

Kombucha Microbiome as a Potential Plant Architecture-Supporting Biostimulant: Metabarcoding Characterization of Its Bacterial and Fungal Community Structure

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Abstract

Identifying novel microbial resources for sustainable agriculture requires characterization of community composition and careful definition of functional and biosafety potential. This study characterized bacterial and fungal communities in the dried cellulosic pellicle (KP) and fermentation liquid (KS) fractions of a Kombucha Microbiome previously associated with plant architecture, pigment metabolism, and redox responses in tomato. Three independent fermentations were pooled within each fraction, and composite KP and KS samples were analyzed by 16S rRNA and ITS metabarcoding, with 18S rRNA as a complementary marker. Final analyses yielded 363, 98, and 30 OTUs in the 16S, ITS, and 18S data sets, respectively. The KS bacterial community was dominated by *Komagataeibacter* (58.81%), whereas KP showed a more heterogeneous profile, with the *Burkholderia-Caballeronia-Paraburkholderia* complex as the strongest classified signal (16.30%). Fungal communities differed more markedly: KS was dominated by *Dekkera* (96.80%), while KP contained *Fusarium* (37.72%), *Aspergillus* (18.02%), *Malassezia* (9.26%), *Penicillium* (8.50%), *Filobasidium* (5.57%), *Clonostachys* (4.85%), and *Lecanicillium* (2.49%). The *Brettanomyces* signal in KS-18S supported the *Dekkera/Brettanomyces* axis, and Kraken2-Bracken cross-checking reproduced dominant taxonomic patterns. Because biological sequencing replicates and a sequenced negative control were unavailable, KP-KS differences were interpreted descriptively. Overall, the two fractions were microbiologically distinct. This inventory does not establish a proven plant growth-promoting product, but provides a taxonomic basis for fraction selection, candidate isolation, functional and colonization studies, biosafety assessment, and development of standardized microbial consortia with potential plant architecture-supporting biostimulant activity.

Highlights

- Pellicle and liquid fractions harbored distinct microbial community structures.
- *Komagataeibacter* and *Dekkera/Brettanomyces* dominated liquid profiles.
- The dried pellicle contained a broader, more heterogeneous microbial assemblage.
- LoTUS2 classifications were cross-checked with Kraken2 and Bracken.
- Plant-associated taxa support a testable architecture-supporting biostimulant hypothesis.

1. Introduction

Microorganisms that support plant development are receiving increasing attention in sustainable agricultural production because of their potential to reduce dependence on synthetic inputs, improve nutrient-use efficiency, and strengthen plant adaptation to environmental conditions. Microbial fertilizers are more commonly associated with nutrient supply and nutrient mobilization, whereas microbial biostimulants are associated with regulation of plant development, stress responses, nutrient-use efficiency, and crop quality. However, this functional distinction is not absolute, particularly in multispecies microbial consortia, because plant responses may be shaped by the combined effects of community composition, formulation, dose, application route, and environmental conditions. Therefore, assessing the suitability of a novel microbial resource for sustainable agriculture requires not only identification of growth-promoting candidates but also resolution of consortium standardization and biosafety issues.

Kombucha is a complex microbial ecosystem generated by fermentation of sweetened tea by a symbiotic community composed primarily of acetic acid bacteria and yeasts and, in some cultures, lactic acid bacteria. Hydrolysis of sucrose by yeasts with production of ethanol and other intermediate metabolites, conversion of these compounds by acetic acid bacteria into acetic, gluconic, and other organic acids, and formation of a cellulosic biofilm by certain bacterial members create a distinct metabolic division of labor within the community (Teoh et al. 2004; Chakravorty et al. 2016; Laureys et al. 2020; Arkan et al. 2020; Nam et al. 2025). The Kombucha Microbiome should therefore be regarded not simply as a culture in which different microorganisms coexist, but as a multicomponent microbe-metabolite system comprising living cells, extracellular matrix components, and metabolites generated during fermentation.

The Kombucha Microbiome is not a uniform community with a fixed composition that is reproduced across all samples. The source of the starter culture, tea or alternative plant substrate, sugar composition, temperature, oxygenation, pH, fermentation duration, and sampling time can substantially alter bacterial and fungal community structure (Marsh et al. 2014; Reva et al. 2015; Coton et al. 2017; Laureys et al. 2020; Yang et al. 2022; Andrade et al. 2025). Comparative studies of commercial and laboratory-scale samples have reported marked variation in both dominant microbial taxa and metabolite profiles among products and production processes.

Although this variability may broaden the potential of Kombucha as a multifunctional biological resource, it also raises challenges related to standardization, reproducibility, and biosafety for agricultural use. Fraction-based community profiling is therefore a first step toward converting a variable fermentation consortium into a standardizable microbial resource for sustainable agriculture.

The cellulosic pellicle fraction and the liquid fermentation phase of Kombucha do not represent the same microbial microenvironment. The pellicle is a structural matrix produced particularly by cellulose-synthesizing acetic acid bacteria, within or on which bacteria and yeasts may be retained. The liquid phase contains suspended cells together with dissolved organic acids, carbohydrate-conversion products, and other metabolites generated during fermentation. Teoh et al. (2004) showed that yeast populations in the liquid phase may change throughout fermentation, whereas populations within the pellicle may remain more stable. Subsequent sequencing studies have likewise shown that the pellicle and liquid phase may display distinct community and metabolite patterns (Arıkan et al. 2020; Lee et al. 2022; Nam et al. 2025). Thus, a metabarcoding profile obtained from a single fraction at a single time point represents the community structure of the sampled fraction at that stage of fermentation rather than the entire Kombucha fermentation ecosystem.

Several microbial and chemical characteristics justify investigation of Kombucha-derived consortia for agricultural applications. Isolation from Kombucha tea of *Acetobacter nitrogenifigens*, for which nitrogen-fixing ability has been experimentally demonstrated, and *Gluconacetobacter kombuchae*, which both fixes nitrogen and produces cellulose, indicates that some Kombucha-derived acetic acid bacteria merit investigation for potential agricultural functions (Dutta and Gachhui 2006, 2007). Kombucha fermentation is also associated with the formation of various organic acids, particularly acetic and gluconic acids, phenolic transformation products, and other bioactive compounds (Laureys et al. 2020; Wang et al. 2022; Bishop et al. 2022; da Silva Júnior et al. 2022). Antimicrobial activity under laboratory conditions has also been reported for some Kombucha cultures or fermentation products (Četojević-Simin et al. 2008; Nyiew et al. 2022). Nevertheless, most of this evidence derives from food-fermentation systems, isolates, or in vitro experiments. Detection of relevant taxa in a particular Kombucha sample does not demonstrate nitrogen fixation, nutrient solubilization, phytopathogen suppression, root colonization, or promotion of plant development. These traits remain biological possibilities that can only be tested through targeted genomic and functional studies.

In our previous tomato study evaluating the agricultural effects of the Kombucha Microbiome, the response pattern generated by the consortium differed from that of the commercial microbial fertilizer Bac-OMET. Bac-OMET was associated with greater increases in fresh and dry biomass, whereas the Kombucha Microbiome was more strongly associated with greater plant height, root and stem length, preservation of photosynthetic pigments, proline accumulation, and CAT, APX, GR, and GST activities. The strong dependence of these responses on formulation, dose, and application route, together with increases in MDA and proline at high application levels, indicated that the effects of the Kombucha Microbiome cannot be interpreted as unidirectional and unlimited growth promotion. These findings supported evaluation of this microbial system as a potential microbial biostimulant exerting context-dependent effects on plant architecture, pigment metabolism, and redox balance rather than as a conventional nutrient-supplying microbial fertilizer (Temizgul et al. 2026).

However, the previous plant experiment was not designed to determine which microbial members or consortium properties might be associated with the observed responses. It remains unknown whether changes in plant architecture and physiological indicators arose through living microorganisms, transient or persistent colonization, extracellular fermentation metabolites, the cellulosic matrix, metabolic complementarity among microbial members, or combined effects of these components. This raises a new research question: can the bacterial and fungal community composition of the Kombucha Microbiome provide biologically plausible explanations for why biostimulant responses characterized more by plant architecture and physiological regulation than by overall biomass increase were observed in tomato?

Taxonomic characterization of the local Kombucha consortium is the first step in addressing this question. Amplicon sequencing of the 16S rRNA and fungal ITS regions enables detection of bacterial and fungal community members that may not be resolved by culture-dependent approaches and allows descriptive comparison of the pellicle and liquid fractions (Reva et al. 2015; Coton et al. 2017; Arıkan et al. 2020; Nam et al. 2025). The 18S rRNA data may additionally contribute a complementary marker-based view of fungal/eukaryotic community structure. Nevertheless, marker-gene metabarcoding demonstrates taxonomic presence; it does not directly establish viability, genomic functional repertoire, gene expression, or a specific metabolic phenotype. Closely related species also cannot always be reliably separated using limited marker regions. Accordingly, metabarcoding results should be used not to assign function directly, but to identify candidate taxa and testable hypotheses that can guide subsequent genomic and functional validation.

The aim of this study was to characterize bacterial and fungal communities in the pellicle and liquid fractions of the Kombucha Microbiome using 16S rRNA and ITS metabarcoding, to evaluate 18S rRNA data as a complementary indicator of fungal community structure, to identify dominant and shared taxa as well as taxa showing different relative representation patterns between fractions, and to interpret the resulting taxonomic structure together with the plant architecture, pigment, and redox responses observed in the previous tomato study. The study was not intended to establish a definitive mechanism or causal relationship for those plant responses. Instead, by identifying microbial groups that may be biologically compatible with the previous phenotypic observations, it aimed to provide a taxonomic basis for evaluating the Kombucha Microbiome as a candidate biostimulant system with plant architecture-supporting potential. Fraction-specific profiling was also intended to distinguish potentially beneficial taxa from signals requiring biosafety validation and thereby establish a research framework for future isolate genomics, targeted functional-gene analyses, metabolomics, culture-based experiments, plant-colonization studies, and safe-consortium design for sustainable agriculture.

2. Materials and Methods

2.1. Study design and sample definition

This study was conducted to descriptively characterize the bacterial and fungal community structure of the liquid and cellulosic pellicle fractions of a local Kombucha Microbiome previously evaluated as a biostimulant candidate in tomato. The fermentation liquid was coded as KS, whereas the cellulosic pellicle formed at the liquid-air interface and subsequently dried was coded as KP. KP was not a cell pellet obtained by centrifugation of the liquid culture, but rather dried biofilm material formed during Kombucha fermentation.

Fermentation was performed in three independent replicates. After fermentation was completed, composite samples were prepared by combining equal volumes of the KS fractions from the three replicates and equal dry weights of the dried KP fractions from the three replicates. Thus, one composite sample representing KP and one representing KS were obtained for each marker region. Because the replicates were pooled before sequencing and were not sequenced as separate libraries, the sequencing data were not analytically treated as $n = 3$; KP-KS comparisons were kept descriptive, and biological-replicate-based alpha diversity, beta diversity, PERMANOVA, or differential-abundance tests were not applied.

The V3-V4 region of the 16S rRNA gene was used for bacterial community profiling, and the internal transcribed spacer (ITS) region was used for fungal community profiling. The 18S rRNA amplicon was evaluated as a complementary eukaryotic marker. *Candida*-targeted PCR using Cand-F/ITS-R was treated as a separate validation screen from broad fungal-community metabarcoding and was not included in community-abundance calculations.

2.2. Development of the stock culture and preparation of the preculture

The Kombucha culture used in previous laboratory studies was used as the stock culture. Development and maintenance of the stock culture were carried out in YPED Broth containing 1% yeast extract, 2% glucose, and 2% peptone, adjusted to pH 4.0. The medium was inoculated with 10% (v/v) stock culture in a sterile cabinet, homogenized after inoculation, and incubated statically at 24°C for 10 days (Temizgul et al. 2026).

2.3. Preparation of the black-tea substrate, fermentation, and sampling

For the fermentation substrate, 10 g of black tea was added to 1 L of boiling water and extracted at 100°C for 15 min. Tea particles were removed using sterile coarse filter paper, and sucrose was added to the filtrate at 70 g L⁻¹ and completely dissolved. The prepared substrate was cooled to 23–27°C before inoculation. Following the 100°C/15 min treatment, the tea substrate was not additionally autoclaved or subjected to another sterilization procedure (Tarhan 2017).

Fermentation was carried out in wide-mouth 5-L glass jars that had been sterilized previously by autoclaving. The cooled sucrose-supplemented black-tea medium was inoculated with 10% (v/v) preculture in a sterile cabinet, and cultures were allowed to ferment statically at 24°C for 10 days.

At the end of the 10-day fermentation, samples were collected under aseptic conditions. The liquid phase constituted the KS sample and was stored at +4°C until DNA isolation. The cellulosic pellicle formed on the surface was separated from the liquid phase, dried

for seven days in an oven set to 30°C, and used as the KP sample. Before sequencing, fraction-specific composite samples were prepared, as described above, by combining equal volumes of KS and equal dry weights of KP from the three independent replicates.

2.4. Isolation and quality assessment of total community DNA

Total community DNA was isolated from the KP and KS fractions using a NucleoGene Genomic DNA Extraction Kit (Cat. No. NGE001; NucleoGene, Istanbul, Türkiye). For the KS fraction, 2 mL of fermented liquid culture was centrifuged at 5000 × g for 10 min, the supernatant was removed, and the cell pellet was resuspended in 100 µL TE Buffer. A total of 400 µL Lysis Buffer and 20 µL Proteinase K were added to the suspension, and samples were incubated at 60°C for approximately 1 h in a shaking incubator. Subsequently, 400 µL Binding Buffer was added, the mixture was transferred to columns, and the columns were centrifuged at 10,000 × g for 2 min. After washing steps were completed according to the manufacturer's protocol, DNA was eluted using Elution Buffer by centrifugation at 10,000 × g for 1 min.

For DNA isolation from the KP fraction, 50 mg of dried cellulosic pellicle from each composite sample was used. The material was transferred to tubes containing homogenization beads and 400 µL Lysis Buffer was added. Samples were mechanically homogenized at maximum speed for 5 min, the resulting liquid phase was transferred to new microcentrifuge tubes, and the isolation procedure was continued from the addition of 20 µL Proteinase K using the incubation, binding, column purification, washing, and elution steps described for the KS fraction. Isolated DNA samples were stored at -20°C.

DNA concentration and purity were evaluated spectrophotometrically by measuring absorbance at 260 and 280 nm to determine the A260/A280 ratio. DNA samples of suitable quality and quantity were used for in-house PCR amplification controls and amplicon sequencing.

2.5. PCR amplification control and *Candida*-targeted screening

Laboratory PCR analyses were used to verify that the DNA samples were amplifiable for the target marker regions and to perform a separate *Candida*-targeted screen. These PCR products were not used as sequencing libraries; isolated total DNA samples were sent to the sequencing service provider (Genoks, Ankara, Türkiye).

For the 16S rRNA V3-V4 region, the forward primer 5'-CCTACGGGNGGCWGCAG-3' and reverse primer 5'-GACTACHVGGGTATCTAATCC-3' were used. Broad fungal ITS screening used ITS1F 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS2R 5'-GCTGCGTTCTTCATCGATGC-3'. For 18S rRNA, the forward primer 5'-AACCTGGTTGATCCTGCCAGT-3' and reverse primer 5'-TGATCCTTCTGCAGGTTACCTAC-3' were used. The separate *Candida*-targeted confirmation PCR used the Cand-F 5'-CCTGTTTGAGCGTCRTTT-3' and ITS-R 5'-TCCTCCGCTTATTGATAT-3' primer pair.

PCR reactions were prepared in a total volume of 25 µL with a total of 1 ng template DNA per reaction. Each reaction contained 2.5 µL 10× PCR buffer, 2.0 µL 25 mM MgCl₂, 1.0 µL 10 mM dNTP mixture, 1.0 µL 10 µM forward primer, 1.0 µL 10 µM reverse primer, and 0.1 µL DNA polymerase (5 U µL⁻¹). DNA volume was adjusted according to stock concentration, and the final volume was brought to 25 µL with nuclease-free water.

Amplification began with an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 53°C for 40 s, and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. PCR products were electrophoresed on 1% agarose gels at 120 V for 2 h. Genesta 100 bp and Thermo Scientific GeneRuler 1 kb DNA markers were used to estimate approximate band sizes. Gels were stained with ethidium bromide and visualized (Arıkan 2018).

2.6. Amplicon library preparation and sequencing

Independently of the in-house PCR control, total DNA obtained from the KP and KS composite samples was sent to the sequencing service provider for amplicon-library preparation. Six libraries were generated for the 16S, ITS, and 18S targets: KP-16S, KS-16S, KP-ITS, KS-ITS, KP-18S, and KS-18S. Library preparation, indexing, and sequencing were performed by the sequencing service provider. Sequencing was conducted on the DNBSEQ-T7 platform using 2 × 150 bp paired-end reads. Primer/adaptor sequences were trimmed by the provider during initial processing, and subsequent bioinformatic analyses were performed using these preprocessed reads.

2.7. Primary bioinformatic processing and OTU generation

The 16S, 18S, and ITS data sets were processed separately by marker using the LoTUS2 v2.34.1 pipeline. Because reads trimmed for primer/adaptor sequences by the provider were used, primer rejection was disabled in the LoTUS2 sdm configuration. Reads passing

quality filtering were clustered into OTUs with VSEARCH v2.29.1 at a 97% sequence-identity threshold; ASV or zOTU approaches were not used in this study (Özkurt et al. 2022; Rognes et al. 2016).

Chimera checking was performed using a VSEARCH/UCHIME-based approach, co-occurrence-based OTU optimization was carried out with LULU, and potential phiX-derived sequences were filtered with minimap2 v2.28. ITSx v1.1.3 was used in the ITS data set to define target-region boundaries and remove off-target amplicons. The final workflow generated representative OTU sequences, OTU abundance tables, taxonomy tables, and phylogenetic trees in Newick format for each marker (Rognes et al. 2016; Frøslev et al. 2017; Li 2018; Bengtsson-Palme et al. 2013).

2.8. Taxonomic assignment and contamination assessment

Taxonomic classification of representative OTU sequences was performed using a BLAST+ v2.16.0+-based lowest common ancestor (LCA) approach. The 16S and 18S data were evaluated against the SILVA SSU 138.1 reference database, whereas ITS data were evaluated against the UNITE v9 fungal reference database (release 25 July 2023). Taxonomic assignments were reported from phylum to species level, but species-level results were interpreted cautiously because of the limited discriminatory power of short marker regions (Camacho et al. 2009; Quast et al. 2013; Nilsson et al. 2019).

Because a true negative extraction/PCR control was not sequenced in the same run, blank-based read subtraction could not be performed. Instead, genera previously associated with reagent/kit-derived contamination were flagged on the basis of the approach of Salter et al. (2014), but were not automatically removed from the data set. These flags were used as quality-control warnings during biological interpretation.

Selected high-abundance but unclassified 16S OTUs from the SILVA-based LoTUS2 analysis were additionally checked manually using web BLAST against the NCBI nt database. This spot-check was performed to evaluate possible close references for representative sequences and classification gaps related to reference-database coverage; the original taxonomic labels in the primary LoTUS2 OTU table were not retrospectively modified.

2.9. Independent algorithmic Kraken2-Bracken cross-check

An independent algorithmic cross-check pipeline was applied to assess the taxonomic reproducibility of the primary LoTUS2 results. Paired-end reads were merged using FLASH v1.2.11, classified with Kraken2 v2.17.1, and subjected to genus-level abundance estimation with Bracken v3.1p1. Kraken2 databases were constructed using SILVA SSU 138.1 reference content for 16S/18S and UNITE v9 reference content for ITS. LoTUS2 and Kraken2-Bracken results were compared particularly with respect to the presence of dominant genera and their relative representation patterns. This approach was not a biologically independent validation, but rather a re-evaluation of the same sequence data using different classification algorithms (Magoč and Salzberg 2011; Wood et al. 2019; Lu et al. 2017).

The 18S data set was treated as a complementary marker in this study. Because the KP-18S library showed marked disagreement in taxonomic resolution between LoTUS2 and Kraken2-Bracken and many reads could not be reliably resolved at genus level, 18S was not used as the primary fungal-community profile; KS-18S was evaluated only as complementary support for the main fungal signals obtained with ITS.

2.10. Phylogenetic analysis and tree visualization

Representative OTU sequences for each marker were subjected to multiple sequence alignment with MAFFT v7.471, and phylogenetic trees were constructed using FastTree2 v2.1.11. OTU identities in the Newick trees provided by LoTUS2 were matched with the abundance and taxonomy tables, while terminal OTU identifiers were retained (Kato and Standley 2013; Price et al. 2010).

For the 16S and ITS trees presented in the main text, relative abundance within KP and KS was first calculated separately for each OTU to prevent differences in library depth from biasing OTU selection toward a single fraction. The arithmetic mean of these two relative abundances $[(KP \% + KS \%)/2]$ was then calculated for each OTU. The 50 OTUs with the highest mean relative abundance for 16S and the 30 highest for ITS were used in the main phylogenetic visualizations. Subtrees were pruned from the complete FastTree2 trees; phylogenetic topology was not re-estimated. Complete trees containing all 16S, ITS, and 18S OTUs were provided in the Supplementary Material. Labels in the main trees displayed the OTU identifier, genus assignment, and mean relative abundance across the two fractions.

For visualization, OTUs were also descriptively classified according to fraction dominance. An OTU was marked as KP-dominant if its relative abundance in KP was more than 1.5 times its value in KS, and as KS-dominant if its relative abundance in KS was more than 1.5 times its value in KP; all remaining OTUs were displayed as shared/balanced. This color coding was not a statistical classification and was used solely for descriptive visualization.

2.11. Relative-abundance calculations and data-interpretation approach

For each marker and sample, relative abundance was calculated by dividing the final read count assigned to the relevant OTU by the total final OTU read count in that sample and multiplying by 100. The summary abundance value used for OTU selection and labeling in the main phylogenetic trees was the arithmetic mean of the relative abundances calculated separately for KP and KS. This prevented differences in library depth from directly weighting the selection of top OTUs.

Because the replicates were pooled before sequencing, no inferential statistics were applied between KP and KS. Results were evaluated descriptively by jointly considering the final OTU tables, within-fraction relative abundances, taxonomic assignments, phylogenetic placements, contamination warnings, and the independent algorithmic Kraken2-Bracken cross-check. Marker-gene metabarcoding was interpreted as evidence of a DNA signal from a taxon, but not as direct proof of microbial viability, metabolic activity, root colonization, pathogenicity, or a specific biostimulant function. Therefore, interpretations of agricultural functions were treated as hypotheses requiring testing in subsequent culture-based, genomic, metabolomic, and plant experiments rather than as validated mechanisms.

The fraction-based experimental sampling, amplicon sequencing, primary LoTUS2 analysis, and Kraken2-Bracken algorithmic cross-check steps of the study are summarized in Fig. 1. All supplementary tables and figures are provided in Supplementary materials.

2.12. Use of artificial intelligence-assisted tools in manuscript preparation

Generative artificial intelligence tools were used only to assist with the visual design and refinement of the graphical abstract and workflow diagram. All scientific content, labels, relationships, and graphical elements were reviewed and verified by the authors for accuracy.

3. Results

3.1. Sequencing outputs and final OTU recovery

The 16S, ITS, and 18S amplicon-sequencing analyses were performed separately for the KP (dried cellulosic pellicle) and KS (fermentation liquid) fractions. Following quality control and LoTUS2 processing, 363 OTUs and a total of 847,748 reads were retained in the final 16S OTU table, 98 OTUs and 1,334,902 reads in the ITS data set, and 30 OTUs and 532,697 reads in the 18S data set.

In the 16S data set, 320,487 and 527,261 final reads were obtained from the KP and KS libraries, respectively. Corresponding values in the ITS data set were 618,666 for KP and 716,236 for KS, while the 18S data set contained 111,326 and 421,371 reads, respectively (Table 1; Fig. 2).

Table 1
Sequencing output, read retention and final OTU summary

Marker	Sample	Raw read pairs	Adapter-trimmed pairs	LoTUS2 QC accepted	Final OTU-table reads	Final/Raw (%)	Final OTU count	Unclassified OTUs	Flagged OTUs	Flagged reads (%)
16S	KP	1,022,108	982,817	852,425	320,487	31.36	363	89	56	6.95
16S	KS	1,559,325	1,496,179	1,313,288	527,261	33.81	363	89	56	6.95
ITS	KP	1,514,979	1,454,098	1,055,942	618,666	40.84	98	0	0	0
ITS	KS	1,158,418	1,109,652	873,231	716,236	61.83	98	0	0	0
18S	KP	269,369	229,901	171,154	111,326	41.33	30	4	0	0
18S	KS	633,241	589,035	495,108	421,371	66.54	30	4	0	0
Note. KP, dried cellulosic pellicle fraction; KS, fermentation liquid fraction; OTU, operational taxonomic unit. Percentages are calculated relative to raw read pairs. Potential-contaminant flags were applied only to the 16S dataset; no sequenced negative control was available.										

During quality control, 89 of the 363 OTUs in the 16S data set could not be classified at genus level. Although these OTUs represented 24.5% of the total OTU count, they accounted for only approximately 6.9% of the total read pool. In the provider's screen for possible reagent/kit contamination, 56 OTUs in the 16S data set, totaling 58,938 reads (6.95%), were flagged as potentially contamination-sensitive signals. These reads were distributed as 48,918 reads (15.26%) in KP and 10,020 reads (1.90%) in KS. Because no sequenced negative control was available, these OTUs were not automatically removed from the data set and were instead evaluated separately in subsequent interpretation.

All 98 OTUs in the ITS data set were taxonomically classifiable, and no ITS OTU was flagged as a potential reagent contaminant in the provider's QC screen. During ITSx control, 58 OTUs and 198,432 reads identified as off-target were excluded from the analysis. Similarly, only four of the 30 OTUs in the 18S data set remained unclassified, containing a total of only 41 reads. No OTU in the 18S data set was flagged as a potential reagent contaminant. Detailed quality-control outputs are provided in Supplementary Tables S4 and S9.

3.2. Bacterial community structure based on 16S rRNA analysis

The 16S data revealed distinctly different bacterial-composition patterns between the KP and KS fractions. In KS, the most dominant genus was *Komagataeibacter*, represented by 310,100 reads and accounting for 58.81% of the total 16S reads. This was followed by *Neisseria* (15.25%), the *Burkholderia-Caballeronia-Paraburkholderia* complex (4.10%), *Haemophilus* (3.25%), *Gemella* (3.08%), and *Acetobacter* (1.77%). In addition, 6.18% of KS reads consisted of sequences that could not be definitively resolved at genus level (?) and 3.52% were assigned to the unclassified category. This overall composition pattern is summarized in Fig. 3 and Table 2.

Table 2
Dominant bacterial genera in KP and KS and Kraken2-Bracken algorithmic cross-check

Genus	KP LoTUS2 reads	KP LoTUS2 (%)	KP Bracken (%)	KS LoTUS2 reads	KS LoTUS2 (%)	KS Bracken (%)	QC status	Interpretive status
<i>Komagataeibacter</i>	25,949	8.10	6.00	310,100	58.81	71.69	Not flagged	Main/secondary result
<i>Burkholderia- Caballeronia- Paraburkholderia</i>	52,231	16.30	18.97	21,617	4.10	1.67	Not flagged	Main/secondary result
<i>Neisseria</i>	4,332	1.35	0.52	80,431	15.25	7.85	Not flagged	Cautious
<i>Staphylococcus</i>	19,791	6.18	3.52	350	0.07	0.08	Not flagged	Cautious
<i>Asaia</i>	18,725	5.84	5.63	1,938	0.37	0.10	Not flagged	Main/secondary result
<i>Escherichia-Shigella</i>	17,319	5.40	5.61	3,747	0.71	0.75	Not flagged	Cautious
<i>Haemophilus</i>	2,634	0.82	1.31	17,135	3.25	3.71	Not flagged	Cautious
<i>Gemella</i>	695	0.22	0.16	16,249	3.08	3.47	Not flagged	Cautious
<i>Methylobacterium- Methylorubrum</i>	7,137	2.23	2.02	1,448	0.27	0.12	Not flagged	Cautious
<i>Acetobacter</i>	6,194	1.93	8.27	9,354	1.77	1.03	Not flagged	Main/secondary result
<i>Delftia</i>	13,487	4.21	0.35	2,761	0.52	0.01	Flagged	QC-only
<i>Kocuria</i>	10,857	3.39	1.33	81	0.02	0	Flagged	QC-only
<i>Cutibacterium</i>	4,157	1.30	0.49	10	0.00	0.00	Flagged	QC-only
Note. KP, dried cellulosic pellicle fraction; KS, fermentation liquid fraction; OTU, operational taxonomic unit. Bracken values are an algorithmic cross-check of genus-level classification and do not constitute biological validation. QC-only taxa were retained but interpreted cautiously.								

The KP fraction exhibited a more heterogeneous bacterial profile. The strongest classified signal belonged to the *Burkholderia-Caballeronia-Paraburkholderia* complex, with 52,231 reads and a relative abundance of 16.30%. This was followed by unclassified sequences (12.59%), sequences unresolved at genus level (11.36%), *Komagataeibacter* (8.10%), *Staphylococcus* (6.18%), *Asaia* (5.84%), the *Escherichia-Shigella* complex (5.40%), *Delftia* (4.21%), *Kocuria* (3.39%), *Methylobacterium-Methylorubrum* (2.23%), and *Acetobacter* (1.93%).

However, because some 16S taxa observed in KP were flagged in the contamination-QC analysis as potential reagent- or laboratory-derived signals, these groups were not directly interpreted as biological community members. OTUs associated particularly with *Delftia*, *Kocuria*, *Cutibacterium*, *Streptococcus*, *Acinetobacter*, *Micrococcus*, *Sphingomonas*, and *Enhydrobacter* were evaluated in this context. Details of flagged OTUs are provided in Supplementary Table S5 and Supplementary Fig. S2. The main 16S phylogenetic placement, based on the 50 OTUs with the highest total abundance when KP and KS were considered together, is shown in Fig. 4; the tree containing all 363 OTUs is provided in Supplementary Fig. S5.

3.3. Fungal community structure based on ITS analysis

ITS analysis revealed a more pronounced separation in fungal composition between KP and KS than was observed for the bacterial profiles. The KS fraction was largely dominated by *Dekkera*. A total of 693,347 reads assigned to this genus accounted for 96.80% of KS ITS reads. *Fusarium* (2.07%), *Malassezia* (0.91%), and *Filobasidium* (0.06%) were detected at lower proportions in KS, while each of the remaining fungal genera was present at or below approximately 0.1%. This distribution is summarized in Fig. 5 and Table 3.

Table 3
Dominant fungal genera in KP and KS and Kraken2-Bracken algorithmic cross-check

Genus	KP LoTUS2 reads	KP LoTUS2 (%)	KP Bracken (%)	KS LoTUS2 reads	KS LoTUS2 (%)	KS Bracken (%)	Interpretive status
<i>Dekkera</i>	1,948	0.31	0.22	693,347	96.80	89.02	Main result
<i>Fusarium</i>	233,348	37.72	31.81	14,858	2.07	0.96	Main result
<i>Aspergillus</i>	111,493	18.02	13.71	121	0.02	0.01	Main result
<i>Malassezia</i>	57,258	9.26	10.76	6,497	0.91	1.55	Cautious secondary result
<i>Penicillium</i>	52,558	8.50	13.12	28	0.00	0.01	Main result
<i>Filobasidium</i>	34,447	5.57	10.17	434	0.06	0.09	Secondary result
<i>Clonostachys</i>	30,008	4.85	2.62	2	0.00	0	Secondary result
<i>Lecanicillium</i>	15,429	2.49	4.12	83	0.01	0	Cautious secondary result
Note. KP, dried cellulosic pellicle fraction; KS, fermentation liquid fraction; OTU, operational taxonomic unit. Bracken values are an algorithmic cross-check of genus-level classification and do not constitute biological validation.							

The KP fraction exhibited a more heterogeneous fungal composition than KS. *Fusarium* showed the highest relative abundance, with 233,348 reads and 37.72%. This was followed by *Aspergillus* (18.02%), *Malassezia* (9.26%), fungal sequences unresolved at genus level (9.23%), *Penicillium* (8.50%), *Filobasidium* (5.57%), *Clonostachys* (4.85%), and *Lecanicillium* (2.49%). *Dekkera* represented only 0.32% of the KP community.

Thus, the ITS data showed that the liquid fraction was characterized predominantly by *Dekkera*, whereas the cellulosic pellicle contained a broader group of fungal taxa including *Fusarium*, *Aspergillus*, *Penicillium*, *Malassezia*, *Filobasidium*, *Clonostachys*, and *Lecanicillium*. The dominant ITS composition pattern is summarized in Fig. 5 and Table 3. Phylogenetic relationships among the 30 ITS OTUs with the highest total abundance when KP and KS were considered together are shown in Fig. 6, while the tree containing all 98 OTUs is provided in Supplementary Fig. S6.

3.4. Evaluation of 18S rRNA data with respect to the fungal community

The 18S rRNA analysis supported the dominant yeast signal in the KS fraction using an independent marker. In KS, 208,030 reads assigned to the genus *Brettanomyces* accounted for 49.37% of the total final 18S reads. Most of the remaining reads consisted of sequences unresolved at genus level (?) (50.63%).

In the KP-18S library, 111,039 of 111,326 reads (99.74%) could not be definitively resolved at genus level, and only 261 reads (0.23%) were classified as *Brettanomyces*. Therefore, KP-18S data were not used to define the quantitative composition of the fungal community. In contrast, the KS-18S result provided independent marker-level support for the dominant *Dekkera/Brettanomyces* axis detected by ITS analysis. The 18S composition is shown in Supplementary Fig. S1, and the phylogenetic tree containing all 30 OTUs is presented in Supplementary Fig. S7. The cross-marker comparison of the *Dekkera/Brettanomyces* axis is summarized in Table 4.

Table 4
Cross-marker support for the dominant *Dekkera*/*Brettanomyces* signal

Marker	Taxon	Sample	LoTUS2 reads	LoTUS2 (%)	Bracken estimated reads	Bracken reported (%)
ITS	<i>Dekkera</i>	KS	693,347	96.80	904,569	89.02
ITS	<i>Dekkera</i>	KP	1,948	0.31	2,952	0.22
18S	<i>Brettanomyces</i>	KS	208,030	49.37	252,288	85.30
18S	<i>Brettanomyces</i>	KP	261	0.23	824	1.01
Note. KP, dried cellulosic pellicle fraction; KS, fermentation liquid fraction; OTU, operational taxonomic unit. <i>Dekkera</i> (ITS) and <i>Brettanomyces</i> (18S) represent the same yeast lineage at different marker assignments. Bracken percentages are reported as provided by the cross-check output and should not be interpreted as directly comparable across markers.						

3.5. *Candida*-targeted PCR screening

In the targeted gradient-PCR screen performed with the Cand-F/ITS-R primer pair, distinct amplification products of approximately 100–200 bp were observed in both KP and KS DNA samples at multiple annealing temperatures. Although band intensities varied according to gradient conditions, an amplifiable DNA signal was obtained with the target primer pair in both fractions (Supplementary Fig. S8).

This targeted PCR finding was evaluated as a complementary screen separate from the broad ITS metabarcoding profile. Because the PCR products were not independently sequenced within the scope of this study, bands observed on the gel were not interpreted as definitive taxonomic confirmation of the genus *Candida* or of any *Candida* species.

3.6. Independent algorithmic cross-check with Kraken2-Bracken

LoTUS2-based taxonomic profiles were compared at genus level using an independent Kraken2-Bracken workflow. Although absolute read values were not directly equivalent between the two methods, the identities of dominant taxa and the main distribution patterns between fractions were generally consistent. The overall cross-check pattern is shown in Fig. 7.

In the 16S-KS data set, Bracken again identified *Komagataeibacter* as the dominant genus, at 71.69%. *Neisseria* (7.85%), *Haemophilus* (3.71%), and *Gemella* (3.47%) were also detected in agreement with the LoTUS2 results. In KP, the *Burkholderia-Caballeronia-Paraburkholderia* complex was again one of the most prominent bacterial groups in the Bracken analysis (18.97%); *Acetobacter* (8.27%), *Komagataeibacter* (6.01%), *Asaia* (5.63%), and *Escherichia-Shigella* (5.61%) were likewise recovered by the independent classification.

In the ITS-KS analysis, Bracken confirmed *Dekkera* as by far the dominant genus, at 89.02%. In KP, *Fusarium* (31.81%), *Aspergillus* (13.71%), *Penicillium* (13.12%), *Malassezia* (10.76%), *Filobasidium* (10.17%), *Lecanicillium* (4.12%), and *Clonostachys* (2.63%) were recovered. Thus, most of the principal fungal taxa in KP were jointly supported by the two independent classification approaches.

In the 18S-KS Bracken analysis, *Brettanomyces* accounted for 85.30% of reads that could be resolved at genus level, whereas this proportion was only 1.01% in KP. However, the high representation in the KP Bracken output of *Ulnaria*, *Myrionecta*, *Platyophryides*, and other non-fungal or target-community-incongruent eukaryotic assignments supported the decision not to use KP-18S data for quantitative evaluation of fungal community structure. Detailed cross-check results are provided in Supplementary Figs. S3-S4 and Supplementary Tables S6-S8.

4. Discussion

4.1. Final metabarcoding profile and fraction-specific community structure

The final service-provider analyses in this study showed that the bacterial and fungal components of the Kombucha system were clearly differentiated according to both marker and sampled fraction. After quality filtering and LoTUS2 processing, 363 OTUs were retained in the 16S data set, 98 OTUs in the ITS data set, and 30 OTUs in the 18S data set. In the bacterial community, strong dominance of the KS fraction by *Komagataeibacter* (58.81%), compared with the joint representation of the *Burkholderia-Caballeronia-Paraburkholderia* complex, *Komagataeibacter*, *Asaia*, and several other groups in KP, indicates that the pellicle and fermentation liquid

are not interchangeable samples of the same community (Table 2; Fig. 4). Likewise, in the fungal community, the near-exclusive dominance of KS by *Dekkera* (96.80%), contrasted with the co-occurrence in KP of numerous genera including *Fusarium*, *Aspergillus*, *Malassezia*, *Penicillium*, *Filobasidium*, *Clonostachys*, and *Lecanicillium*, demonstrates that fractionation is even more pronounced for fungal structure (Table 3; Fig. 6).

This fraction-specific pattern is consistent with the view in the Kombucha literature that the pellicle and liquid phase constitute distinct microbial niches. Marsh et al. (2014), Reva et al. (2015), Coton et al. (2017), Arıkan et al. (2020), Lee et al. (2022), and Nam et al. (2025) showed that bacterial and fungal composition may vary with starter, substrate, and sampling fraction. Lee et al. (2022) additionally reported that using pellicle or culture liquid as the starter can alter dominant bacteria and metabolite trajectories. Accordingly, the KP-KS separation observed here is not merely a technical sampling difference; it suggests that the fermentation ecosystem may be ecologically partitioned between the biofilm and liquid phase. Nevertheless, because the three fermentation replicates were pooled to create a single composite library per marker, this distinction is descriptive; differences between KP and KS were not statistically tested using independent biological replicates.

4.2. Bacterial core: the *Komagataeibacter* axis, pellicle heterogeneity, and QC sensitivity

The dominance of *Komagataeibacter* in KS strongly agrees with previous studies identifying this genus as one of the most reproducible bacterial cores of Kombucha. *Komagataeibacter* species have been associated with acetic acid metabolism and bacterial cellulose formation and have been reported as dominant or core members in different Kombucha systems (Marsh et al. 2014; Arıkan et al. 2020; Nam et al. 2025). In the present study, independent Kraken2-Bracken classification again identified *Komagataeibacter* as the dominant genus in KS (71.69%), supporting the algorithm-independent taxonomic reproducibility of the LoTUS2 result. In addition, independent NCBI nt BLAST checking of high-abundance OTU11, which remained unclassified in LoTUS2, showed a complete match with *Komagataeibacter*, further supporting this core signal in KS. However, a BLAST match does not demonstrate organismal viability or metabolic activity.

The lower relative abundance of *Komagataeibacter* in KP than in KS does not exclude an important role for this genus in pellicle formation. Relative amplicon abundance is not directly equivalent to the amount of cellulose previously synthesized by a taxon or to its historical contribution to biofilm formation. DNA-extraction efficiency, marker copy number, distribution of cells within the biofilm, and the community structure of the dried pellicle at the time of sampling may all influence the observed relative abundances. Therefore, the 8.10% representation of *Komagataeibacter* in KP should not be interpreted as low functional importance for the cellulose matrix. The combined use of isolation, genomic confirmation, and metabolite measurement for *Komagataeibacter saccharivorans* by Jang et al. (2026) provides a useful example of the evidence chain required to move from taxon detection to actual functional conclusions.

In contrast, the KP 16S profile requires more cautious interpretation with respect to quality control as well as biological heterogeneity. In the provider's QC workflow, 56 OTUs in the 16S data set, representing 6.95% of total reads, were flagged for potential reagent/kit contamination; the corresponding burden was 15.26% in KP and 1.90% in KS. Consequently, some signals associated with *Delftia*, *Kocuria*, *Cutibacterium*, *Acinetobacter*, *Micrococcus*, *Sphingomonas*, and *Enhydrobacter* were excluded from the main biological interpretation. In particular, the complete match of LoTUS2-unclassified OTU193 to *Cutibacterium acnes* in the independent BLAST check clearly demonstrates that high taxonomic similarity does not establish a biological origin in a data set lacking a negative control. Flagging potential contaminants rather than automatically deleting them allowed these signals to be separated from biological claims and supported a more conservative interpretation. This approach is consistent with recommendations showing that reagent and laboratory contamination may affect sequence-based microbiome studies, particularly in low-biomass samples (Salter et al. 2014).

4.3. Fungal core: *Dekkera/Brettanomyces* dominance in KS and the broad fungal reservoir in KP

The most pronounced fungal-community result was the 96.80% dominance of KS by *Dekkera*. This structure was confirmed by the independent Kraken2-Bracken analysis, in which *Dekkera* remained dominant at 89.02%. Moreover, the *Brettanomyces* signal in the 18S-KS library, representing 49.37% in LoTUS2 and approximately 45% of the Bracken input pool, showed that the same yeast axis was supported by a second marker (Table 4). The *Dekkera/Brettanomyces* group is among the yeasts frequently reported in Kombucha fermentation and has been associated with sugar conversion, ethanol production, and metabolic cross-feeding with acetic acid

bacteria (Teoh et al. 2004; Marsh et al. 2014; Coton et al. 2017). The strong yeast dominance in KS therefore indicates that the local culture studied here contains a core structure consistent with classical Kombucha fermentation ecology.

The fungal structure of KP differed markedly from that of KS. *Fusarium* (37.72%), *Aspergillus* (18.02%), *Malassezia* (9.26%), *Penicillium* (8.50%), *Filobasidium* (5.57%), *Clonostachys* (4.85%), and *Lecanicillium* (2.49%) together formed a more heterogeneous profile. Recovery of most of these taxa in the Bracken analysis indicates that the signal was not dependent on a single classification algorithm. However, this finding should not be generalized as evidence that the pellicle is rich in beneficial fungi. In particular, the genus *Fusarium* includes many pathogenic and non-pathogenic species and lineages; an ITS-level genus assignment does not show that the detected sequences belong to a tomato pathogen or represent viable infective propagules. Similarly, genus-level taxonomic presence of *Aspergillus*, *Penicillium*, or *Malassezia* is insufficient for species- or strain-level conclusions regarding safety or function. If agricultural use is intended, culture-based isolation, species confirmation, toxigenicity/pathogenicity screening, and viability assessment of these groups will be essential.

Although *Candida* did not emerge as a dominant or high-confidence LoTUS2 genus signal in the broad ITS metabarcoding profile, targeted gradient PCR with Cand-F/ITS-R primers produced amplification products of approximately 100–200 bp in both KP and KS DNA (Supplementary Fig. S8). One possible explanation for this difference is that targeted PCR may more readily detect low-abundance targets or targets preferentially enriched by the primers than broad community metabarcoding. However, because the PCR products were not sequenced, band presence alone does not constitute genus- or species-level confirmation of *Candida*. The *Candida*-associated signal should therefore be regarded as a complementary finding requiring future validation by Sanger sequencing of the target amplicons, species-specific qPCR, and, where possible, culture isolation.

Some lower-abundance fungal groups detected in KP are noteworthy for agricultural hypothesis generation. *Clonostachys rosea* has been shown to support root development in tomato through auxin production (Han et al. 2022); *Penicillium olsonii* TLL1 has been reported to solubilize phosphate and support root development under phosphate deficiency (Suraby et al. 2023); and *Lecanicillium saksenae* has been reported to exhibit endophytic growth, plant growth promotion, and various PGP traits in rice (Naveena et al. 2025). *Filobasidium wieringae* has also shown biocontrol potential against *Alternaria porri* in onion (Abo-Elyousr et al. 2024). However, these publications concern specific species and strains. The presence in our data of ITS signals belonging only to the same genera does not demonstrate that these functions occur in our Kombucha sample; these taxa can only be prioritized as candidates for isolation and functional testing.

4.4. Relationship to previous tomato responses: the 'plant architecture-supporting' biostimulant hypothesis

The agricultural relevance of this metabarcoding study is better understood in the context of the phenotypic findings from the previous tomato study conducted with the same Kombucha Microbiome. In that study, the Kombucha Microbiome was associated less with producing the highest fresh and dry biomass than the commercial microbial fertilizer and more with plant height, root and stem length, photosynthetic pigments, and redox responses such as CAT, APX, GR, and GST; the effects were shown to depend on dose, formulation, and application route (Temizgul et al. 2026). This pattern supports positioning the Kombucha Microbiome not as a conventional, unidirectional 'fertilizer-like' growth enhancer, but as a context-dependent microbial biostimulant candidate that may contribute to plant architecture and physiological adjustment.

Although the present taxonomic results do not provide a direct mechanism explaining this phenotype, they define several testable biological axes. The association of *Komagataeibacter* and other acetic acid bacteria with organic-acid production, the contribution of the dominant *Dekkera/Brettanomyces* yeast axis to fermentation metabolites, the presence in KP of fungal genera such as *Clonostachys*, *Penicillium*, and *Lecanicillium* for which some species have been associated with plant growth-promoting traits, and the ability of the cellulosic matrix to retain microorganisms and fermentation products within the same material strengthen the hypothesis that the observed plant response may arise from a multicomponent microbe-metabolite-matrix system rather than from a single taxon. Wang et al. (2022) showed that microbial composition can alter metabolite output in defined Kombucha consortia, whereas cross-kingdom synthetic-community studies have shown that combined bacterial and fungal members can produce different and, in some cases, stronger functional outcomes in tomato than single-kingdom communities (Zhou et al. 2022). However, such parallels with the literature do not demonstrate synergy or a specific mechanism in the present study.

In particular, the concurrent increase in proline and MDA at high doses in the previous tomato study suggested that the effect of Kombucha material was nonlinear and that a threshold may exist between biostimulation and biological/chemical load. The present

community structure provides a plausible framework for this dose dependence when considering that living cells, the cellulosic matrix, and fermentation-derived metabolites were applied together. Nevertheless, comparison of a live consortium, cell-free supernatant, washed cells, cellulosic matrix, and unfermented-tea controls will be required to determine the relative contribution of each component to the plant response.

4.5. Nitrogen fixation and nutrient mobilization: a more conservative reassessment of the initial expectation

The isolation from Kombucha of nitrogen-fixing *Acetobacter nitrogenifigens* and the nitrogen-fixing, cellulose-producing *Gluconacetobacter kombuchae* has justified investigation of this ecosystem in relation to agricultural nutrient cycling (Dutta and Gachhui 2006, 2007; Yilmaz et al. 2025). However, our final data set does not support a direct claim of nitrogen fixation. Some 16S signals, including *Bradyrhizobium* and *Paenibacillus*, that initially appeared agriculturally interesting were flagged in the provider's contamination-QC list and were therefore not used as evidence of nitrogen fixation. The presence of *Acetobacter* alone likewise does not establish the presence of *nif* genes or active nitrogen fixation. The safest interpretation for the present study is therefore that nitrogen fixation is not a validated trait, but rather a hypothesis that may be tested in the future using *nifH*-targeted PCR/qPCR, isotopic or acetylene-reduction assays, and genomic analyses.

A similar limitation applies to phosphate or micronutrient solubilization. Although *Penicillium* and certain acetic acid bacteria have been associated in the literature with organic-acid production or phosphate solubilization, phosphate-solubilization phenotype, organic-acid profile, and related genes were not measured in this study. Community composition can therefore guide selection of candidate taxa for nutrient mobilization, but it does not permit the functional conclusion that the 'Kombucha Microbiome solubilizes phosphorus' or 'fixes nitrogen' (Suraby et al. 2023).

4.6. Level of evidence for independent classification, phylogenetic visualization, and biosafety

One strength of this study is that LoTUS2 taxonomy was checked using an independent FLASH-Kraken2-Bracken workflow. Recovery by both algorithmic approaches of major signals such as *Komagataeibacter* and the *Burkholderia-Caballeronia-Paraburkholderia* complex in 16S, *Dekkera*, *Fusarium*, *Aspergillus*, *Penicillium*, *Filobasidium*, *Clonostachys*, and *Lecanicillium* in ITS, and *Brettanomyces* in 18S-KS supports the classification reproducibility of the dominant taxa. However, Kraken2-Bracken analysis is not an independent biological replicate; it represents assessment of the same sequence data using a different classification approach. Thus, agreement between methods increases taxonomic confidence but does not independently validate ecological reality, viability, or function. Bracken validation was also primarily at genus level, and species-level functional inferences should not be based on it.

The phylogenetic visualizations in the main text show the 50 16S OTUs and 30 ITS OTUs with the highest overall abundance (Figs. 4 and 6), while trees containing all 363 16S, 98 ITS, and 30 18S OTUs are provided in Supplementary Figs. S5-S7. These trees were used to visualize phylogenetic structuring of the community and fraction-associated distribution patterns; definitive species-level phylogenetic identities were not inferred from short amplicon sequences.

A similar hierarchy of evidence is required for biosafety. The absence of ITS OTUs flagged by the provider as reagent contaminants does not demonstrate that all fungal signals are viable and safe. Species/strain-level identification and viability assessment are required for *Fusarium*, *Aspergillus*, and other environmental genera, while repeated sequencing with negative extraction and PCR controls is needed for potentially undesirable bacterial signals. Particularly when the objective is development of an agricultural product, community composition must be standardized not only for efficacy but also for biosafety. As emphasized by Andrade et al. (2025) and the Kombucha standardization literature, attributing beneficial traits to identified and validated strains or controlled consortia rather than to the name of a fermented product is a safer approach.

4.7. Study limitations and validation roadmap

The principal limitation of this study is that the three fermentation replicates were pooled by fraction before sequencing. Consequently, independent biological sequencing replicates are unavailable for KP and KS; inferential community statistics such as alpha diversity, beta diversity, PERMANOVA, or differential abundance cannot be applied, and relative-abundance differences must be interpreted descriptively. Second, because no true negative control was sequenced, blank-read subtraction could not be performed, and contamination could only be evaluated through flagging of known reagent/kit contaminants, sample-distribution patterns, and biological plausibility. Third, amplicon data demonstrate the presence of DNA and do not provide direct information on viability,

absolute cell numbers, transcriptional activity, or colonization. Fourth, the 16S/18S/ITS primers and reference databases may limit discrimination among closely related species.

The 18S-KP library is a marker-specific example of these limitations. In LoTUS2, almost all KP reads remained unresolved at genus level, whereas the Kraken2-Bracken output contained high proportions of assignments inconsistent with the target fungal community or assigned to non-fungal eukaryotes. Therefore, rather than being completely excluded as an 'erroneous' library, 18S-KP was retained as exploratory/QC data but was not used for quantitative interpretation of the fungal community. In contrast, the *Brettanomyces* signal in 18S-KS supported the ITS-based dominance of *Dekkera* at an independent marker level.

The next validation stage should prioritize: (i) separate 16S and ITS sequencing of independent fermentation replicates; (ii) negative extraction and PCR controls; (iii) culture-based isolation of dominant and agriculturally interesting taxa; (iv) species-level genomic confirmation and targeted functional genes for selected bacteria; (v) species confirmation, pathogenicity/toxigenicity testing, and PGP phenotypes for fungal candidates; (vi) absolute-abundance measurement by qPCR or ddPCR; (vii) organic-acid and untargeted metabolomic analyses; and (viii) separate testing of the effects of living cells, cell-free supernatant, and the cellulosic matrix on plants. This strategy would move the present metabarcoding inventory from a correlative community description toward a mechanism-oriented and standardizable biostimulant-development program.

For translation to sustainable agriculture, the central implication of this roadmap is that the whole Kombucha consortium should not be treated directly as a plant growth-supporting microbial product without standardization. Fraction-specific profiles show that KP and KS are distinct biological inputs, and the same inventory simultaneously reveals potentially beneficial candidates and signals requiring biosafety validation. Rational product development should therefore proceed through fraction selection, isolation of candidates or reconstruction of defined consortia, validation of plant growth-supporting phenotypes and metabolites, root colonization, batch-to-batch stability, and dose-response analyses. Such an approach offers an innovative biotechnological framework for converting local fermentation microbiomes into environmentally friendly agricultural applications; however, the current metabarcoding data alone do not constitute evidence of product efficacy or field applicability.

4.8. Overall synthesis

In summary, the final metabarcoding profile shows that the Kombucha Microbiome investigated here is not a uniform mixture of microorganisms but a bacterial-fungal consortium structured distinctly by fraction. Dominance of the *Komagataeibacter* and *Dekkera/Brettanomyces* axes in KS supports a classical Kombucha fermentation core, whereas KP appears to represent a broader microbial reservoir requiring careful biosafety evaluation. When considered together with the plant architecture, pigment, and redox responses observed in the previous tomato experiment, this structure justifies investigation of the Kombucha Microbiome as a 'plant architecture-supporting' biostimulant candidate. However, the present data do not identify any specific taxon as the cause of the plant response. The strongest outcome of this study is the establishment of a local Kombucha Microbiome inventory that simultaneously makes potentially beneficial and undesirable candidates visible, is supported by an algorithmic cross-check, and can guide development of standardized consortia based on fraction selection. In this respect, the study identifies an innovative candidate resource for the use of plant growth-supporting microorganisms in sustainable agriculture while positioning functional and biosafety validation as mandatory steps to be completed before product development.

5. Conclusion

This study revealed the bacterial and fungal community structure of a local Kombucha Microbiome, whose agricultural-use potential had previously been observed in tomato, using 16S rRNA, ITS, and complementary 18S rRNA metabarcoding approaches. Final analyses identified 363, 98, and 30 OTUs in the 16S, ITS, and 18S data sets, respectively, and showed that the dried cellulosic pellicle (KP) and fermentation liquid (KS) are distinctly different microbial fractions rather than equivalent representations of the same community. KS was characterized particularly by the *Komagataeibacter* and *Dekkera/Brettanomyces* axes, whereas KP represented a more heterogeneous microbial reservoir requiring biosafety validation. The findings do not show that the Kombucha Microbiome is a ready-to-use or functionally proven plant growth-supporting product. Instead, the fraction-specific taxonomic inventory provides the necessary first step for selecting candidates that may underlie the previously observed plant architecture and physiological responses, separating potentially beneficial taxa from undesirable or uncertain signals, and developing defined, safe, and reproducible microbial consortia. Translation of a Kombucha-derived biostimulant into sustainable agriculture will require independent biological replicates, sequenced negative controls, culture- and genome-based validation, metabolomic profiling, root-colonization studies, dose-response analyses, and biosafety testing in an integrated framework.

Declarations

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Author contributions

Sumeyye Temizgul: Conceptualization, Investigation, Methodology, Project administration, Funding acquisition, Writing - original draft.

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Akife Dalda Sekerci: Investigation, Validation, Writing - review & editing.

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All authors read and approved the final manuscript.

Competing interests

The authors declare no financial or non-financial competing interests relevant to the content of this study.

Data availability

The raw amplicon sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA1512441. The associated BioSample accessions are SAMN62382234 (KP) and SAMN62382235 (KS). The records are currently scheduled for public release on 5 September 2027.

Ethics approval

This study did not involve human participants or experimental animals; therefore, approval from a human or animal research ethics committee was not required.

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Figures

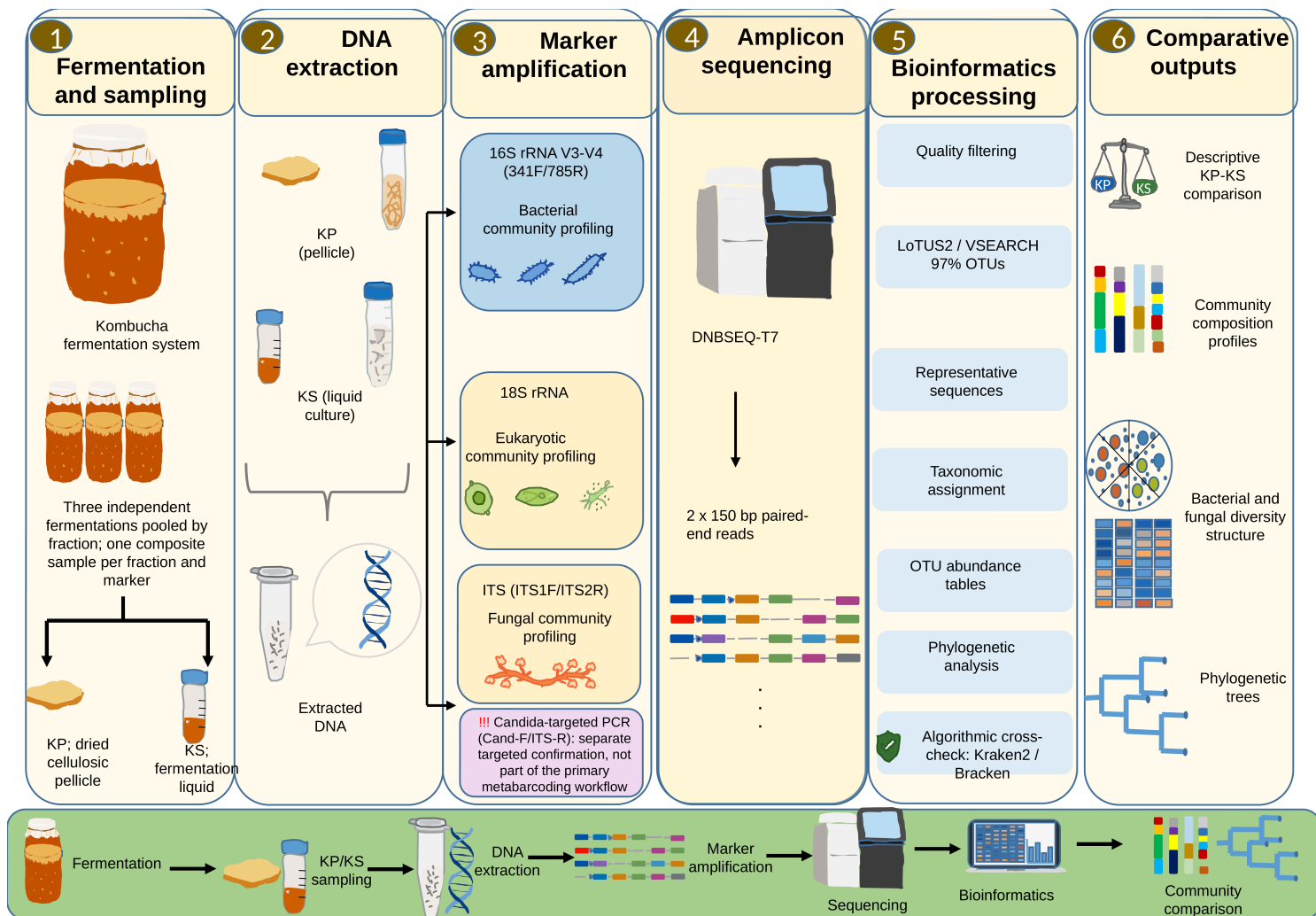


Figure 1

Experimental and bioinformatic workflow for fraction-resolved metabarcoding of the Kombucha microbiome. Three independent fermentations were pooled by fraction to generate one composite dried pellicle (KP) and one composite fermentation-liquid (KS) sample per marker. In-house PCR was used only for amplifiability control and separate *Candida*-targeted confirmation; sequencing libraries were prepared by the service provider. The Kraken2-Bracken analysis was an algorithmic cross-check of the same reads rather than biological validation. Because biological replicates were pooled before sequencing, KP-KS comparisons were descriptive



Figure 2

Sequencing read retention across the six amplicon libraries. Bars show raw paired-end reads, provider adapter-trimmed reads, LoTUS2 quality-control accepted reads, and reads retained in the final OTU tables for KP and KS across 16S, ITS, and 18S datasets

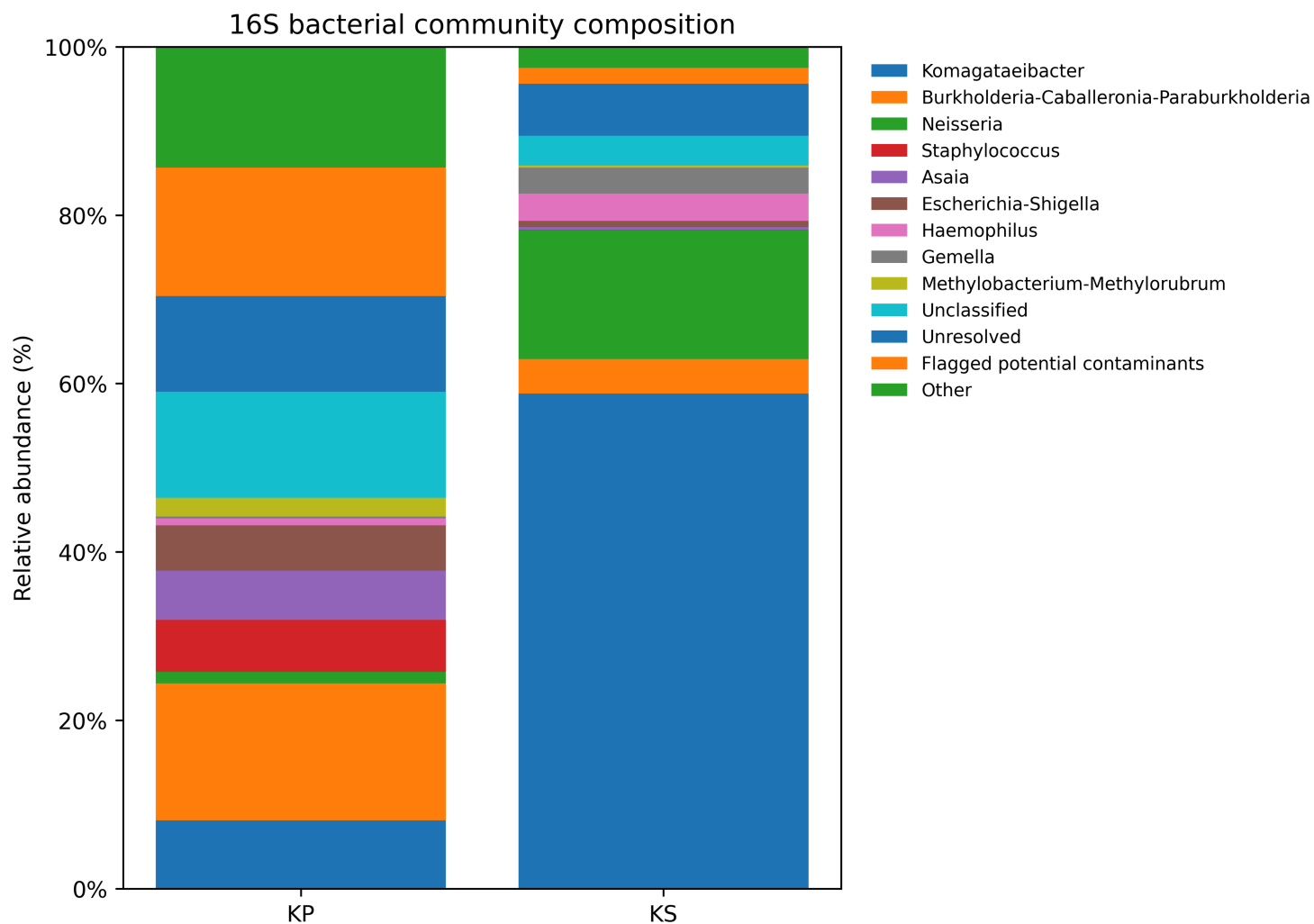


Figure 3

Relative abundance of the bacterial community in KP and KS based on the final 16S LoTUS2 OTU table. Dominant genus-level assignments are shown together with unresolved, unclassified, and provider-flagged potential-contaminant read categories. Percentages are descriptive within each composite library

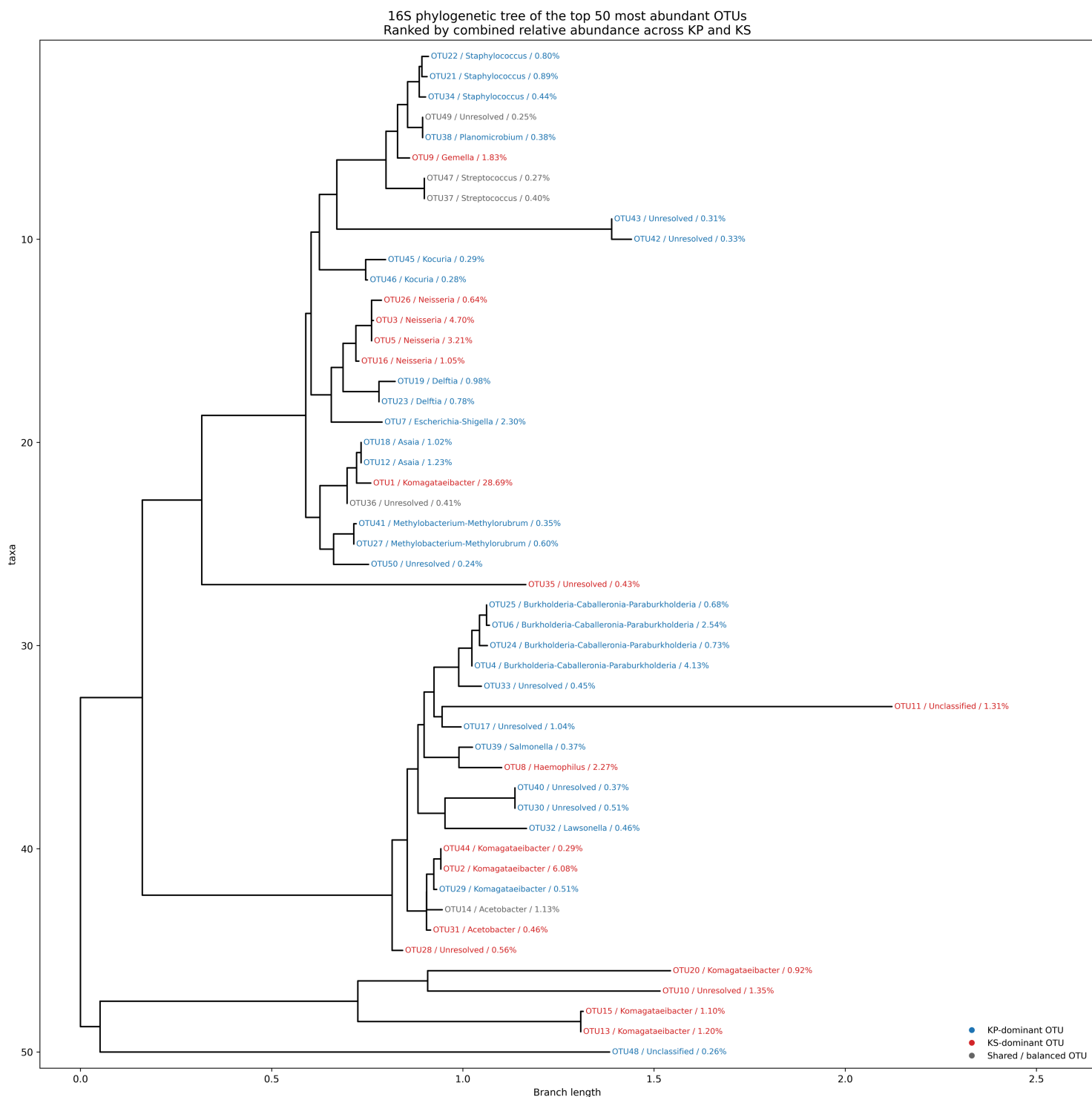


Figure 4

Phylogenetic relationships among the 50 most abundant 16S OTUs, ranked by combined read abundance across KP and KS. Terminal labels show OTU identifier, genus-level assignment, and combined relative abundance. Blue labels indicate OTUs with at least 1.5-fold greater within-fraction relative abundance in KP, red labels indicate OTUs with at least 1.5-fold greater relative abundance in KS, and gray labels indicate less than a 1.5-fold difference between fractions. The complete 363-OTU 16S tree is provided in Supplementary Fig. S5

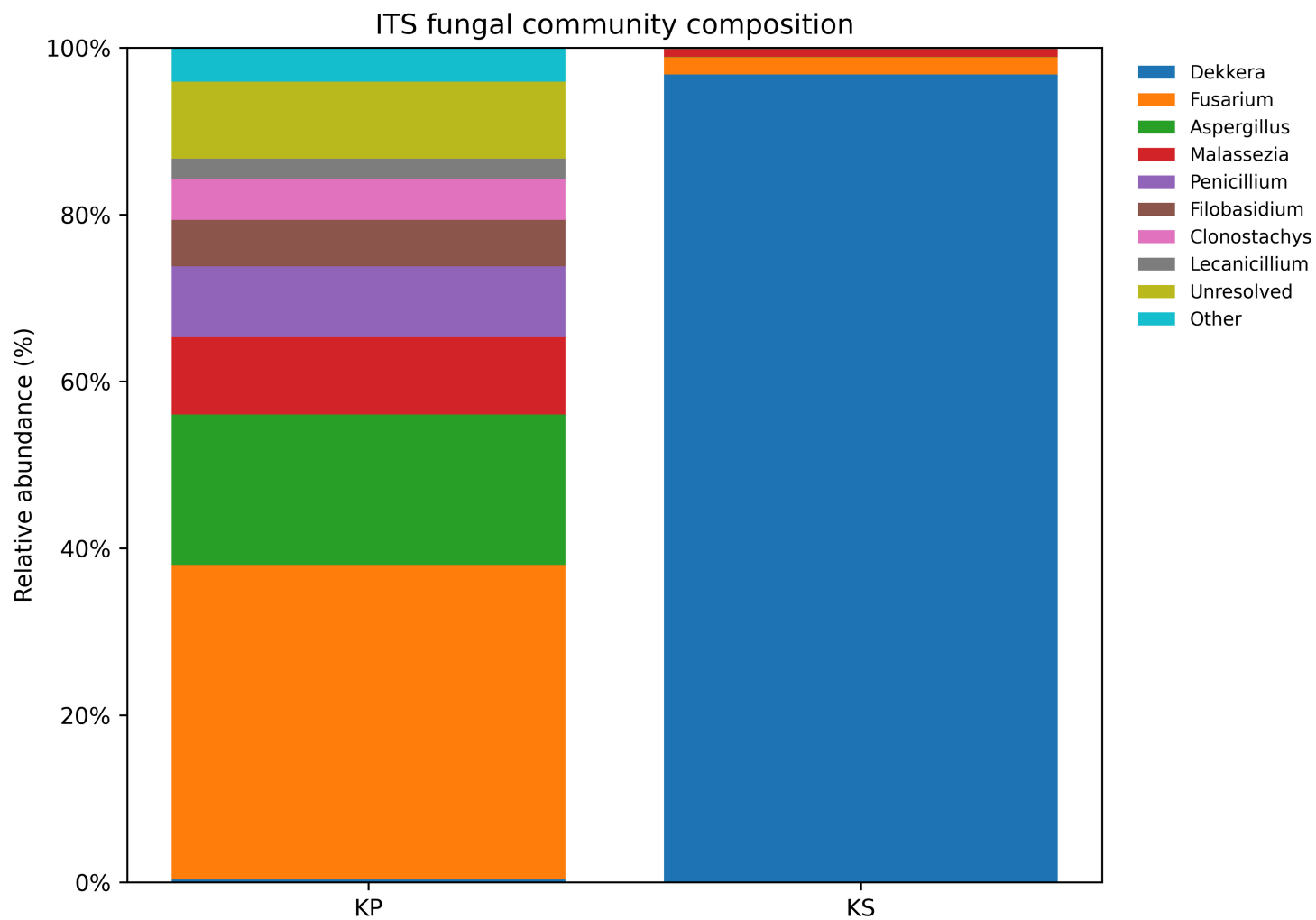


Figure 5

Relative abundance of the fungal community in KP and KS based on the final ITS LoTUS2 OTU table. The KS fraction was dominated by *Dekkera*, whereas KP contained a broader fungal assemblage including *Fusarium*, *Aspergillus*, *Penicillium*, *Malassezia*, *Filobasidium*, *Clonostachys*, and *Lecanicillium*

ITS phylogenetic tree of the top 30 most abundant OTUs
Ranked by combined relative abundance across KP and KS

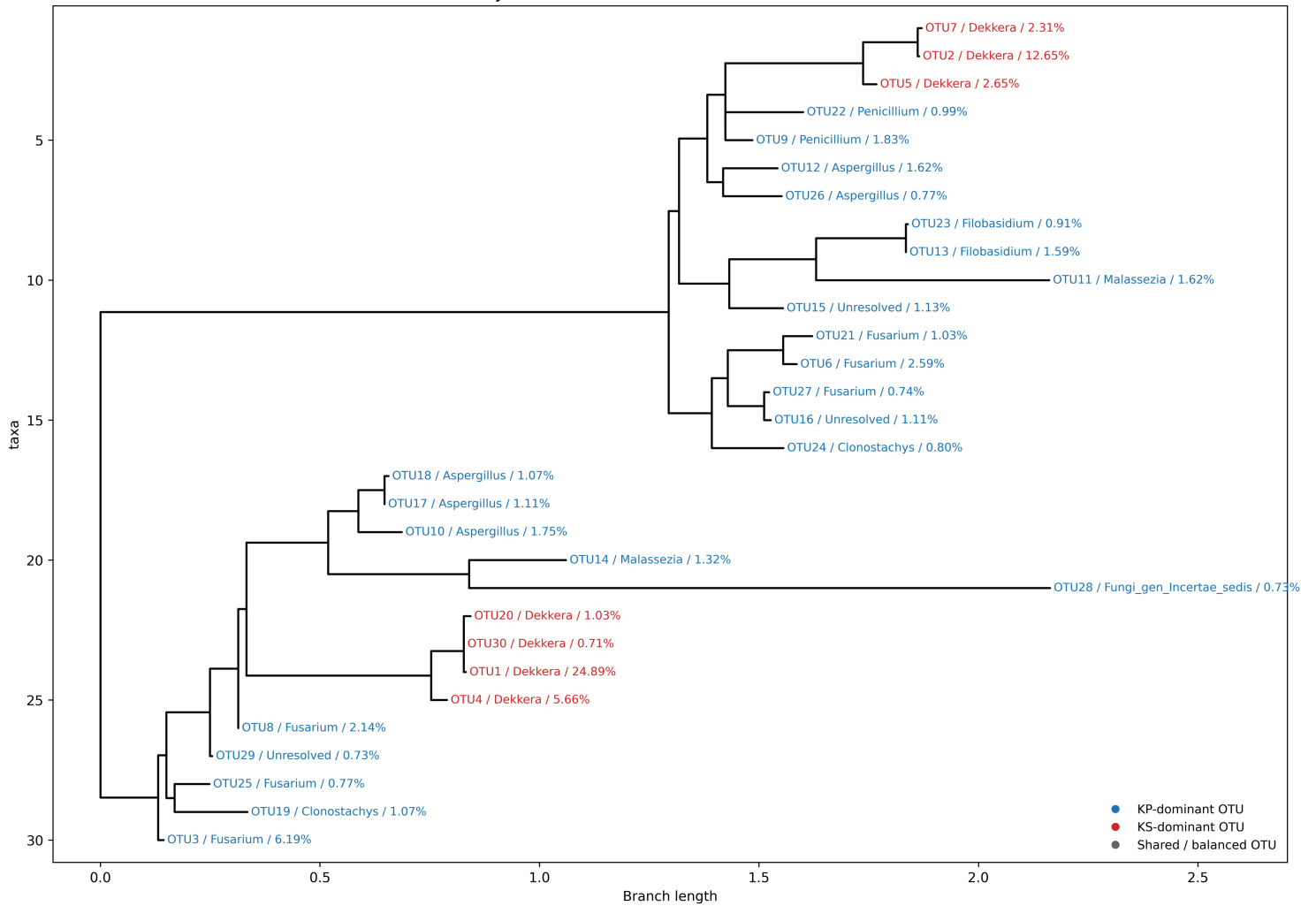


Figure 6

Phylogenetic relationships among the 30 most abundant ITS OTUs, ranked by combined read abundance across KP and KS. Terminal labels show OTU identifier, genus-level assignment, and combined relative abundance. Blue labels indicate OTUs with at least 1.5-fold greater within-fraction relative abundance in KP, red labels indicate OTUs with at least 1.5-fold greater relative abundance in KS, and gray labels indicate less than a 1.5-fold difference between fractions. The complete 98-OTU ITS tree is provided in Supplementary Fig. S6

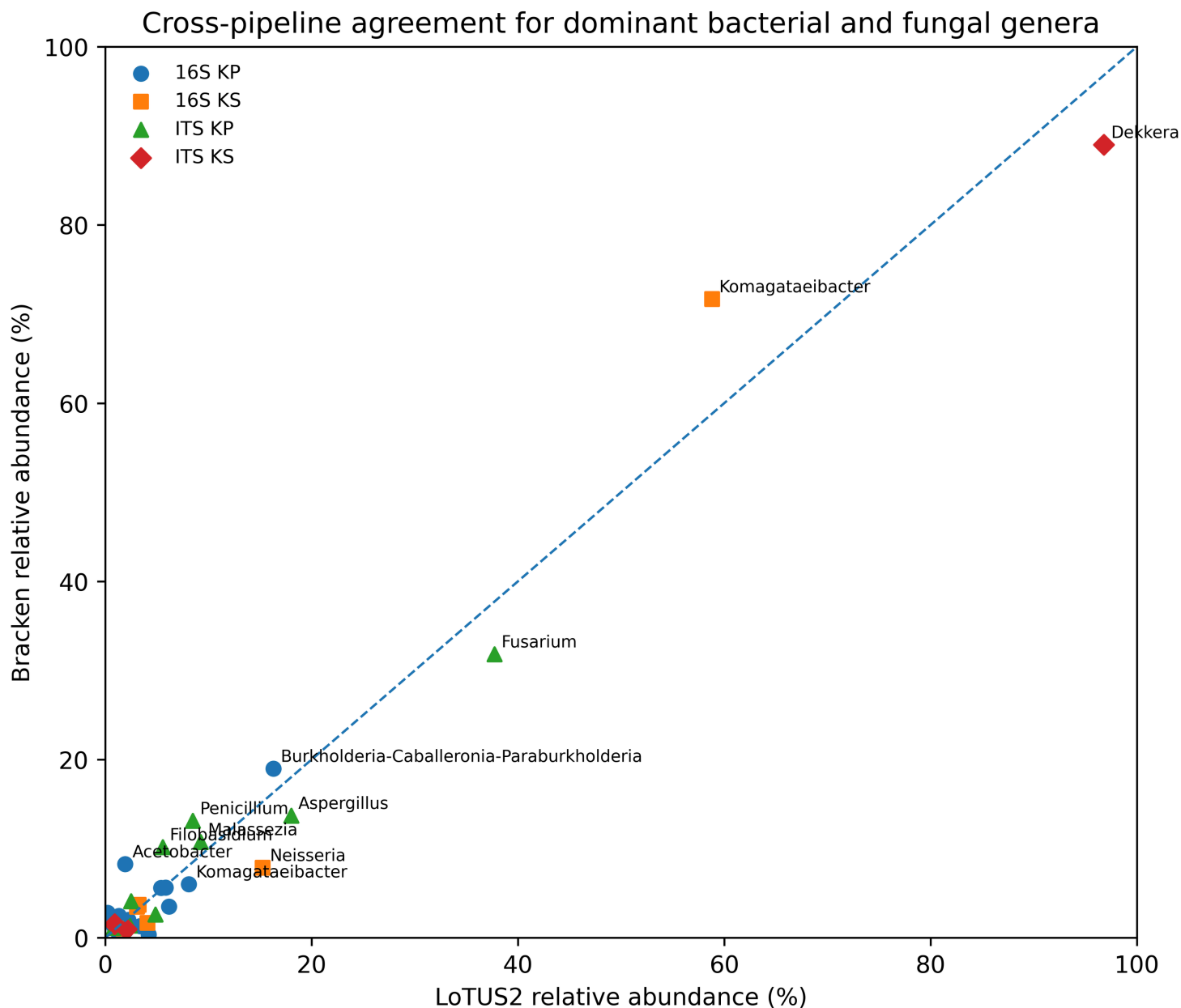


Figure 7

Cross-pipeline comparison of dominant bacterial and fungal genera detected by LoTUS2 and the independent Kraken2-Bracken workflow. Points represent genus-level relative abundance estimates for 16S-KP, 16S-KS, ITS-KP, and ITS-KS. The diagonal line indicates equal relative abundance between workflows. Agreement is interpreted as support for taxonomic reproducibility rather than evidence of organism viability, metabolic activity, or absence of contamination

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [06SupplementaryMaterial.pdf](#)