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Shaping the Microbiome: A DNA Metabarcoding Analysis of Bacterial Diversity in Gluten-Free Sourdough Fermented with Modified Cassava Flour (MOCAF)

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ABSTRACT

This study investigates the bacterial diversity in spontaneously fermented sourdough using a novel substrate, modified cassava flour (MOCAF), through DNA metabarcoding based on next-generation sequencing (NGS) of the 16S rRNA gene. Three sourdough formulations were evaluated: pure MOCAF sourdough (M1), a composite of MOCAF and wheat flour sourdough (M2), and wheat-based flour sourdough (M3). MOCAF, a gluten-free substrate, created a highly selective fermentation environment, enriching substrate starch-adapted bacterial specialists such as *Lactiplantibacillus plantarum* and *L. paraplantarum*, which were present in all treated sourdough. Notably, *Holzapfeliella floricola* was exclusively detected in MOCAF-based sourdough and absent in wheat-based sourdough (M3). Despite its low leavening capacity, M1 exhibited high acidity (pH 3.64) and a distinct sour aroma (total titratable acidity (TTA) 4.79%), indicating intense metabolic activity by lactic acid bacteria. While the wheat sourdough exhibited slightly higher acidity (pH 4.38-3.04; TTA 4-4.97%), the acidification in the M1 was substantial enough to generate the characteristic sour aroma observed. Cluster analysis and alpha diversity indices revealed that M1 harboured the highest species richness but with low evenness, suggesting dominance by a few acid-tolerant taxa. The findings underscore the potential of MOCAF as an innovative fermentation substrate that can shape distinct microbial ecosystems. This offers novel opportunities for the development of gluten-free and functional bakery products characterized by unique fermentation dynamics and flavor profiles.

1. INTRODUCTION

Sourdough fermentation is initiated by mixing flour and water, where spontaneous fermentation under acidic and nutrient-stressed conditions promotes the growth of acid-tolerant lactic acid bacteria (LAB) and resilient yeasts. Through successive backslopping, a stable and functionally adapted microbial community emerges, contributing to the distinctive biochemical and sensory characteristics of sourdough [1-3]. The metabolic activities of these microorganisms lead to acidification, flavor development, and dough leavening [4]. The composition of sourdough microbiota is influenced by endogenous factors (e.g., flour type and equipment) and exogenous factors (e.g., temperature and incubation time), resulting in mature sourdough dominated by LAB, while yeasts are present in lower quantities [4, 5]. The microbial composition of sourdough varies with the ingredients and production conditions. Spontaneous sourdough made from a single flour type typically contains fewer than three LAB species [3, 6]. The most commonly found LAB groups are *Lactobacillus fructivorans*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Lactobacillus reuteri* [7, 8]. The presence of *L. sanfranciscensis* is linked to specific environmental conditions, whereas *L. plantarum* and *L. reuteri* are influenced by metabolic capacity and environmental stress resistance [9].

LAB are gram-positive, non-spore-forming, and highly fermentative members of the *Firmicutes* phylum that dominate fermented foods. In sourdough, LAB are typically more prevalent than yeast species [8]. In stable sourdough, the heterofermentative LAB community is the dominant bacterium found in the system, especially the *Lactobacillus* genus [10-12]. The less dominant genera included *Weisella*, *Pediococcus*, and *Leuconostoc*, whereas homofermentative species such as *Enterococcus*, *Lactococcus*, and *Streptococcus* were more abundant than *Weisella* spp. [13]. Flour is the primary source of sourdough microbiota. Different flour types create selective pressures on microbial communities, producing unique profiles [14, 15]. Previous research has explored the use of gluten-free substrates in sourdough production, such as quinoa, rye, legumes, and cereals [16-19]. However, the application of modified cassava flour (MOCAF) as a substrate for spontaneous sourdough fermentation remains underexplored. Consequently, MOCAF, as a local

carbohydrate source, has a unique starch matrix that offers a novel substrate that may influence microbial diversity and fermentation dynamics of sourdough microorganisms because of performing in a specific environment, warranting further investigation.

MOCAF is produced through fermentation and supports food security by providing a local calorie source [20, 21]. It is created through microbial cell modification, offering advantages such as high starch bioavailability, reduced cyanogenic glycosides, and enhanced water-binding capacity [22]. Its neutral flavours make it suitable for fermentation [23], but its unique biochemical composition may impose novel selection pressures on sourdough microbiota [24, 25]. Understanding the influence of MOCAF on microbial behavior is crucial for optimizing its use in baking. LAB diversity regulates acidification rate, exopolysaccharide production, and enzymatic breakdown of starch and proteins, impacting texture, shelf life, and sensory attributes [26]. Given the distinctive properties of MOCAF, employing advanced methodologies such as 16S rRNA sequencing is crucial for elucidating its effects on sourdough microbiota.

16S rRNA sequencing enables high-resolution microbiome analysis by targeting hypervariable regions for species identification [27]. The use of 16S rRNA with Oxford Nanopore Technology, and analyzed using the centrifuge classifier represent a significant advancement in microbial community profiling. Oxford Nanopore Technologies (ONT) allows for the sequencing of full-length 16S rRNA genes, which is crucial for accurate taxonomic resolution at the species level [28, 29]. Microbial community composition was determined using Oxford Nanopore sequencing followed by direct taxonomic classification through the Centrifuge classifier. Consequently, the analytical unit used herein refers to taxonomically classified reads mapped to the NCBI RefSeq database, providing a direct representation of microbial abundance. DNA metabarcoding overcomes culture-based biases, revealing rare taxa and successional dynamics [30, 31]. It effectively distinguishes flour-derived microbiota from environmental contaminants [3, 12, 30]. Sourdough substrate such as MOCAF is shaping unique microbial profiles [5, 14, 15]. Moreover, contamination from the processing environment may occur [32-35], and both equipment and sanitation practices significantly influence microbial growth [36]. While wheat-based sourdough microbiomes are well-characterized, those derived from alternative flours, particularly gluten-free substrates like MOCAF, remain underexplored. Given the unique characteristic of MOCAF and its underexplored potential in sourdough fermentation, this study aims to employ 16S rRNA gene sequencing to elucidate the impact of MOCAF as a single or combined matrix on the bacterial community structure, diversity, and function of spontaneously fermenting sourdough, thereby providing a comparative analysis with traditional wheat sourdough. It explores how MOCAF influences microbial composition and fermentation dynamics, providing insights for developing local gluten-free material and functional bakery products with tailored attributes.

2. MATERIAL AND METHODS

The study carried out three types of flour substrates to prepare sourdough: pure MOCAF, a composite mixture of MOCAF and wheat flour, and pure wheat flour. The sourdough formulations were designated according to section 2.1.

High-protein wheat flour was sourced from a local supplier in Indonesia. Throughout the 17-day fermentation period, all sourdough samples were monitored daily for leavening capacity: it was evaluated on a daily basis by quantifying its rise in the jar: the initial height was documented on day 0, followed by measurements taken immediately before and 24 hours after each propagation [37], pH, total titratable acidity (TTA): 1 gram of the sample was homogenized with 10 milliliters of distilled water. This mixture was then titrated with 0.1 normal sodium hydroxide (NaOH) solution using 1% phenolphthalein (PP) as an indicator. The endpoint of the titration was identified by the persistent faint pink color that appeared [18], and growth rate: cell counts were calculated as growth rates using de Man, Rogosa, and Sharpe maltose agar. Samples were diluted appropriately and grown (plated) on MRS agar media and incubated anaerobically at 32°C for 48 hours [38].

On day 17, further analyses were conducted to assess organic acid profiles using Fourier Transform Infrared Spectroscopy (FTIR), simple sugar content using high-performance liquid chromatography (HPLC), and bacterial community composition through 16S rRNA gene sequencing. In microbiome analysis, DNA extraction was performed on each biological replicate. Then, equal concentrations of DNA from the three replicates of each treatment were pooled to create a single composite sample for high-throughput sequencing. This approach ensured a representative profile of the microbial community.

2.1 Sourdough preparation and propagation

Three sourdough variants were prepared using spontaneous fermentation and conventional backslopping techniques over a 17-day period to establish stable microbial communities. The first variant (M1) utilized MOCAF as the sole substrate, initiated by mixing 50 g MOCAF with 50 mL warm water (40°C) at a 1:1 (w/v) ratio. The second variant (M2) employed a composite flour blend of MOCAF and high-protein wheat flour (1:1), while the third variant (M3) used only high-protein wheat flour. All mixtures were incubated at ambient temperature (approximately 27°C) for 24 hours under static conditions to promote indigenous microbial growth.

Each sourdough underwent daily backslopping, where 50% of the fermented mixture was replaced with fresh flour and warm water in a 1:2:2 (w/v/w) ratio. The MOCAF-based sourdough (M1) included an initial three-day aeration phase before transitioning to daily propagation. Throughout the fermentation process, pH and ambient temperature were monitored daily to assess microbial activity and fermentation progression. This standardized approach facilitated the development of distinct microbial consortia influenced by substrate composition.

2.2 Organic acid and simple sugar analysis

The analysis of organic acids in sourdough samples was conducted using FTIR, covering a spectral range of 400 to 4000 cm^{-1}, with scans performed at a resolution of 6 cm^{-1}. The detected spectra were subsequently examined utilizing OMNIC software. Monosaccharide concentrations in sourdough samples were quantified using HPLC equipped with a 20 mL automatic injection loop and a refractive index detector (RID 156, Beckman) [39]. Separation was performed on an ion-exclusion ORH-801 column (300 mm × 6.5 mm,

Interaction Chromatography, France) using 0.001 N H_2SO_4 as the mobile phase at a flow rate of 0.7 mL/min. Quantification was carried out using the external standard method based on calibration curves generated from pure standards. The column temperature was maintained at 45℃, and analyses were conducted under ambient conditions.

2.3 Metagenome 16S rRNA sequencing

Genomic DNA was extracted using the Quick-DNA MagBead Plus Kit (Zymo Research). DNA quality and concentration were assessed via NanoDrop and Qubit fluorometry. Full-length 16S rRNA genes were amplified using universal primers (27F/1492R) and visualized by agarose gel electrophoresis. Sequencing libraries were prepared with Oxford Nanopore kits and sequenced on a MinION platform. Basecalling was performed using Dorado, and quality control was conducted with NanoPlot and NanoFilt. Taxonomic classification was carried out using a centrifuge with reference indices from the NCBI 16S RefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/).

2.3.1 Relative abundance of the microbiota in sourdough

The relative abundance for each sample was calculated based on the total species observed at the phylum and genus levels of taxonomic classification.

2.3.2 Alpha diversity of sourdough bacterial microbiota

Relative abundance for each sample was calculated based on the total number of observed taxa at the phylum and genus levels. Alpha diversity indices, including Shannon, Simpson, and Inverse Simpson, were computed using taxonomic assignments and abundance data to evaluate species richness and evenness.

Shannon Index (H')

The Shannon diversity index accounts for both the abundance and evenness of species in a community [40].

$$H' = \sum(p_i \times \ln p_i) \quad (1)$$

where, p_i is the proportion of species i relative to the total number of species

Simpson's Index (D)

Simpson's Index measures the probability that two individuals randomly selected from a sample belong to the same species [40].

$$D = \sum(p_i) \quad (2)$$

The value of D ranges from 0 to 1, where 0 indicates infinite diversity and 1 indicates no diversity.

Inverse Simpson Index (1/D)

The Inverse Simpson Index is the reciprocal of Simpson's Index, providing a measure of diversity where higher values indicate greater diversity [40].

$$1/D = 1/\sum(p_i) \quad (3)$$

2.4 Data analysis

Microbial diversity was assessed using Chao and Simpson indices, while species evenness and richness were visualized via Pavian and RStudio (v4.2.3). Multivariate analyses, including PCA and PCoA, were performed on normalized LAB data to explore microbial community structure. Spearman correlation analysis was applied to dominant bacterial taxa (≥ 5% relative abundance) and sourdough physicochemical parameters (pH, TTA, leavening capacity, LAB growth rate). All statistical analyses were conducted using R and Python environments.

3. RESULTS AND DISCUSSION

3.1 Leavening capacity

Leavening capacity, a key indicator of dough gas retention and final bread texture, was lowest in MOCAF sourdough (M1) due to the absence of gluten, which limits CO_2 entrapment despite high acid production. In contrast, wheat flour sourdough (M3) showed the highest volume expansion, supported by gluten and fermentable sugars (Figure 1), while the composite sourdough (M2) exhibited a moderate rise, reflecting the combined effects of both substrates [41, 42]. The presence of gluten in wheat flour allows the formation of an elastic matrix that effectively retains gas, leading to significant dough rise [43].

However, MOCAF relies on the hydration of its less elastic fiber to provide structure, rather than forming an elastic gluten network. Its high fiber content may slow down or inhibit microbial growth [44] and affect the availability of simple sugars for fermentation. Simple sugars such as glucose serve as effective substrates for yeast, and their limited presence may restrict CO_2 production. Inadequate CO_2 retention in MOCAF sourdough results in insufficient dough rise. Consequently, combining MOCAF with wheat flour helps form a gluten matrix that improves dough volume, though not as ideal as pure wheat sourdough; it performs better than MOCAF alone.

3.2 pH and total titratable acidity

Lactic acid bacteria (LAB) produce organic acids such as lactic and acetic acid, which lead to a gradual decrease in pH during the fermentation process (Figure 2). Conversely, TTA tends to increase over time due to the accumulation of these acids (Figure 3). The most significant pH reduction was observed in the MOCAF sourdough (M1), likely due to the dominance of heterofermentative LAB known for their high acid production capacity [45, 46]. Organic acids not only lower pH but also contribute to dough structure enhancement through protein denaturation and starch gelatinization [47, 48].

MOCAF sourdough (M1) exhibited rapid acidification due to its modified starch structure, which initially provided fermentable sugars and later retrograded into resistant starch, leading to pH stabilization near the pK_a of lactic acid [37, 49]. In contrast, wheat flour sourdough (M3) showed slower acidification and greater microbial diversity due to intact starch and gluten [50, 51], while the composite sourdough (M2) demonstrated moderate acidification and buffering effects from wheat components, reflecting substrate-driven microbial ecology and selective enrichment in MOCAF sourdough [4, 50-52].

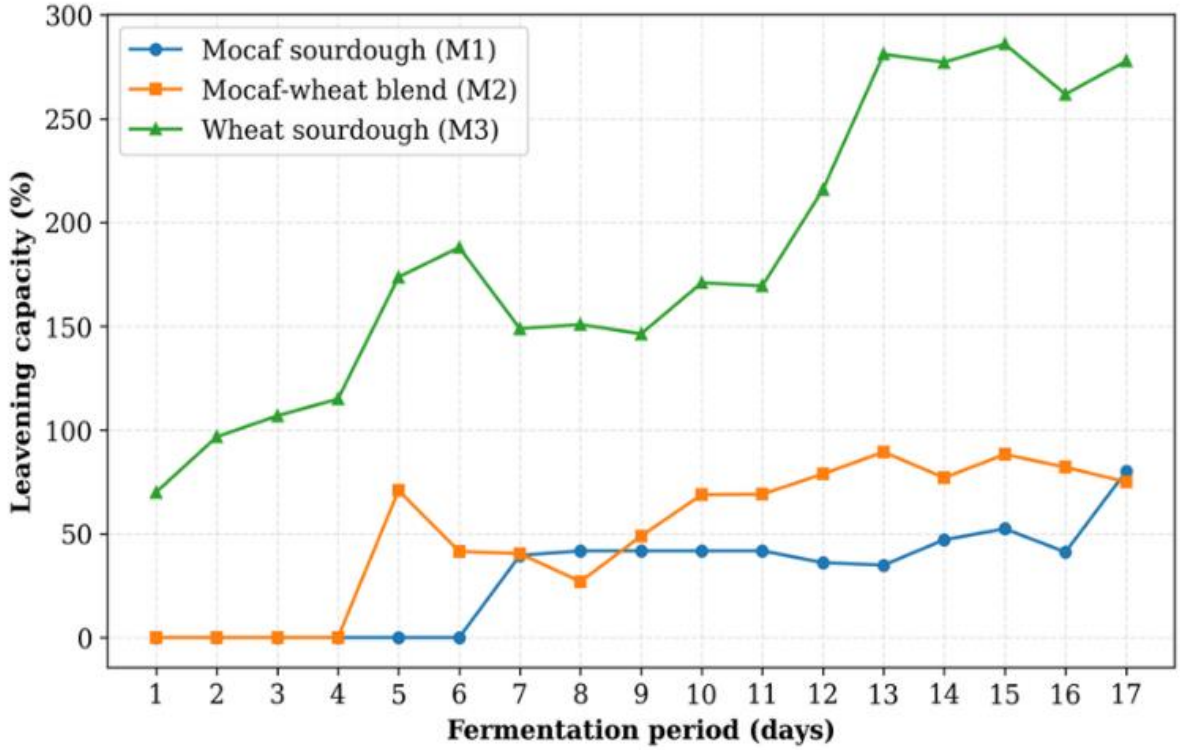


Figure 1. Leavening capacity during the spontaneous fermentation sourdough period (17th day)
M1: MOCAF sourdough, M2: composite MOCAF and wheat flour (1:1), M3: wheat flour sourdough

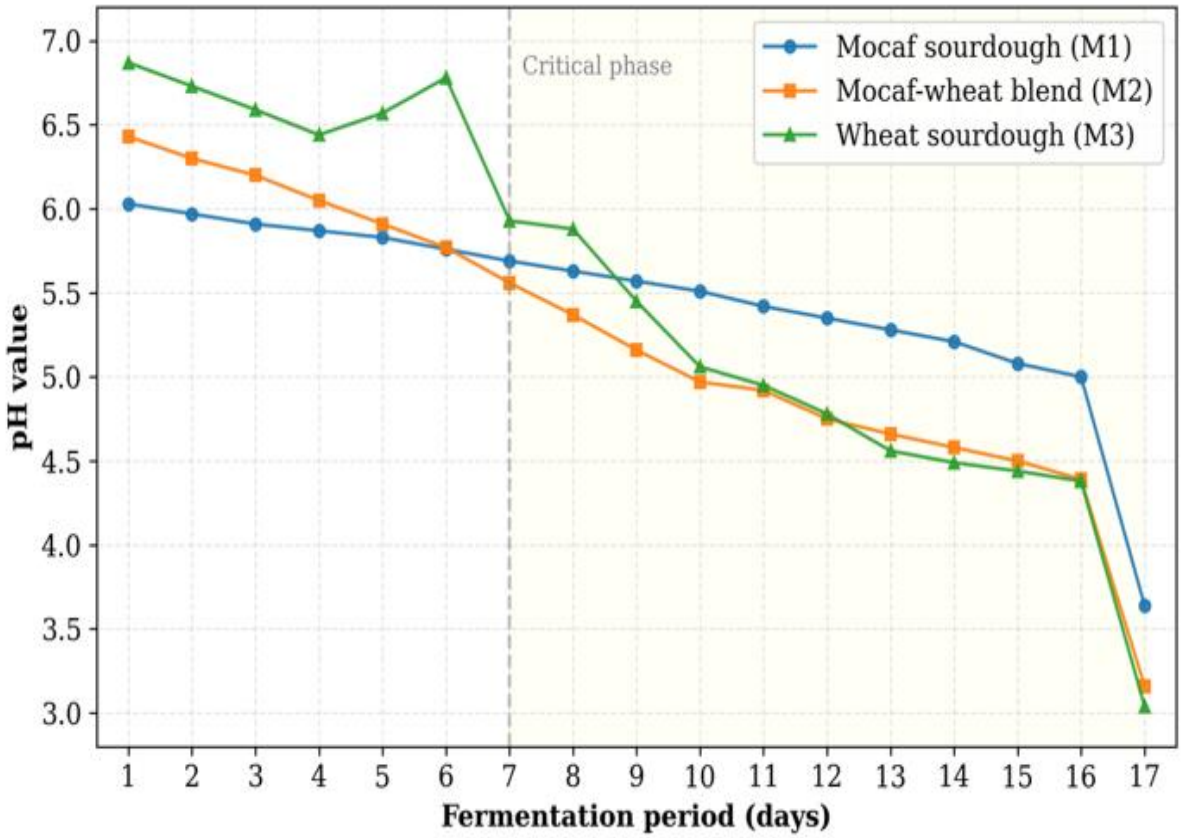


Figure 2. pH value of sourdough during the fermentation period (17th day)

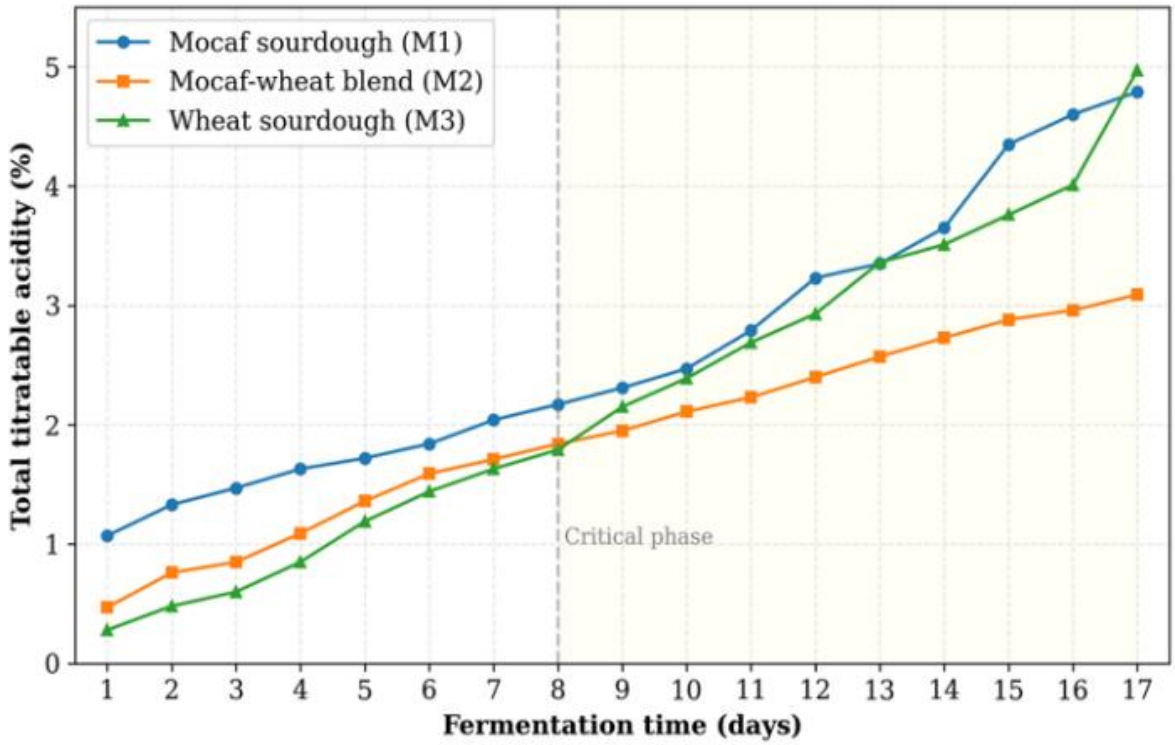


Figure 3. Total titratable acidity (%) of sourdough during the fermentation period (17th day)
M1: MOCAF sourdough, M2: Composite MOCAF and wheat flour (1:1), M3: wheat flour sourdough

3.3 Growth rate

The growth curve of total lactic acid bacteria (LAB) in all sourdough formulations exhibited distinct lag, exponential, and stationary phases (Figure 4). In the M1 formulation, LAB proliferation was not observed until 72 hours, after which exponential growth occurred between 72 and 96 hours, reaching 11.62 × 10^8 CFU/mL. The population peaked at 12.70 × 10^8 CFU/mL at 240 hours and remained stable at 12.78 × 10^8 CFU/mL until 384 hours. In the M2 formulation, microbial growth was detected as early as 24 hours (3.52 × 10^8 CFU/mL), followed by exponential growth until 72 hours (11.86 × 10^8 CFU/mL), peaking at 12.98 × 10^8 CFU/mL at 264 hours and stabilizing at 12.97 × 10^8 CFU/mL. M3 exhibited a faster onset of exponential growth at 24 hours (7.28 × 10^8 CFU/mL), reaching a peak of 13.37 × 10^8 CFU/mL at 264 hours, and stabilizing at 13.43 × 10^8 CFU/mL.

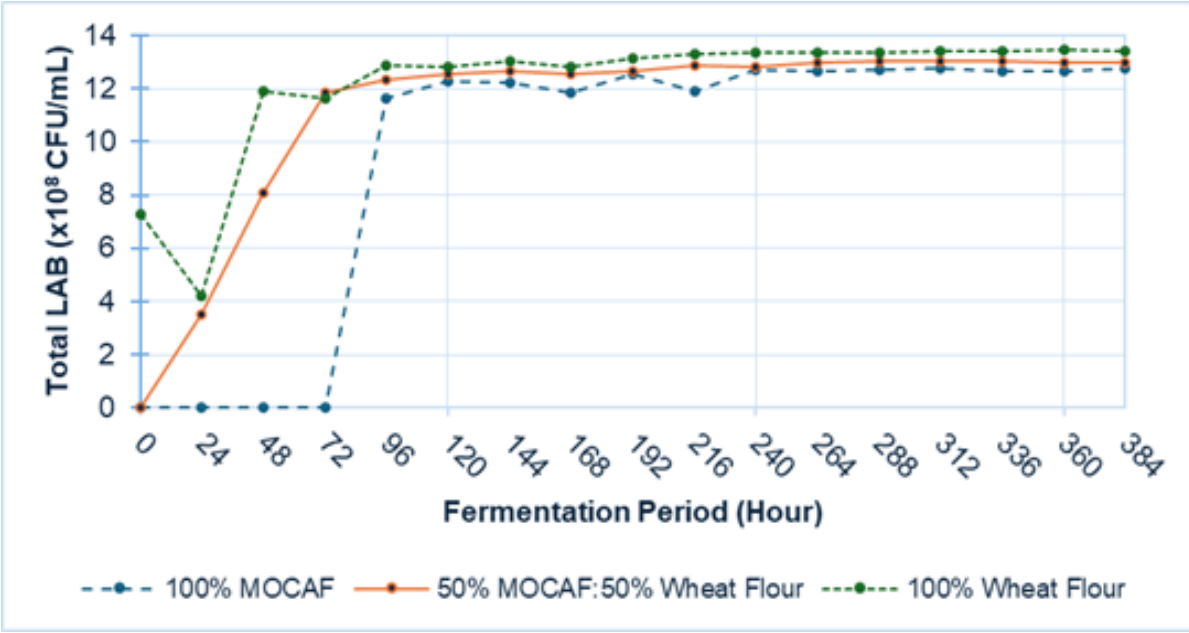


Figure 4. Total lactic acid bacteria of three types of spontaneous fermented sourdough (17th day)

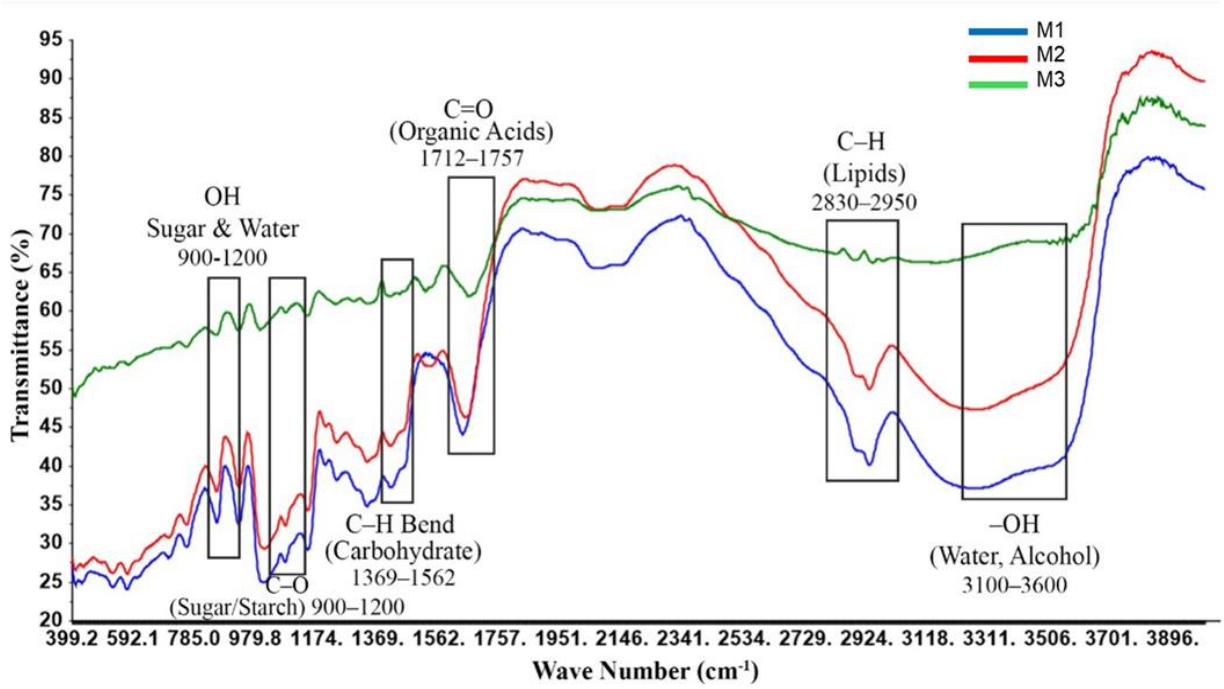


Figure 5. FTIR spectra of three types of spontaneously fermented sourdough (17th day)

The extended lag phase observed in MOCAF sourdough indicates microbial adaptation to the gluten-free substrate, requiring time for hydrolytic enzyme synthesis [53-56]. In contrast, wheat-based sourdoughs accelerate LAB proliferation due to the presence of readily fermentable sugars, proteins, and peptones, which are rapidly converted into amino acids, supporting initial microbial growth [57-59]. The accumulation of organic acids such as lactic acid and acetate contributes to pH reduction, which inhibits the growth of competing microorganisms and stabilizes the LAB population. This pH decline is directly correlated with increased bacterial abundance [54, 52].

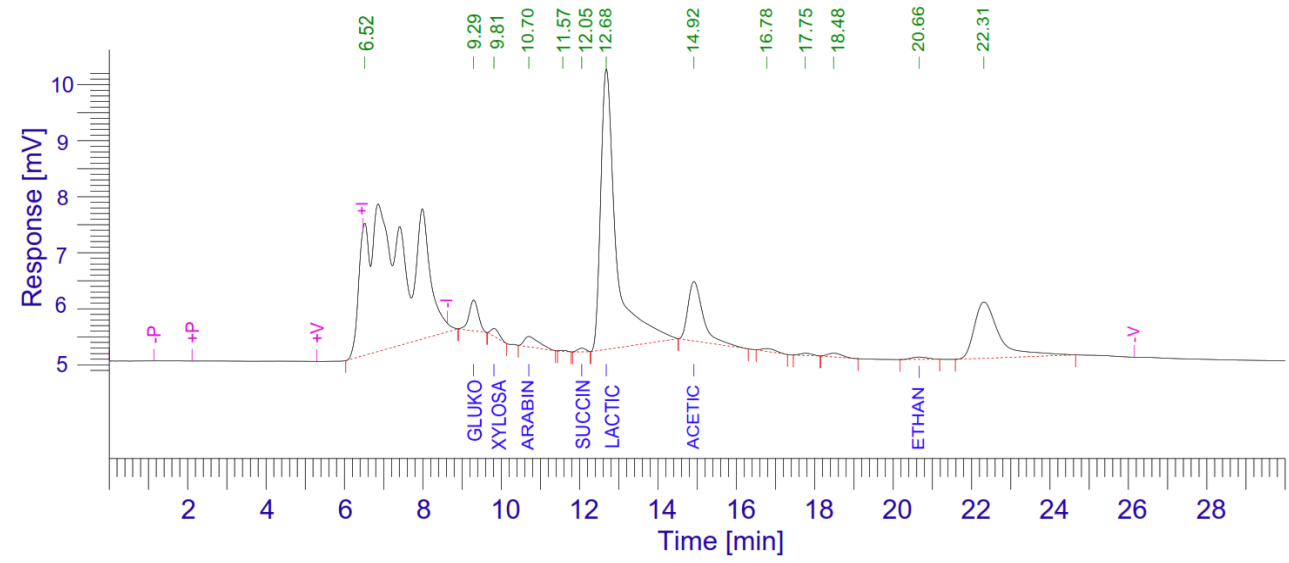
3.4 Organic acids

FTIR spectroscopy revealed distinct chemical differences among sourdough samples, particularly in organic acid, carbohydrate, and hydroxyl group content (Figure 5). All samples exhibited characteristic peaks at ~1700 cm^{-1}, 1400 cm^{-1}, and 1100 cm^{-1}, corresponding to carboxylic acid carbonyl groups, COO^{-}/CH_3 bending, and C–O stretching, respectively—indicative of lactic and acetic acid presence during fermentation [60, 61]. MOCAF sourdough displayed the highest peak intensity at 1700 cm^{-1}, suggesting a greater accumulation of carboxylic acids, particularly lactic and/or acetic acid [62, 63]. The composite sourdough showed intermediate peak intensities, while wheat sourdough exhibited the lowest. This trend was consistent across other key spectral regions (1400 cm^{-1} and 1100 cm^{-1}), supporting the hypothesis that MOCAF promotes higher organic acid production. These spectral findings were corroborated by HPLC analysis, which quantified lactic acid at 75%, acetic acid at 15%, and succinic acid at 0.5% in MOCAF sourdough, confirming its elevated acid content.

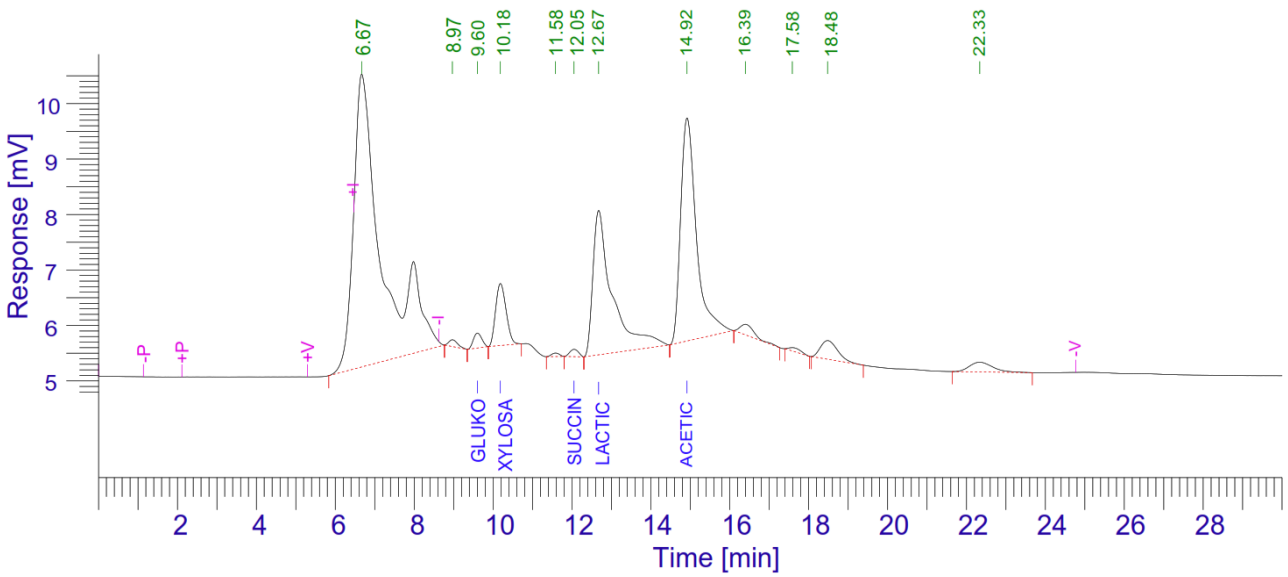
These findings align with those validated by HPLC analysis (see Figure 6). This research indicates that lactic acid constitutes 75%, acetate about 15%, and succinic acid 0.5% in MOCAF sourdough. Additionally, the combination of sourdough between MOCAF and wheat flour sourdough exhibited lactic acid concentrations of 39%, acetic acid at 49%, and succinic acid at 0.9%. The minimal concentrations were recorded in wheat flour sourdough, with lactic acid at 14%, acetic acid at 24%, and succinic acid at 0.8%. The overlay of spectra from all sourdough-treated samples highlights the disparities in chemical profiles, with each sourdough sample forming distinct clusters, mostly influenced by the 1700–1400 cm^{-1} area, which is the main factor in the differentiation among groups [37, 64].

3.5 Simple sugar

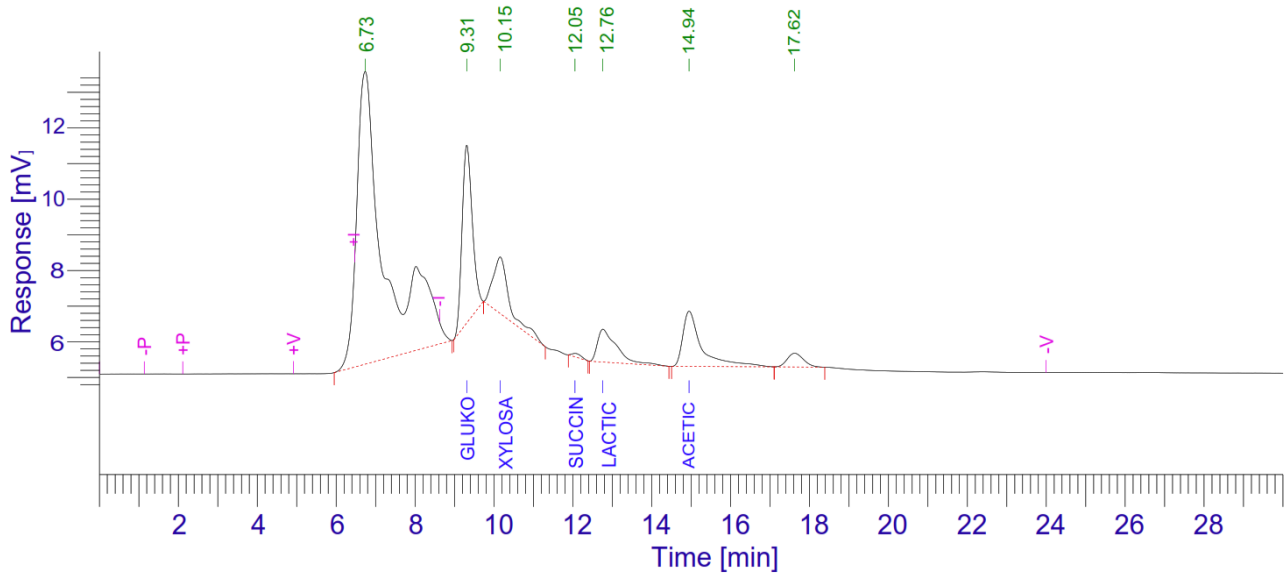
The unique simple sugar compositions identified through HPLC analysis (Figure 6) profoundly influence the fermentation dynamics and organic acid generation in all treated sourdoughs. The predominance of glucose (4.84%) in MOCAF sourdough (Table 1), along with reduced levels of xylose (1.12%) and arabinose (2.62%), indicates a rapid enzymatic breakdown of cassava starch, facilitating swift microbial glycolysis [49]. The accessible glucose stimulates vigorous homolactic fermentation [65], as demonstrated by the rapid acidity of MOCAF increase over the initial five days and a subsequent pH decline to 3.5. The FTIR spectra further validate this process, with pronounced C=O peaks (1712–1757 cm^{-1}) affirming the predominance of lactic acid. The depletion of simple sugars by mid-fermentation, indicated by the plateauing TTA percentage at around 2.2 mL (0.1M NaOH/10 g sample) by day 8, highlights MOCAF sourdough metabolic constraint; the lack of complex carbohydrates, such as arabinoxylans, limits prolonged leavening capacity beyond day 7 [41, 66, 67].



M1



M2



M3

Figure 6. HPLC chromatogram analysis of sourdough monosaccharides and main organic acids of sourdough fermentation

Table 1. Simple sugar in sourdough with different flour treatments

Simple Sugar	MOCAF Sourdough	Composite MOCAF and Wheat Flour (1:1)	Wheat Flour Sourdough
Glucose (%)	4.84	1.76	40.96
Xylose (%)	1.12	9.07	20.24
Arabinose (%)	2.62	-	-

In contrast, wheat sourdough demonstrates remarkably elevated levels of glucose (40.96%) and xylose (20.24%), reflecting the abundant starch and hemicellulose content of wheat [50]. Notwithstanding this abundance, acidification commences gradually, characterized by an extended critical pH phase (around 4,5 - 5.0; days 4–8) that postpones microbial succession. According to research [66, 67], the slow acidification phase in sourdough is essential for microbial succession. The pH range of 4.5 to 5.0 is sustained for several days, facilitating microbial adaptation and the formation of a stable microbiota. This delay is likely due to catabolic inhibition or microbial adaptation to intricate substrates.

Upon initiation, the successive consumption of glucose (early phase) and pentoses such as xylose (middle-late phase) maintains heterofermentative activity, producing various organic acids (wide FTIR C=O bands). As a result, wheat sourdough attains the highest final TTA (5% by Day 17; Figure 3) and increasing acidification growth, facilitated by prolonged sugar availability [51, 68].

The composite flour of MOCAF and wheat sourdough establishes a metabolic equilibrium, incorporating glucose from MOCAF (1.76%) and xylose from wheat (9.07%). The absence of arabinose suggests a preference for the utilization of pentoses by the microbiome. This synergy enables simultaneous homolactic (glucose-driven) and heterofermentative (xylose-driven) pathways, leading to a stable acidification rate and optimal TTA (3%) without reduction. FTIR data confirm this balance, indicating intermediate C=O peak widths between MOCAF wheat sourdough, which signifies a balanced lactic and acetic acid ratio. M2 accelerates through the critical pH phase more rapidly than wheat flour sourdough alone, while also preventing the unnecessary metabolic exhaustion observed in MOCAF sourdough. This highlights how the blend contributes to stabilizing fermentation kinetics.

3.6 Bacterial diversity of MOCAF sourdough

All treated sourdough samples (M1, M2, and M3) were analysed, as illustrated in Figure 7, the successful amplification of the full-length 16S rRNA gene from the extracted gDNA, producing bands of the anticipated size (approximately 1500 bp, as indicated by the 27F-1492R primers). The results indicate that the gDNA from each sample has been successfully amplified and is prepared for the subsequent stage, specifically sequencing. Effective amplification is crucial for ensuring the quality and reliability of the data obtained from this study.

This study employed 16S rRNA gene-based next-generation sequencing (NGS) to characterize the bacterial communities in spontaneously fermented sourdoughs prepared from M1, M2, and M3. The concentration and yield of the extracted genomic DNA are summarized in Table 2. Qubit dsDNA high sensitivity allows for accurate quantification of the DNA concentration in the range of 0.1 ng/µL to 120 ng/µL. This measurement method was highly selective for dsDNA, ignoring the presence of contaminants such as salts, free nucleotides, solvents, detergents, or proteins.

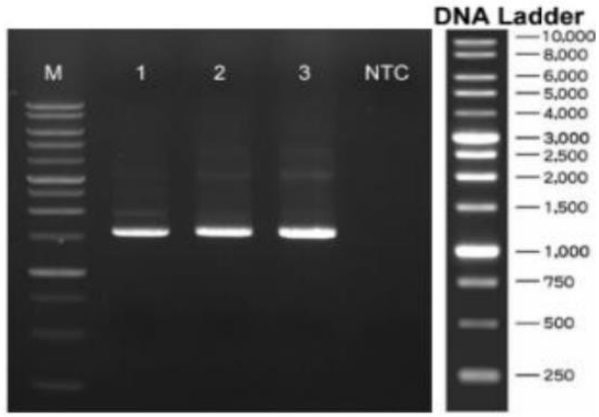


Figure 7. Agarose gel electrophoresis photo of the PCR product
1 = MOCAF sourdough, 2 = composite flour sourdough, and 3 = wheat sourdough

Table 2. Nanodrop and qubit DNA quantification of treated sourdough

Sample	Sample Code	Volume (µl)	Nano Drop (ng/µL)	Qubit (ng/µL)
M1	N1261-1	40	49.4	39.4
M2	N1261-2	40	17	13.7
M3	N1261-3	40	83	8.52

Note: M1: MOCAF sourdough, M2: composite MOCAF and wheat flour (1:1), M3: wheat flour sourdough.

The concentrations of the extracted DNA ranged from 17 to 83 ng/µL, while the total DNA yield was between 8.52 and 39.4 ng/µL. Microbial genomic analysis using metagenomics and RNA sequencing reveals that the type of flour used to make sourdough affects the presence of microorganisms, including the diversity and number of microorganisms in the final product [3, 69, 70].

Nanopore sequencing generated an average of 97,647 reads per sample. To ensure the quality of the data, NanoFilt was employed to retain high-quality full-length sequences. The filtering criteria included a minimum quality score threshold of 10 (Q > 10) and a minimum read length of 1,000 bp. As a result, a high-quality dataset was obtained, with an average quality score of 15 and an average read length of 1,641 bp.

3.6.1 The bacterial abundance community

The bacterial community across all sourdough samples was predominantly composed of the phylum *Bacillota* (formerly *Firmicutes*), with *Bacilli* as the dominant class. Minor variations in relative abundance were observed between MOCAF and composite flour sourdoughs, suggesting substrate-specific ecological influences (Figure 8). While *Alphaproteobacteria* and other minor classes (e.g., *Actinobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, *Flavobacteria*) were detected in low abundance, their presence indicates subtle environmental differences. At the order level, *Lactobacillales* were most abundant, particularly in wheat sourdough. *Pseudanabaenales* appeared exclusively in

MOCAF and composite sourdoughs, whereas *Acetobacterales* was more prominent in the composite variant. The family *Lactobacillaceae* was consistently dominant, with *Lactiplantibacillus* and *Levilactobacillus* prevailing across all samples. Lacticaseibacillus was more prevalent in composite and wheat sourdoughs, but less so in MOCAF, indicating that the type of substrate influences the microbial selection process.

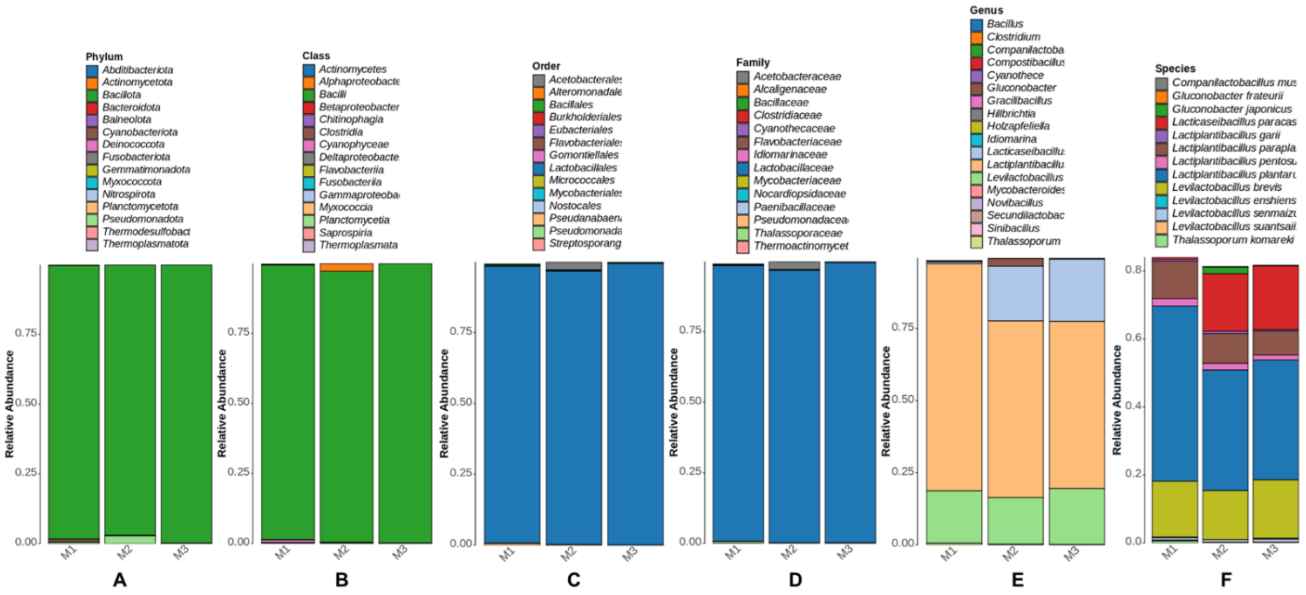


Figure 8. The top 10 taxonomic compositions of bacterial communities: A. Phylum-level, B. Class-level, C. Order-level, D. Family-level, E. Genus-level, F. Species-level

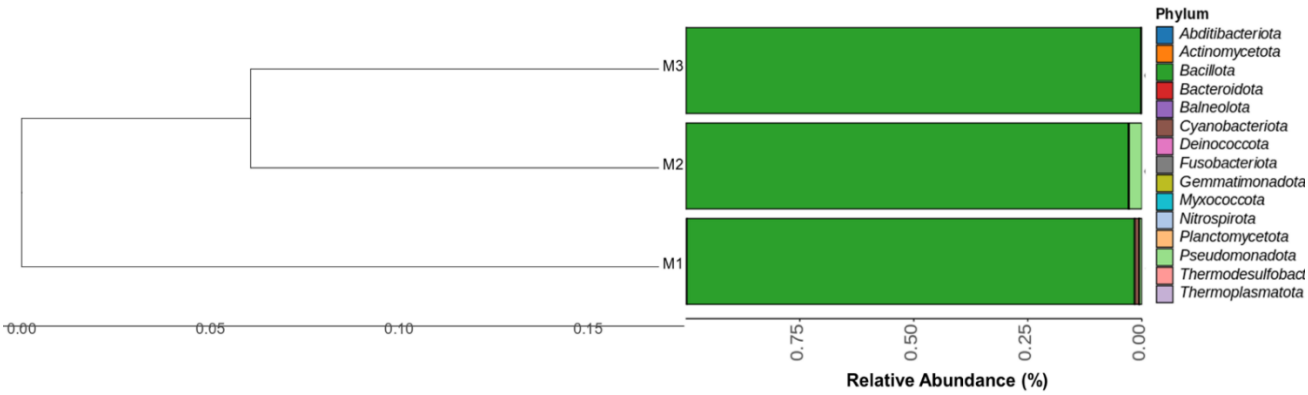


Figure 9. UPGMA cluster tree along with species relative abundance at the phylum level from sourdough (M1: MOCAF sourdough; M2: composite MOCAF and wheat flour sourdough; M3: wheat flour sourdough)

This taxonomic research revealed that although all treated sourdoughs exhibit comparable bacterial compositions at broader taxonomic levels, notable differences emerge at deeper taxonomic levels, especially within genera. These findings indicated that sourdough may preferentially support specific taxa of sourdough bacteria. In contrast, the greater diversity of the bacterial community in sourdough likely facilitates a more intricate variety of nutrients as carbon sources within the ecosystem [4, 50, 71, 72].

Lactiplantibacillus plantarum, *Lactiplantibacillus pentosus*, and *Levilactobacillus brevis* were dominant in MOCAF and composite sourdoughs, indicating that cassava-based substrates selectively promote these lactic acid bacteria. This supports previous findings on the metabolic adaptability of *L. plantarum* in carbohydrate-rich environments such as cassava [11, 71-73]. The exclusive presence of *Holzapfeliella floricola* in MOCAF samples indicates a substrate-specific association. *Holzapfeliella floricola* is a Gram-positive, catalase-negative, non-motile rod-shaped bacterium. This process employs a limited spectrum of carbohydrates, including glucose and fructose, through homofermentative metabolism, a characteristic feature of lactic acid bacteria [74, 75]. In contrast, *Lacticaseibacillus paracasei* and *Companilactobacillus musae* were more abundant in wheat sourdough, likely due to the higher availability of fermentable sugars and proteins in wheat flour [17, 59, 76]. These microbial differences have functional implications, influencing fermentation kinetics, acidification, and the sensory attributes of the final product. Understanding the microbial ecology of alternative flours like MOCAF is essential for developing gluten-free flours with enhanced shelf life, nutritional, and sensory qualities [77].

3.6.2 Similarity among sourdough samples

The unweighted pair group method with arithmetic mean (UPGMA) dendrogram on the left shows the clustering of

three samples based on their microbial community composition at the phylum level (Figure 9). The samples are grouped according to their similarities; samples that are more similar cluster closer together. In the dendrogram, two samples are more similar to each other, while the third sample is distinct, joining the cluster at a larger distance.

The observed pattern indicates that two of the samples exhibit highly comparable microbial community structures, while the third sample contains a distinctly different community. Based on the UPGMA cluster tree and the relative abundance chart at the phylum level, it is evident that the microbial communities in the three samples have low diversity, with one phylum overwhelmingly dominating all samples. However, the bar charts reveal that all samples are primarily composed of the same dominant phylum, with only minor contributions from other phyla.

This pattern suggests a highly selective environment where only certain microbial groups can thrive, leading to a lack of evenness and richness at the phylum level. Such dominance may indicate functional specialization within these communities but also reflects potential vulnerability to environmental changes, as low diversity can reduce ecosystem resilience. Overall, the combination of clustering analysis and relative abundance profiles demonstrates that while there may be minor compositional differences, the microbial communities are largely uniform and dominated by a single phylum [78-81].

3.6.3 Alpha diversity analysis

The effect of the extraction methods on alpha diversity was assessed by Chao1 (nonparametric indices used to estimate species richness in microbial communities), Abundance-based Coverage Estimator (ACE), Shannon, and Simpson indices. For the evaluation of the species richness, ACE and Chao1 indices were estimated (Figure 10). The diversity indices Shannon and Simpson (these indices provide insights into the complexity and balance of microbial communities within sourdough) were also calculated for each isolate [82]. The rarefaction curves plateaued, signifying that the sequencing depth was sufficient.

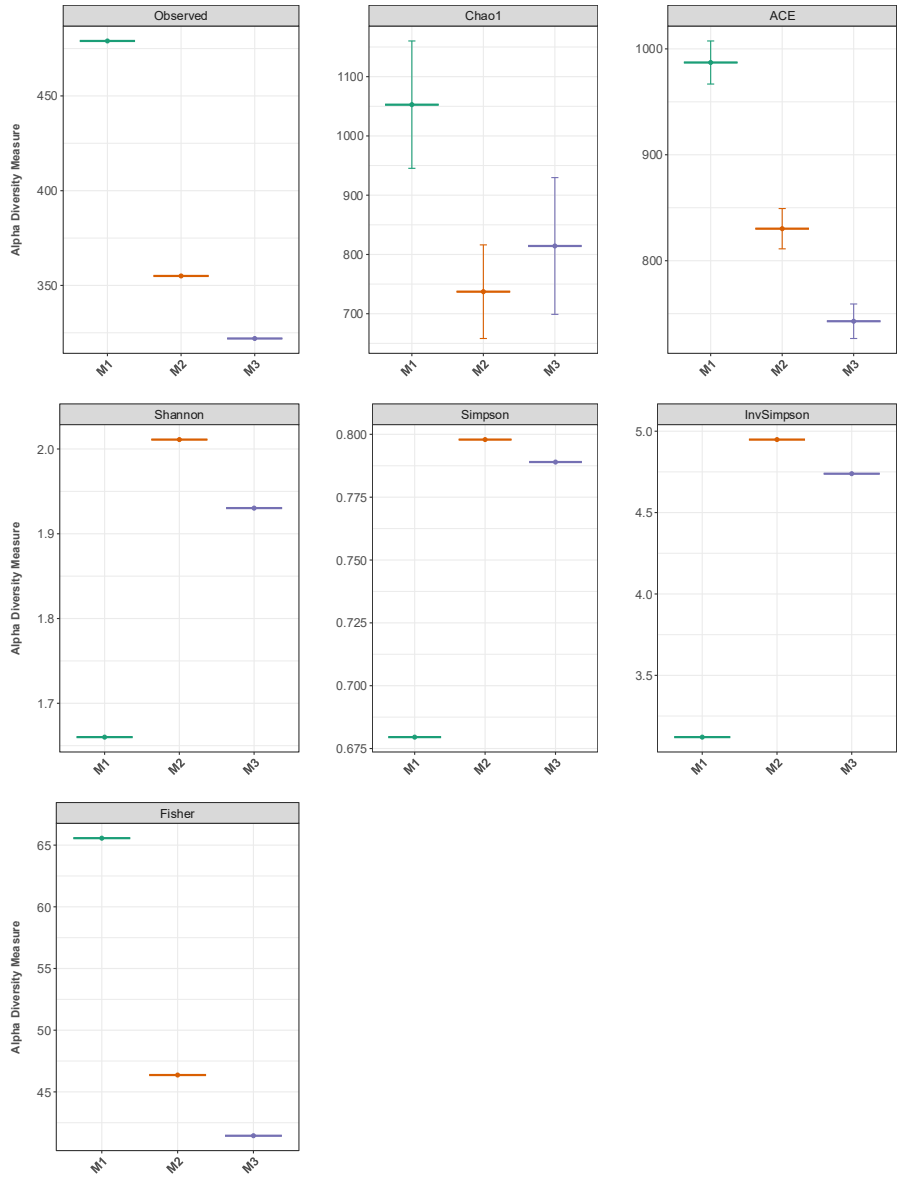


Figure 10. Comparative analysis of microbial richness and evenness on M1, M2, and M3
Observed species (Observed), richness estimators (Chao1, ACE), and diversity/evenness indices (Shannon, Simpson, InvSimpson, Fisher)

Alpha diversity analysis revealed differences in microbial richness and evenness among the sourdough variants, with MOCAF sourdough (M1) exhibiting the highest species richness (Observed = 479; Chao1 = 1052.77; Fisher's alpha = 65.56), followed by the composite (M2) and wheat (M3) sourdoughs. Despite its richness, M1 displayed lower evenness, suggesting dominance by a few acid-tolerant taxa, notably *Lactiplantibacillus plantarum*. The observed difference in richness and evenness in M1 indicates a functional specialization, whereas dominance drives ecosystem activity towards significant acidification instead of encompassing multifunctionality [82, 83]. In contrast, M2 showed the highest diversity indices (Shannon = 2.01; Simpson = 0.8), indicating a more balanced microbial community structure. The composite substrate likely supports a synergistic microbial environment due to the combined availability of fermentable carbohydrates and proteins from both MOCAF and wheat flours [77]. This balanced diversity enhances fermentation stability, acid and gas production, and potentially improves leavening performance and resilience through functional redundancy [58, 82]. Conversely, the high gluten content in wheat flour favors species such as *Lacticaseibacillus paracasei*, contributing to a more uniform but less diverse community [58].

3.6.4 Beta diversity and community

The Bray-Curtis approach was employed for beta diversity analysis, which was subsequently visualised through principal coordinates analysis (PCoA). Beta diversity analysis using PCoA (Figure 11) revealed distinct clustering of microbial communities across sourdough samples, corresponding to flour type [70, 84]. MOCAF sourdough (M1) exhibited the most distinct microbial profile, clustering separately along the primary PCoA1 axis, which accounted for 75.03% of the total variation. Composite sourdough (M2) occupied an intermediate position, while wheat sourdough (M3) clustered along the PCoA2 axis (24.97% variation), indicating more subtle differences. These findings suggest that flour type exerts a strong influence on microbial community structure, with MOCAF and wheat flours imposing distinct selective pressures on microbial colonization and succession [69]. Principal component analysis (PCA) further supported these results, with PC1 and PC2 explaining 87.03% and 12.97% of the variance, respectively. MOCAF sourdough demonstrated a uniquely differentiated microbial composition, while composite and wheat sourdoughs showed more nuanced distinctions, likely due to differences in substrate composition [43, 54].

3.7 Correlation between bacterial diversity and sourdough MOCAF characteristic

Spearman correlation analysis revealed significant associations between dominant bacterial species and key physicochemical parameters of sourdough, including leavening capacity, pH, and TTA [71]. Based on Figure 12, among the 30 most abundant taxa, nine species demonstrated strong correlations with these parameters. *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, and *Gluconobacter japonicus* exhibited strong positive correlations with pH and leavening capacity, but negative correlations with TTA, suggesting their role in moderating acidity.

These *Lacticaseibacillus* species are known for their probiotic potential and tolerance to mildly acidic environments [43]. *G. japonicus*, an acetic acid bacterium, may influence pH and microbial dynamics through acetic acid production [85]. These findings underscore the importance of microbial composition in shaping sourdough fermentation outcomes and highlight the potential of MOCAF as a substrate for modulating microbial behavior.

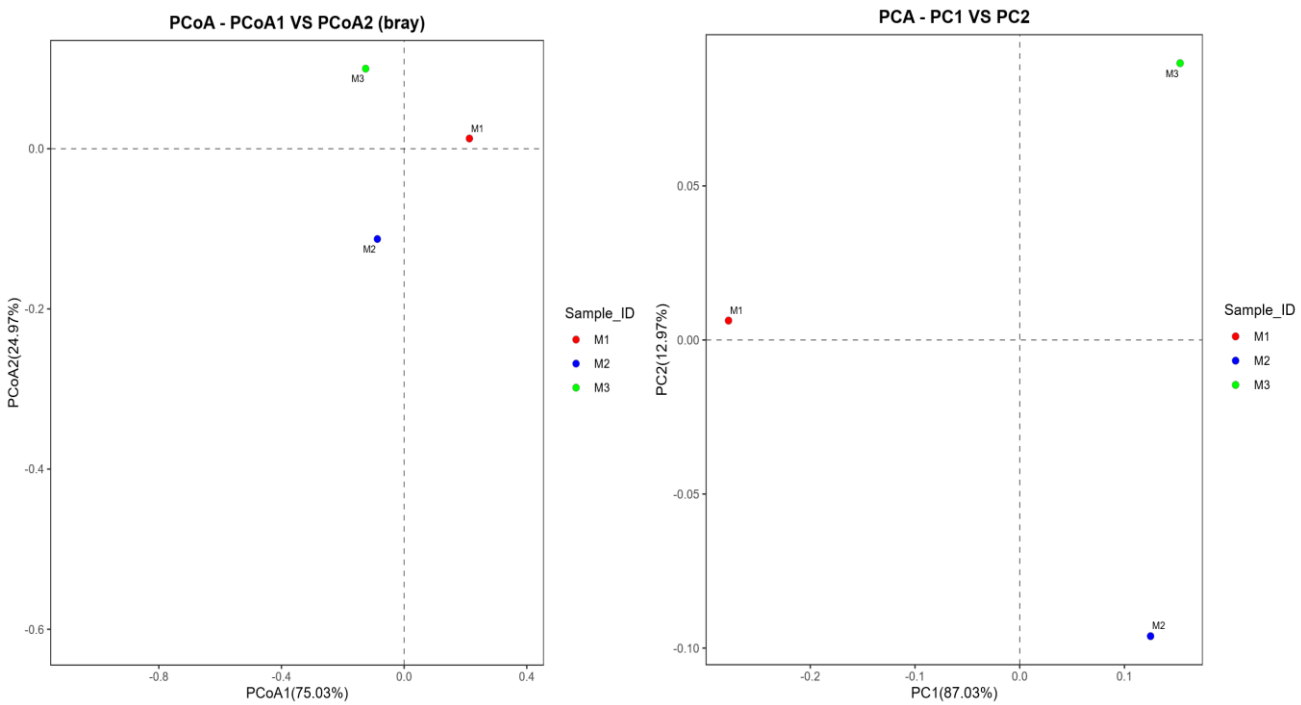


Figure 11. Principal coordinate analysis (PCoA) and principal component analysis (PCA) on M1, M2, and M3

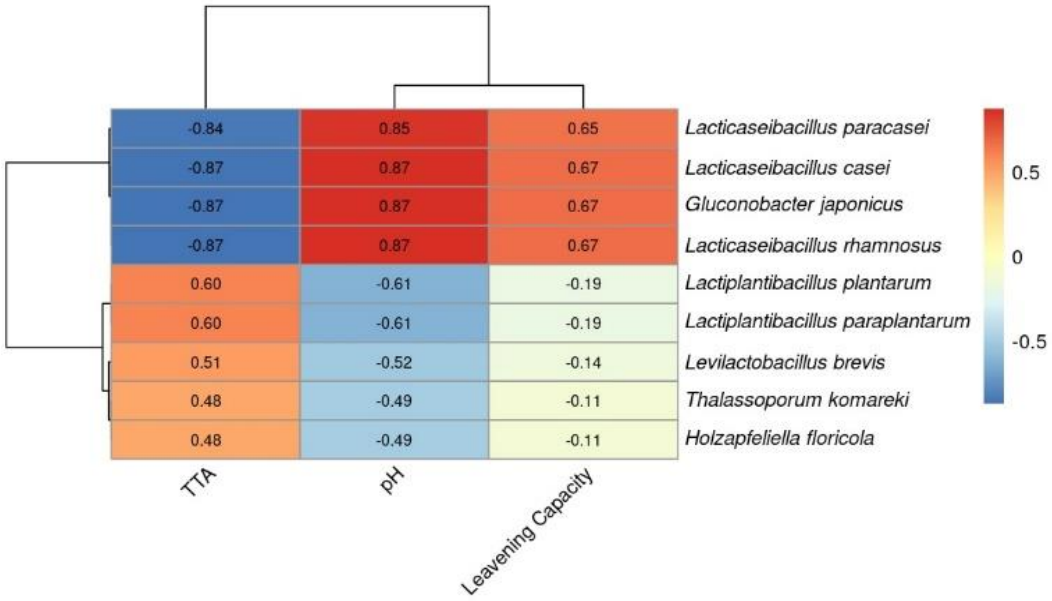


Figure 12. Spearman correlation analysis between significant species and sourdough characteristics (pH, total titratable acidity, and leavening capacity)
Significant correlations (FDR-adjusted $p < 0.05$)

L. plantarum and *L. paraplantarum* exhibited strong negative correlations with pH and positive correlations with TTA, highlighting their central role in sourdough acidification [86]. *L. plantarum*, a dominant and acid-tolerant lactic acid bacterium, is enriched in MOCAF-based sourdough due to its enzymatic capacity to degrade cassava starch, facilitating rapid acidification. Similarly, *Levilactobacillus brevis* demonstrated a preference for acidic environments and contributed significantly to acid production during later fermentation stages [87, 88]. The resulting pH decline not only favors acidophilic taxa such as *Lacticaseibacillus* spp. but also alters the overall microbial community structure [89, 90]. In contrast, *Lacticaseibacillus paracasei*, *Lacticaseibacillus casei*, *Lacticaseibacillus rhamnosus,* and *Gluconobacter japonicus* were positively associated with leavening capacity. These species, particularly *G. japonicus*, enhance CO_2 production via acetic acid synthesis, potentially stimulating yeast activity and improving dough expansion. Their prevalence in composite and wheat sourdoughs suggests synergistic interactions that support balanced fermentation dynamics.

Conversely, *L. plantarum*, *L. paraplantarum*, and *L. brevis* showed weak to moderate negative correlations with leavening, likely due to their homolactic metabolism, which limits CO_2 output [4, 87, 89, 91]. While MOCAF sourdoughs exhibit high acidification and complex flavor profiles due to elevated acetic acid levels, their low gluten content and limited gas retention result in denser textures. In contrast, wheat-based sourdoughs benefit from a balanced production of acids and gases, which promotes better dough structure and volume. Overall, the microbial composition plays a crucial role in determining the physicochemical, sensory, and functional properties of sourdough.

4. CONCLUSIONS

This study demonstrates that flour type is a key determinant of microbial diversity and fermentation dynamics in spontaneously fermented sourdoughs. MOCAF-based sourdoughs selectively enriched acid-tolerant *Lactiplantibacillus* species, particularly *L. plantarum*, contributing to pronounced acidification but limited leavening capacity due to homolactic fermentation and low CO_2 production. Notably, *Holzapfeliella floricola* was uniquely identified in MOCAF sourdough. In contrast, composite sourdoughs (MOCAF and wheat flour) supported a more balanced microbial community, enabling both homolactic and heterofermentative pathways through the combined availability of glucose and xylose. This resulted in improved leavening and acidification performance. These findings highlight the potential of MOCAF as a gluten-free substrate for sourdough fermentation, offering distinct microbial profiles and flavor characteristics.

While this study focused on bacterial communities, future research should explore yeast populations and their interactions in MOCAF-based systems. Additionally, isolating key bacterial strains may facilitate the development of targeted starter cultures for optimized sourdough functionality and product quality, especially in bakery products. MOCAF sourdough demonstrates potential as a starter for producing high-quality, shelf-stable gluten-free bread for individuals with celiac disease, attributed to its significant acidification that guarantees enhanced microbial stability.

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