Ferrigo et al. 2016; European Food Safety Authority et al. 2017). However, since viability testing was not performed, these signals could represent the presence of *Fusarium* DNA from raw materials such as malted barley, wheat, or rye rather than active contamination. Nevertheless, these observations support the need for systematic raw-material control of incoming grain lots to complement microbiome screening.

Importantly, the functional categorization suggested low overall abundance of explicitly harmful bacteria, with a subset of "mixed-function" taxa flagging candidates for targeted follow-up. Together, these findings underscore that "usual suspect" culture panels may miss emergent or low-abundance hazards and that HTS provides an efficient first-pass screen to prioritize confirmatory testing (Bokulich and Mills 2013; Wolfe and Dutton 2015).

Methodological considerations and next steps

The comparatively lower fungal richness and weaker clustering likely reflect both biological and methodological constraints. Many fungal reads in food matrices remain unassigned beyond the phylum level or are classified to taxa with mixed or unclear ecological roles. This aligns with challenges posed by variable ITS lengths complicating denoising and merging, copy-number variation, and the under-representation of food-associated fungi in reference databases (Schoch et al. 2012; Nilsson et al. 2019). For critical interpretations, long-read amplicons (e.g., full-length ITS) could improve resolution and reduce classification drop-off (Bokulich and Mills 2013).

Several additional considerations are important when interpreting DNA metabarcoding-based food product assessments: (i) DNA signals do not establish viability or metabolic activity of detected taxa; (ii) marker biases and copy-number variation can confound absolute abundance estimates; (iii) the compositional nature of the data limits direct interpretation of observed increases or decreases without internal standards; and (iv) taxonomic assignment depends on database coverage (Gloor et al. 2017), with conserved markers generally limiting resolution below the genus level (Snipen et al. 2021). While metabarcoding effectively monitors broad community trends, batch consistency, and unexpected taxa, it is less suitable for quality frameworks requiring species- or strain-level resolution (Binda et al. 2020).

To strengthen molecular tools in the food industry, complementary approaches should be considered: targeted qPCR/dPCR for priority taxa (e.g., LAB strains or toxin-associated fungi), viability-PCR (e.g., PMA/EMA) or RNA-based assays for live/dead discrimination, and periodic high-resolution shotgun metagenomics for strain-level surveillance and functional genes (e.g., bacteriocins in *Lactiplantibacillus plantarum* or mycotoxin biosynthesis clusters) (Nocker and Camper 2009; Hill et al. 2014; Srinivas et al. 2022). Functional assignments from metagenomic data can further characterize microbial communities by identifying secondary metabolites, bacteriocin gene clusters, complex metabolic pathways, and interactions between pathways, which are inaccessible via metabarcoding alone (Srinivas et al. 2022). Because bacterial–fungal interactions can shape fermentation trajectories and downstream product characteristics, integrating multi-kingdom network analysis alongside simple process covariates (substrate lot, temperature, pH) can help link community shifts to functional and quality outcomes (Wolfe and Dutton 2015).

Conclusion

In this study, the microbial profiles of rapidly commercialized fermented beverages across seven manufacturers and two production batches were characterized. Metabarcoding provided a broad view of community composition and identified taxa linked to fermentation functions. Significant differences were observed between manufacturers and, in some cases, between batches, reflecting the influence of production practices and raw materials. Despite this variability, certain taxa were consistently dominant across batches and manufacturers, illustrating reproducibility under controlled production conditions.

To integrate high-throughput sequencing into beverage monitoring, a tiered system is proposed: (1) baseline compliance testing using standard culture assays for indicators and pathogens; (2) microbiome profiling with periodic 16S/ITS metabarcoding to track community trends, monitor batch-to-batch drift, detect out-of-panel taxa, and flag microbial groups of potential functional interest; and (3) targeted verification using strain-specific qPCR or viability assays for label-claimed probiotics and rapid LC–MS/MS screening for mycotoxins when cereal-associated fungi are detected (Ferrigo et al. 2016; European Food Safety Authority et al. 2017). Combining metabarcoding with targeted molecular and long-read or shotgun sequencing approaches provides complementary insights, enabling reproducible monitoring, detection of emergent taxa, and assessment of functional potential across production batches.

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Supplementary material 1

High-throughput microbiome profiling of fermented grain drinks: insights across manufacturers and batches: supplementary figures and tables

Authors: Michelle Scriver, Anastasija Zaiko, Rūta Gudaitytė, Aurelija Samuilovienė

Data type: docx

Explanation note: This file contains all supplementary figures and tables referenced in the manuscript, along with additional data supporting the study and underlying datasets.

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Supplementary material 2

Supplementary appendix S1 – bacterial ASVs with functions and microbial categorical classifications

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Data type: xlsx

Explanation note: The appendix includes all assigned classifications for each ASV in the bacterial 16S rRNA metabarcoding dataset based on functional assignment. Categorization was performed according to the criteria defined in the Methods. Any modifications to the classifications and verification of assignments were based on a review of the literature. The table also includes relevant references and the rationale supporting each assignment.

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Supplementary material 3

Supplementary appendix S2 – fungal ASVs with functions and microbial categorical classifications

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Data type: xlsx

Explanation note: The appendix includes all assigned classifications for each ASV in the fungal ITS2 metabarcoding dataset based on functional assignment. Categorization was performed according to the criteria defined in the Methods. Any modifications to the classifications and verification of assignments were based on a review of the literature.

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Additional information

Conflict of interest

Sequench Ltd conducted this study using anonymised samples provided by one of the authors, who is affiliated with a manufacturer included in the study. Sequench Ltd received funding from this manufacturer to complete the study. Both Sequench Ltd and the affiliated manufacturer were involved in study design and manuscript preparation. All samples were anonymised throughout the study. All authors declare no competing interests.

Ethical statement

No ethical statement was reported.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.
No AI tools were used in the preparation of this manuscript.

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Author contributions

Conceptualization and project design: AZ, RG. Sample collection and curation: RG, AS. Funding acquisition: RG. Laboratory analysis and methodology: AS, MS. Data analysis, visualization, and interpretation: MS. Writing – original draft: MS. Writing – review and editing: all authors.

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Data availability

Raw sequence reads have been submitted to the NCBI Short Read Archive under accession number PRJNA1347471. All supplementary figures, tables, and Appendices 1 and 2 are available in the supplementary files provided.