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Microbial Diversity Using a Metataxonomic Approach, Associated with Coffee Fermentation Processes in the Department of Quindío, Colombia

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Abstract: Coffee fermentation is a complex process, mainly involving bacteria and yeasts, whose interaction influences beverage quality. The way this process is conducted affects the interactions between these microorganisms. To identify microbial diversity in fermenting coffee, samples were collected from 20 farms in the Department of Quindío, Colombia. Metataxonomic analyses using high-throughput sequencing and volatile organic compound identification in green coffee beans were performed with HS-SPME and GC-MS. Potential relationships between some families and genera with different fermentation types and coffee quality were evaluated. In our results, samples presented with high richness and diversity were greater for bacteria than for yeast/fungi. The Enterobacteriaceae family dominated at the beginning of fermentation, while *Leuconostoc*, *Lactobacillus*, *Gluconobacter*, and *Acetobacter* genera dominated at the end, a finding related to pH reduction and final coffee quality. Overall, 167 fungal families were identified, but Saccharomyceaceae dominated from the beginning. Alcohols and esters were the main chemical classes identified in green coffee bean samples from these fermentations. These results will facilitate the identification process conditions that influence the presence and abundance of microorganisms related to quality as well as contributing to the design of strategies to conduct fermentations to improve the final quality of coffee.

Keywords: coffee fermentation processes; *Coffea arabica* L.; microbial diversity; high-throughput sequencing; lactic acid bacteria; yeast; coffee quality

Citation: Peñuela-Martínez, A.E.; Velasquez-Emiliani, A.V.; Angel, C.A. Microbial Diversity Using a Metataxonomic Approach, Associated with Coffee Fermentation Processes in the Department of Quindío, Colombia. *Fermentation* **2023**, *9*, 343. https://doi.org/10.3390/fermentation9040343

Academic Editors: Wanping Chen and Bin Wang

Received: 13 March 2023
Revised: 26 March 2023
Accepted: 27 March 2023
Published: 29 March 2023

1. Introduction

Coffee continues to be the second-most-consumed beverage in the world after water and represents the second-most-traded and highest-valued global commodity on the market after oil. For the 2021/22 coffee year, an increase of 3.3% in consumption was projected compared to the previous year, while the total world production of 60 kg bags of green coffee was 167.2 million [1]. Colombia remains the third or fourth largest coffee-producing country, accounting for nearly 7 to 9% of world production, depending on the year, with the country producing between 12 and 14 million 60 kg bags per year between 2017 and 2021. Colombian coffee stands out in the international market for its permanent fresh product availability due to its softness and beverage quality, which are essentially due to the combination between the planted varieties of the Arabica species (*Coffea arabica* L.) and the wet process method [2–4]. This method consists mainly of removing different parts of the coffee fruit, such as the exocarp (pulp) and mesocarp (mucilage), to obtain the endosperm (green bean), which is covered by the endocarp (parchment). The processed beans are subjected to solar or mechanical drying until reaching a humidity between 10% and 12% (dry parchment coffee) and then are subjected to the subsequent processes of threshing, roasting, grinding, and beverage preparation. To remove the mucilage, which is a gelatinous substance with a high water content that is rich in

polysaccharides, reducing and non-reducing sugars [5,6], the method that is traditionally used is fermentation, in which microorganisms carry out the transformation of complex compounds into simpler substances that are removed from the bean by washing with clean water [7–9]. Isolation, identification, and characterization of pectinolytic yeasts for starter culture in coffee fermentation have shown the potential to obtain, in this case, pectinase enzymes (pectin lyase, polygalacturonase, and pectin methyl esterase) to accelerate the fermentation process [6], and to improve antioxidants, flavonoids, and polyphenols. A previous study using a commercial formulated pectin-lyase obtained from *Aspergillus niger* fungi accelerated degradation of coffee mucilage, with no effects on quality [10]. Native or genetically engineered microbiota are alternatives to improve fermentations, as shown for second-generation sugars to generate energy in biorefineries using non-traditional crops as raw material [11]. A complete recent review on coffee fermentation by Elhalis et al. [5] explored the possible ways to convert the traditional process carried out by microorganisms into a more controlled and industrialized process, presenting several fungi and bacteria as contributors to fermentation and quality when they are starter cultures or inoculated.

In coffee farms, the time and the way to carry out this fermentation process are variable and dependent mainly on the coffee grower, who is influenced by empirical knowledge and market stimuli. The way in which fermentation is performed affects not only the microbial community's behavior in the process but also has an impact on the coffee quality, given the change in the bean's chemical composition [3,9,12–14]. Due to the above, the study of microorganisms that participate in this fermentation stage, their population, and their temporal dynamics has become the focus of several studies, and these are key aspects to understand the influence of microorganisms on the coffee beverage quality of this product and their potential to maintain or improve chemical and sensory quality attributes. Studies using microorganism-independent culture techniques have provided significant technological advances in recent decades. Technologies such as high-throughput sequencing (HTS) of the nucleic acids in these microorganisms have been commonly used to determine the taxonomic structure and microbial community profiles in food fermentation [15–18]. This technique, also known as metagenomics, involves sequencing specific amplified regions of DNA or amplicons, allowing for taxonomic classification at the genus level of the populations involved in these processes, which contributes to an understanding of the role played by them in fermentation or other processes [6,16,19]. Additionally, with these identification techniques, progress has been made in knowledge about the role of microorganisms as modifiers of coffee beans' chemical composition, which is reflected in the final beverage quality [5,8]. From the previously generated information, it is also known that coffee fermentation can be considered as a closed system, in which microorganisms coexist from the beginning to the end of the process and may or may not be identified, depending on their relative abundance [20,21].

The microbial diversity present in a coffee fruit depends on the crop variety, the processing method, the environmental factors of the region where it is cultivated, and on cultivation practices [21,22]. In this diversity, lactic acid bacteria, both homofermentative and heterofermentative, have been identified as important and predominant, especially the *Lactobacillus* and *Leuconostoc* genera, respectively, along with yeasts, such as the *Pichia* and *Candida* genera [3,9,13,14,23,24]. On the other hand, in the same producing region, coffee is obtained with different quality characteristics, influenced by predominant factors, such as agroecological conditions, crop characteristics, such as variety and handling, and the type of postharvest process. In addition, several aspects can influence microbial composition and load present in the fermentation process, including the fruit's ripeness and handling, and the logistics at harvest and postharvest. Likewise, they can favor cross-contamination throughout the harvest, processing, and milling processes due to contact with utensils, equipment, fermentation tanks, and water used in the process [9,21,25].

Several studies have been carried out examining fermentation types, based on a single coffee variety, origin, or a specific farm, so the results are specific in that regard [21,22,26–29]. Consequently, there are still discrepancies regarding the role of predominant microorgan-

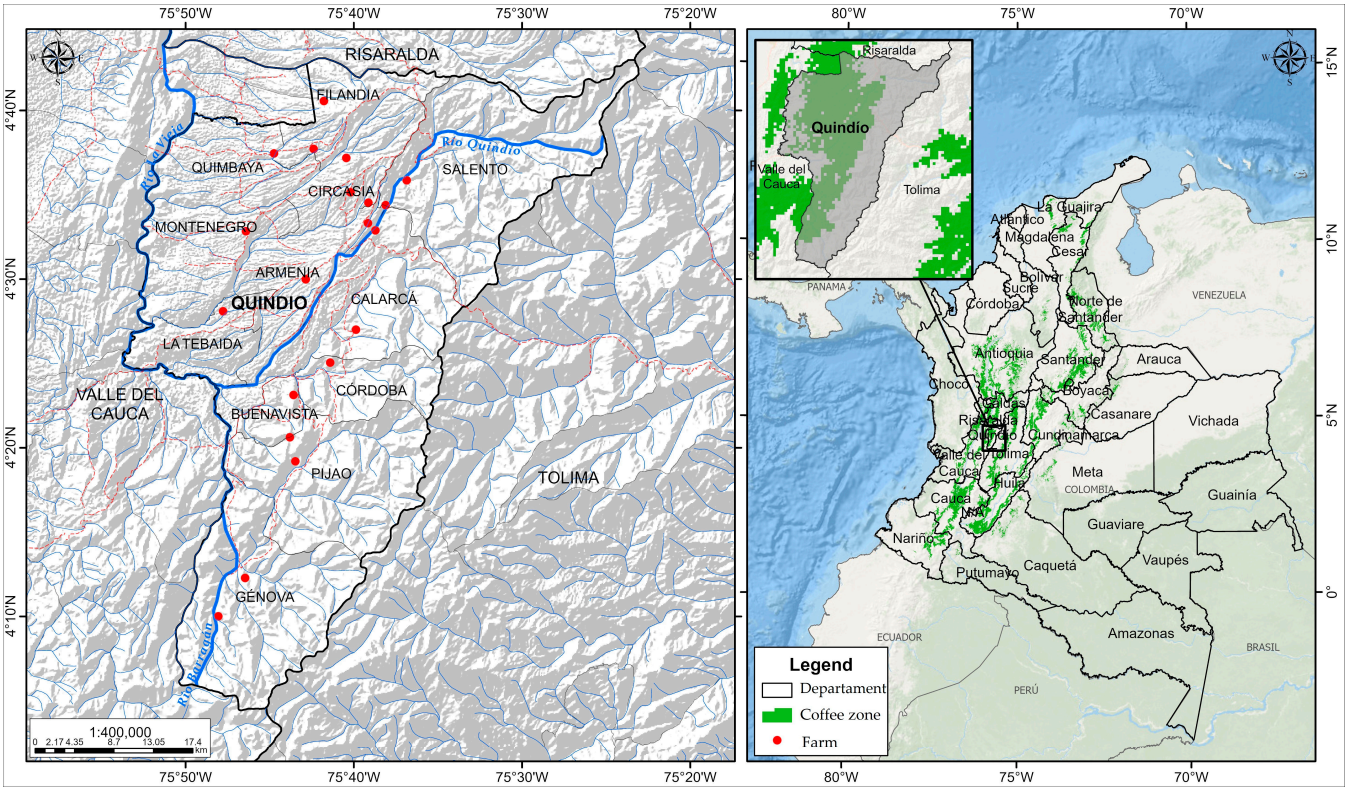
isms in fermentation [9,13,14,20]. The general conclusion of these studies is the need to expand the information on the diversity, richness, abundance, and dynamics of the microbiota involved in coffee fermentation, in different regions and production conditions or contrasting ones, and to perform wider taxonomic characterizations.

In this study, metataxonomic sequencing was applied to describe the attributes of diversity and richness of microbial communities in the coffee fermentation process, produced on different farms in the Department of Quindío, Colombia's central coffee zone. The objective was to establish and describe the existing microbiota and their relationship with fermentation processes carried out in selected farms and the consequences on coffee beverage quality, considering the variability in factors that affect the microbial composition of this process.

2. Materials and Methods

2.1. On-Farm Coffee Fermentation and Sampling

Sampling was carried out in selected coffee-producing farms, growing the variety *Coffea arabica* L. Castillo®, whose processing method includes spontaneous fermentation to obtain washed coffee. Samples of coffee and mucilage were collected at the beginning and at the end of fermentation during the harvest season of the second semester of 2021, and the resulting dry parchment coffee was also sampled. In total, we studied 20 representative farms (sampling units) located in 12 municipalities of the Department of Quindío, in the central coffee zone of Colombia (Figure 1).



(**A**) (**B**)

Figure 1. Geographic location of the study area in the Department of Quindío (**A**), in the central coffee zone of Colombia (**B**).

The location and mean annual average environmental conditions of each farm [30] are presented in Table 1. Additionally, to describe the fermentation type for each farm,

information was recorded using a structured format with variables related to the process (Table S1).

Table 1. Information on type of samples collected from selected coffee farms in 12 municipalities of the Department of Quindío, Colombia.

Sample-ID	Municipality	Altitude (m)	Temperature Mean Annual Average. (°C)	Relative Humidity Mean Annual Average. (%)
F01	La Tebaida	1179	22.0	76
F02	Salento	1642	19.3	81
F03	Filandia	1623	19.3	80
F04	Calarcá	1550	20.4	79
F05	Córdoba	1610	19.4	80
F06	Buenavista	1695	18.7	81
F07	Génova	1593	19.8	80
F08	Circasia	1646	19.1	81
F09	Quimbaya	1356	21.0	77
F10	Armenia	1599	19.5	80
F11	Armenia	1317	21.2	77
F12	Génova	1832	18.1	83
F13	Circasia	1600	19.5	80
F14	Filandia	1558	19.9	80
F15	Montenegro	1288	21.4	77
F16	Armenia	1551	19.9	80
F17	Pijao	1721	18.5	82
F18	Salento	1561	19.8	80
F19	Calarcá	1467	21.1	78
F20	Buenavista	1329	21.1	77

2.2. pH and Temperature Measurement

The pH of coffee bean mass and the fermentation temperature were determined in two sample types, at the beginning and at the end of fermentation, using a wireless pH meter with automatic temperature compensation (HI 10532/Halo®). The equipment was calibrated prior to each use with pH 7.0 and pH 4.0 buffer solutions (HI 7004L/C; HI 7007L/C; HANNA®). The pH values were recorded by directly immersing the electrode in the coffee fermentation mass at each sampling moment.

2.3. Coffee Beverage Sensory Quality

From the dry parchment coffee samples from each farm, the parchment was removed through a threshing process to obtain green coffee beans, and the beans were roasted and ground, followed by beverage preparation, according to the Specialty Coffee Association (SCA) procedure [31]. Samples were sensorily evaluated by an expert panel in coffee sensory analysis, formed by three taster raters with Q-Grader certification [32]. The evaluation protocol is internationally recognized, and it considered ten attributes for the coffee beverage sensory profile, including: fragrance/aroma, uniformity, cleanliness, sweetness, flavor, acidity, body, aftertaste, balance, and overall impression. Each attribute was evaluated on a scale up to 10 points, and the sum of the values of these attributes generated each sample qualification; the higher the score, the better the beverage quality. This scale made

it possible to identify alterations in the attributes that reveal unpleasant flavors, identified as quality defects, whose total cup scores are less than 60 points [31].

2.4. Volatile Organic Compound Determination

The green coffee beans were cryogenically ground and stored at −20 °C in plastic containers until analysis. The method used to determine volatile organic compounds was carried out by means of headspace solid-phase microextraction (HS-SPME) in conjunction with gas chromatography coupled with mass spectrometry (GC-MS), according to the procedure of Peñuela-Martínez et al. [7], with modifications as follows: for the extraction of volatile compounds, 1.0 g of ground coffee was placed in solid-phase microextraction (SPME) vials. Samples were placed for 50 min in an oven at 60 °C to reach sample headspace equilibrium. Compounds were trapped using a polydimethylsiloxane/divinylbenzene (PDMS/DVB)-type 65 µm SPME fiber assembly, Stableflex 23 Ga Autosampler (pink) (Sigma Aldrich, Milwaukee, WI, USA). The desorption process was conducted in a GC injection port for 2.9 min at 250 °C in splitless mode. The separation process for chromatography analysis was performed with a DB-FFAP J&W GC column measuring 60 m × 0.25 mm in an Agilent Trace 1300 Gas Chromatographer, coupled with an Agilent TSQ duo Mass Spectrometer, where detection was performed. The carrier gas was helium with a 1.0 mL/min flow. From 3.5 min onwards, column temperature was programmed from 40 °C to 180 °C at 5 °C/min ramp, and then at 6 °C/min until reaching 240 °C, for a total running time of 60 min. Compound identification was performed according to the mass spectrum comparison with the recognized databases Wiley 275 Mass Spectral Data and NIST 98 Mass Spectral Library [33]. Furthermore, compound concentrations were related to the area under the curve of each chromatographic peak.

2.5. DNA Extraction and Quantification

Samples of 50 g of coffee beans and mucilage were collected at the beginning (CD) and at the end of fermentation (CF), in triplicate, in Falcon™ DNase/RNase-Free sterile conical tubes, from each of the sampling units (farms), and they were labeled and stored at −80 °C until genetic material extraction. For genomic DNA (gDNA) extraction, a commercial QIAGEN kit, DNeasy Powerlyzer Powersoil was used according to the Qiagen protocol (Hilden, Germany) [34]. At the end of the extraction process, DNA quantification was performed by means of the light absorbance method at 260 nm and 280 nm using a NanoDrop™ 2000-Thermo Scientific™, and purity was estimated based on its relationship: A260 nm/A280 nm. The subsequent gDNA samples were normalized to a 30 ng/µL concentration, then frozen at −20 °C for massive sequencing processes. This procedure was performed with the service provider Centro Nacional de Secuenciación Genómica—CNSG (Medellín, Colombia).

2.6. Illumina MiSeq Amplicon Sequencing of Bacterial 16S rRNA Gene and of Fungal ITS Region

Extracted DNA samples were used for microbial diversity (metataxonomic) analyses for bacteria and fungi. Sequencing was performed by service provider Macrogen Inc. (Seoul, Republic of Korea). For the deep sequencing of bacterial 16S ribosomal genes, a fragment containing the V3 and V4 variable regions was amplified using the oligonucleotide primers Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-GACTACHVGGGTATCTAATCC-3′) [35]. For fungal diversity, amplification of the ITS2 region was performed using the primers ITS3F (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′) [36]. Deep sequencing was performed on the Illumina MiSeq platform, generating 300 base-paired (bp) reads each. Sequence reads were trimmed with a Q30 quality threshold, with a probability of incorrect base calling of 1 in 1000, representing an accuracy of 99.9%. Using bioinformatics routines, unpaired sequences (singletons) and sequences with a length of less than 200 bp or those with ambiguous bases were eliminated using the Cutadapt version 3.5 program [37]. Subsequently, 20,000 sequences were randomly selected to be included in bioinformatic analyses. Sequence

quality and classification analyses were performed using MOTHUR version 1.44 platform, using default parameters [38]. Sequences with homopolymers greater than six, chimeric sequences, and contaminants belonging to other lineages, such as chloroplasts, mitochondria, and eukaryotes, were eliminated.

2.7. Microbiome and Statistical Analysis

The sequencing data were normalized with the *normalize.shared* command of the MOTHUR package using default parameters. Sequences of each case were grouped into operative taxonomic units (OTUs), considering a limit distance between sequences of 0.03 (97% similarity), using the *dist.seqs* command. Sequences for fungi and bacteria were taxonomically assigned according to the SILVA v138 database [39], using the VSEARCH method [40]. Fungi and bacteria phylogenetic classification was performed using the *Classifier* tool from the RDP (Ribosomal Database Project) database, with a bootstrap of 80. To identify the richness and diversity of OTUs in coffee samples at the beginning and at the end of fermentation in the different sampling units (farms), the Rstudio version 2022.07.0 program was used with Phyloseq [41] and Microbiome [42] packages.

For alpha diversity, richness, and abundance, the Observed Species (S_{obs}) and Chao1 [43] indexes were estimated, and Shannon and inverse Simpson diversity indexes were calculated [44,45]. A comparison of taxonomic abundances in the studied groups was performed using Kruskal–Wallis non-parametric tests [46], with a p value < 0.05. Analysis of beta diversity or the relationship between the species diversity and sample type was performed with PERMANOVA [47], with 999 permutations ($p < 0.05$), utilizing RStudio version 2022.07.0, using the distance or Bray–Curtis dissimilarity [48] and represented graphically with *ggplot* version 3.3.2, by means of non-metric multidimensional scaling (NMDS) analysis [49]. Additionally, Spearman's correlation coefficient was estimated to explore relationships between factors associated with sampling, such as cultivation altitude, pH, type and time of fermentation, abundance of genera and families of dominant bacteria and yeasts, and coffee beverage quality rating by SCA.

3. Results

3.1. Coffee Fermentation Processes and Sample Characteristics

The characterization of processes in the wet method identified in the sampled farms showed five ways of carrying out fermentation, as described in Table 2, which also presents variable times to finishing fermentation. The traditional or spontaneous process was the most frequently identified, followed by fermentation in a mixture of batches, and performed underwater. For all samples, pH values at the beginning of fermentation were between 4.34 and 5.95, typical for mucilage from recently depulped coffee, even for the cases in which there was a waiting time for the fruit. The end of fermentation was indicated by a decrease in pH, and pH values varied between 3.00 and 4.51 (Table S1). In the latter case, the observed wider variation is due to different fermentation times, which depended on the process carried out under non-controlled fermentation; the longer the time, the greater the acidity. A pH decrease is indicative of the metabolic activity of these microorganisms, and an increase in temperature is also generated. The temperature values of samples corresponding to the beginning of the process were between 19.1 °C and 26.5 °C, and at the end of the fermentation process, they were between 17.7 °C and 26.3 °C (Table S1). This behavior could be related to the environmental temperature when the samples were collected. Higher values at the beginning of fermentation were influenced by the "field heat" that the fruits possessed when they were harvested during the day. At the end of fermentation, the temperature decreased, usually overnight, trying to equilibrate to the atmosphere in the early-morning hours, as occurred in most cases and farms. Only for prolonged fermentations, the final temperature increased above the initial one due to heat accumulation generated by many reactions presented throughout this process. Data for each farm (sampling unit) are presented in Table S1.

Table 2. Description of fermentation types, time, and frequency in sampled farms.

Fermentation Type	Description	Time (h)	Frequency (%)
Traditional	Fermentation occurs spontaneously until the mucilage is broken down into water-soluble compounds, to be removed from the grains by washing. It usually happens over one night.	12–16	55
Prolonged	After mucilage is degraded, fermentation is terminated after an additional time, arbitrarily defined by the coffee farmer.	24–72	10
Mix of de-pulped coffee batches	Fermentation is carried out with batches of de-pulped coffee from different days of harvest. The amounts of mixed coffee are not controlled.	48–72	15
Underwater	This corresponds to traditional fermentation with the addition of water until the de-pulped coffee mass is completely covered. The relationship between the volume of water (L) and the amount of coffee (kg) is not controlled.	14–24	15
Induced Anaerobic	Coffee fruits or berries are placed in closed containers to avoid contact with air. Generated lixiviates are collected and periodically added to the coffee mass.	72–96	5

3.2. Coffee Sensory Quality and Volatile Organic Compounds in Green Coffee Beans

Sensory beverage analysis of coffee samples from different fermentation processes showed that 80% of them had no defects (clean cup) (Table S1), which indicates SCA scores higher than 80 points, describing the representative and characteristic quality of Colombian mild coffee, in addition to being classified as specialty coffee according to the SCA category scale [31]. Most of these samples (66.7%) presented scores between 80 and 81 SCA points, whereas 26.7% had scores between 82 and 83, and the remaining 13.3% had scores higher than 83 points. Samples with scores below 60 points (20%) presented sensory defects, identified as earthy, phenol, and fermented; the latter is related to uncontrolled fermentation, as one of the causes generating it.

Regarding volatile organic compound determination, 45 compounds were identified in green coffee bean samples, which were grouped into 8 chemical classes: alcohols (9), esters (12), ketones (2), lactones (4), aldehydes (5), furans and pyrazines (5), amines and amides (5), and pyridines and pyrrolidines (3). Figure 2 shows the proportions of chemical classes and predominant compounds in green coffee samples (compound-specific values are presented in Table S2). The first five classes were products of microbial metabolism; the remaining three classes were products of thermal reactions in postharvest. Descriptive differences in compound proportions were identified, possibly associated with differences in fermentation types. Compounds, such as phenylethyl alcohol and methyl anthranilate, were present in all samples, while 2,3-butanediol, ethyl acetate, and 3-hydroxybutanone were only present in samples that received the highest SCA cupping scores. The high concentration of 2,3-butanediol was only found in sample F20 (Table S2).

3.3. Microbial Richness and Diversity Using Illumina-Based Amplicon Sequencing

In total, 40 samples with replicates, corresponding to the beginning and the end of fermentation, were collected from 20 coffee-producing farms in the Department of Quindío and analyzed to determine their fungal and bacterial abundance and diversity. To establish bacterial diversity, 22,308,962 reads were obtained from high-quality sequencing, with an average per sample of 187,470 ± 24,791 reads, ranging between 126,600 and 262,032 reads. Reads were clustered into a mean number of 184 ± 95 OTUs (ranging 69–563 OTUs) (Table S3). Likewise, to estimate fungal diversity from the same number of samples, 20,262,224 reads were generated, with an average of 168,713 ± 28,548 reads (range from 97,110–269,076 reads), which were grouped into 80 ± 44 OTUs, ranging 27–324 OTUs (Table S4). Coverage analysis of sequences showed a value greater than 91% (Tables S3 and S4), indicating that they were representative, sufficient, and had appropriate

characteristics to study bacterial and fungal diversity in those samples at the beginning and at the end of the coffee fermentation process.

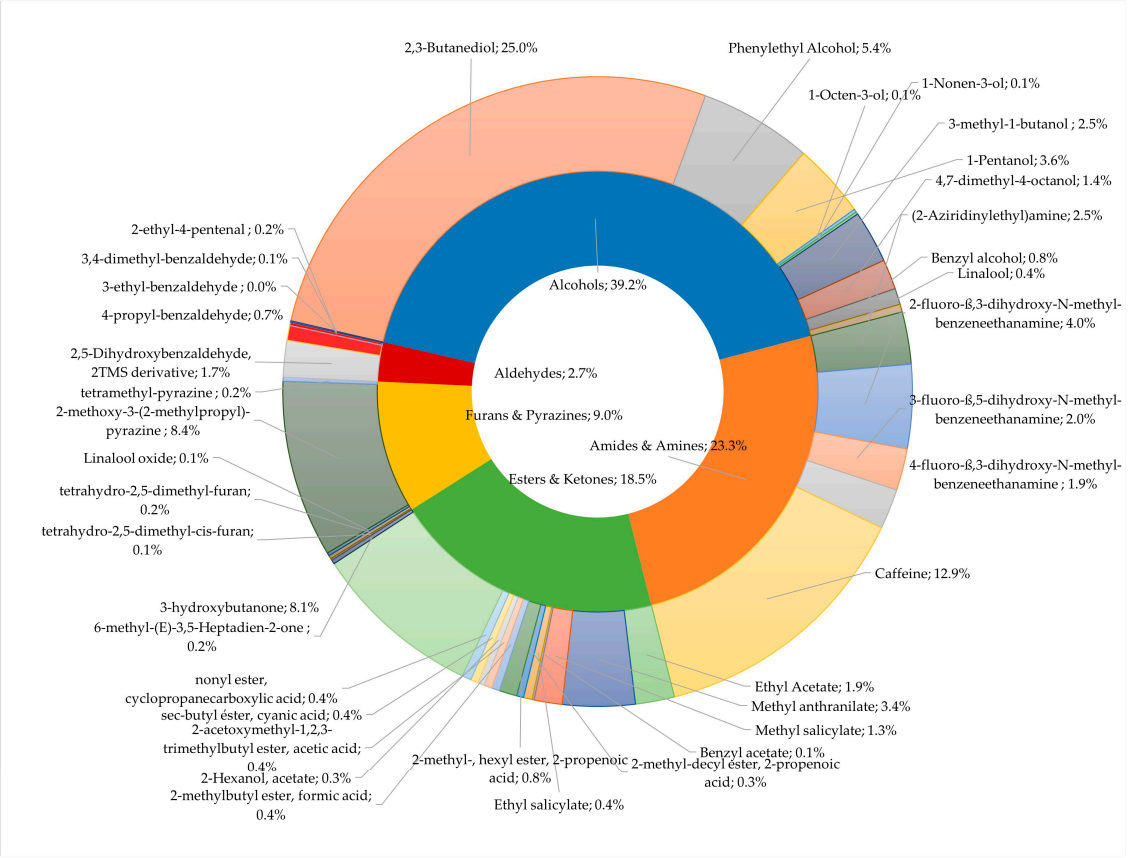


Figure 2. Volatile organic compounds found in green coffee bean samples in selected studied farms in Department of Quindío, Colombia.

Richness and diversity estimations were carried out by estimating the observed (S_{obs}), Chao1, Shannon, and inverse Simpson indexes (Figure 3). Results based on Kruskal–Wallis tests indicated that the effective number of OTUs, according to the S_{obs} index, presented significantly greater differences ($p < 0.05$) for bacteria and yeast (fungi) microbiota in coffee beans at the beginning of fermentation (CD). They were significantly more abundant ($p < 0.05$), according to the Chao1 index, with respect to the samples from the end of fermentation (CF). Likewise, from bacterial and fungal diversity index estimation, it was determined that samples corresponding to the beginning of fermentation (CD) had a greater number of equally frequent, abundant, and diverse OTUs (Shannon and Inverse Simpson) in comparison to samples from the end of fermentation (CF).

Observed OTUs for both bacteria and yeast/fungi presented significant differences with respect to farms ($p < 0.05$). Bacterial diversity between farms was statistically equal, according to Kruskal–Wallis test results for Shannon and inverse Simpson indexes ($p > 0.05$), contrary to what was obtained for fungal diversity, which presented statistical differences ($p < 0.05$). These results, indicating different levels of diversity, were possibly related to differences in the fermentation processes performed on the farms included in this study (Figure S1).

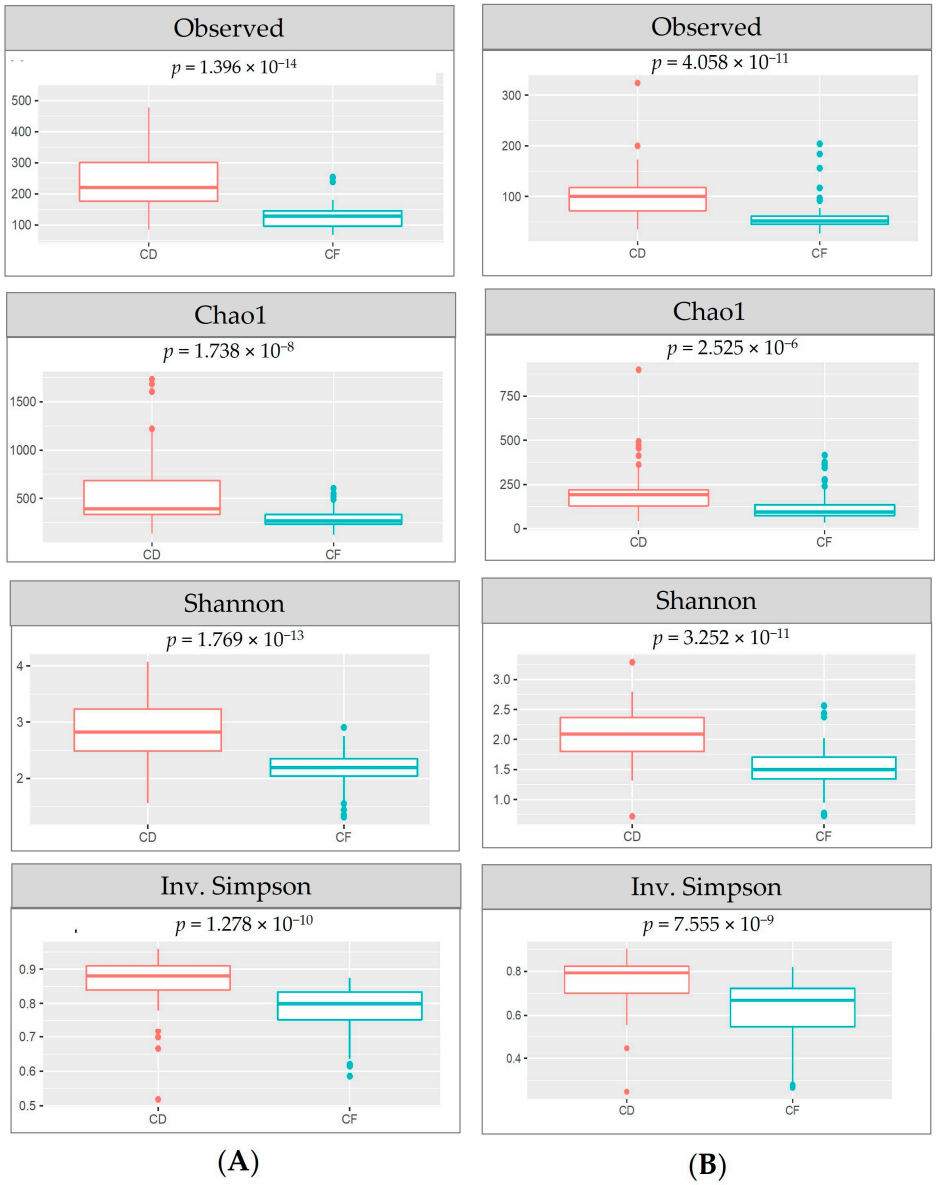


Figure 3. Boxplot of alpha diversity indexes, namely Observed, Chao1, Shannon, and inverse Simpson, for bacterial communities (**A**), as well as for yeast/fungal microbiota (**B**), for the two sample types. Samples are colored according to sample type: pink for samples at the beginning of fermentation—CD—and light blue for the end of fermentation—CF.

3.4. Taxonomic Composition and Relative Abundance of the Microbial Community in Coffee Fermentation Samples

Taxonomic assignments for 16S rRNA gene sequences were mainly distributed among 36 bacterial phyla, 465 families, and 1349 genera. In general, the Proteobacteria phylum was the most abundant (66.22%), followed by Firmicutes (31.22%). The phyla Bacteroidetes and Actinobacteria represented about 1.0%. At the family level, Enterobacteriaceae was the most predominant (34.78%), followed by Acetobacteraceae (23.71%) and Leuconostocaceae (23.05%). The Lactobacillaceae (6.46%) and Pseudomonadaceae (1.96%) families were identified in lower proportions (Table S5).

Bacterial genera listed in Figure 4 represented 92% of the total obtained sequences, where 22.1% corresponded to the *Leuconostoc* genus, followed by OTUs of unclassified genera, the Enterobacteriaceae (UC) family (19.9%), acetic bacteria, such as *Gluconobacter* (11.4%) and *Acetobacter* (9.5%), enterobacteria belonging to *Tatumella* (10.4%), *Lactobacillus*

(6.4%), and the *Pantoea*, *Pseudomonas*, *Rosenbergiella*, *Frateuria*, *Lactococcus*, *Zymomonas*, and *Weisella* genera, at proportions less than 3.0%.

Figure 4. Relative abundance of sequences (%) at different taxa levels (family and genera), obtained via metataxonomic analysis from 16S data, for samples of coffee at the beginning of fermentation (**A**) and at the end of fermentation (**B**). Detected OTUs below 0.5% are indicated as "Others".

The relative abundance of predominant genera OTUs varied according to the sampling unit (farm), process, and sample types. In general, *Leuconostoc*, *Acetobacter*, *Tatumella*, and *Lactobacillus* genera populations increased at the end of fermentation, while there was a significant decrease in Enterobacteriaceae family OTUs compared to those identified at the beginning of this process. Data from samples collected in units 6 and 20 were highlighted, in which *Tatumella* and *Lactobacillus* populations increased from 4.1 to 48.9% and from 45.1 to 79.3%, respectively (Figure 4). By contrast, OTUs of Enterobacteriaceae family populations decreased in most samples at the end of fermentation (CF), from 26.7 to 15.3% on average (Figure 4B). Similarly, genera with a relative abundance classified in "Others" decreased their proportion to less than 3% on average, due to performed fermentations (Table S6).

Regarding fungal diversity at the beginning and at the end of coffee fermentation, as assessed by rRNA internal transcribed spacer–ITS sequences, 6 phyla, 167 families, and 279 fungal genera were identified, 85.97% of which were grouped within the Ascomycota phylum, while 10.90% were not classified at the phylum level (Fungi_UC). The Saccharomycodaceae family was the most predominant (44.28%), followed by the Pichiaceae family (13.16%) and non-classified families within the Saccharomycetales order (UC) (11.28%) (Table S5).

Figure 5 represents the relative abundance for 94.9% of the total ITS sequences, among which *Pichia* (13.2%), *Candida* (10.0%), and *Hanseniaspora* (1.8%) were identified at the genus level. The genera *Meyerozyma*, *Wickerhamomyces*, *Metschnikowia*, and *Debaryomyces* had less than 1% relative abundance. At the end of fermentation (Figure 5B), genera from the Saccharomycedaceae family predominated, as their relative abundance increased from 33 to 52%, on average, for all fermentations. Genera classified as "Others" decreased in all samples, at the end of fermentation, going from 7.9%, on average, to 2.2% (Table S6).

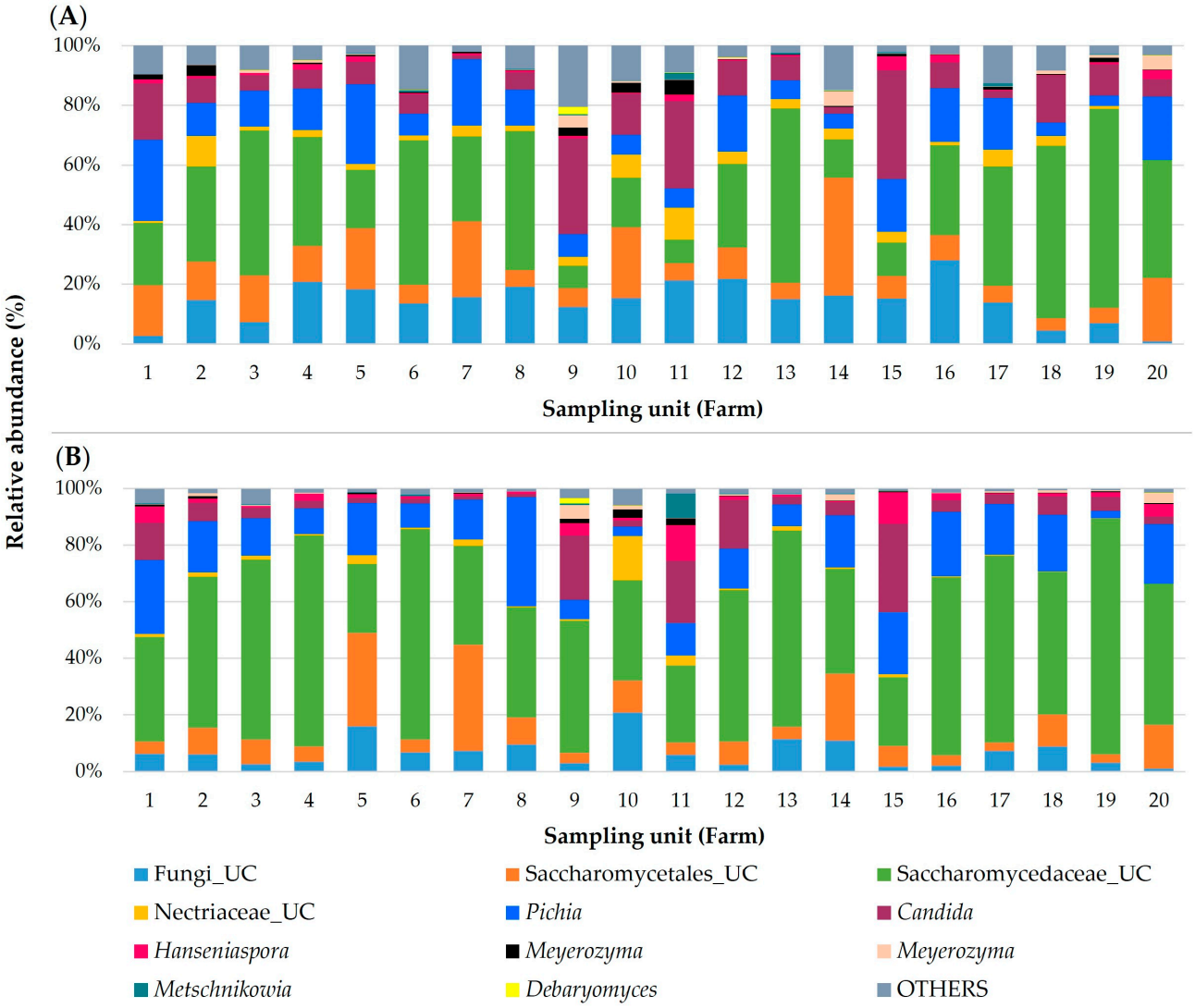


Figure 5. Relative abundance of sequences (%) at different taxa levels, obtained via metataxonomic analysis from ITS data for samples of coffee at the beginning of fermentation (**A**) and at the end of fermentation (**B**). Detected OTUs below 0.5% are indicated as "Others".

3.5. Microbial Beta Diversity

A Beta diversity analysis using the Bray–Curtis distance was performed to qualitatively estimate the presence of microbial patterns among samples (Figure 6). PERMANOVA analysis indicated significant statistical differences for both bacterial and fungal diversity ($p < 0.05$), and it showed four separate groups, which were characterized by differences in the relative abundance of the dominant genera. *Leuconostoc* dominated the first group (G01); *Acetobacter*, *Gluconobacter*, *Leuconostoc*, OTUs of the Saccharomycedaceae_UC family, and *Pichia* dominated the second group (G02); Enterobacteriaceae_UC, *Tatumella*, and Saccharomycedaceae_UC dominated the third group (G03); and *Lactobacillus* dominated the fourth group (G04). These groups were representative of a relationship with coffee

quality, with G01 and G03 showing a clean cup, G02 showing a greater number of sensory defects, and G04 having higher SCA quality scores. The variability in what individuals contribute to quality, according to the groups, is explained by 35.1% and 13.6% of bacterial and fungal diversity, respectively, leaving unexplained a high percentage of variability in the four groups, which may be related to other factors that were not included in this study.

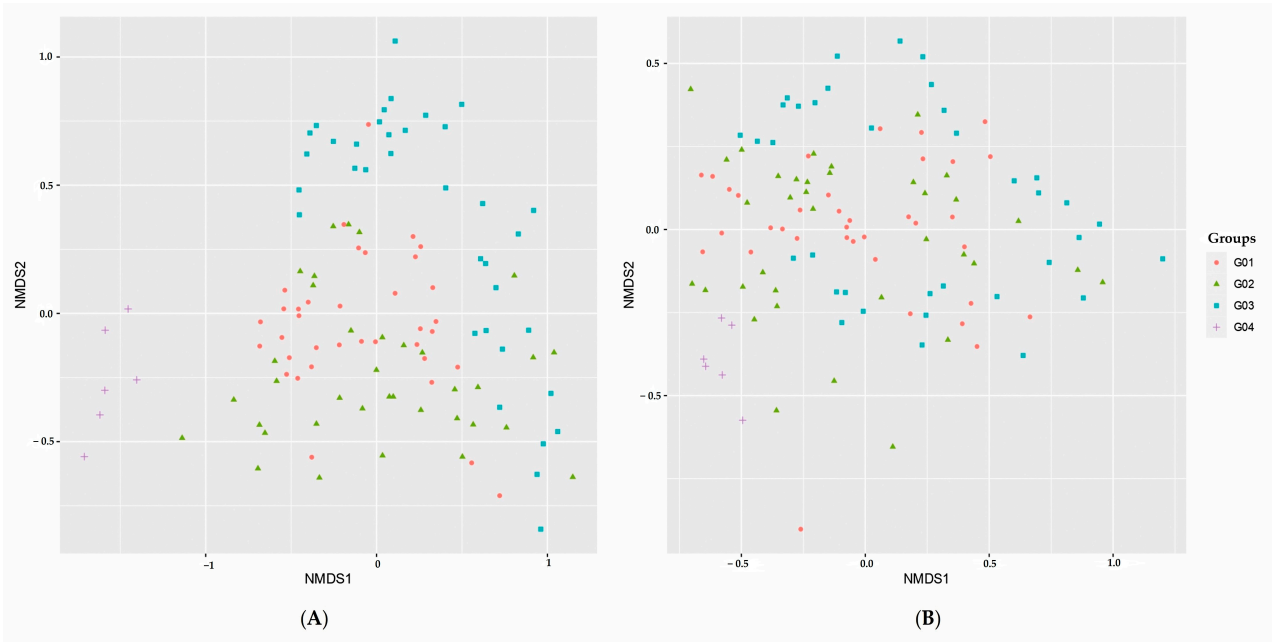


Figure 6. Non-metric multidimensional scaling (NMDS) plot for microbial beta diversity among coffee fermentation samples, regarding bacterial beta diversity (**A**) and fungal beta diversity (**B**), according to the Bray–Curtis distance test.

Relationships between different fermentation factors are represented by a heatmap of the obtained Spearman correlation coefficient values (Figure 7). Strong direct correlations are observed between *Lactobacillus* relative abundance, fermentation duration time, and fermentation type, with R^2 = 0.826 and 0.771, respectively. An inverse correlation was observed between the crop´s altitude and the abundance of *Hanseniaspora* (R^2 = −0.765). In general, with larger *Gluconobacter*, *Acetobacter*, *Candida*, *Hanseniaspora*, and *Meyerozyma* populations, moderate inverse correlations were observed with the SCA quality score, maintaining the behavior between families of these genera. It was also observed that the final fermentation pH had no influence on cup quality (R^2 = 0.079), but lower pH values were related to a greater bacteria abundance, such as *Gluconobacter* (R^2 = −0.62).

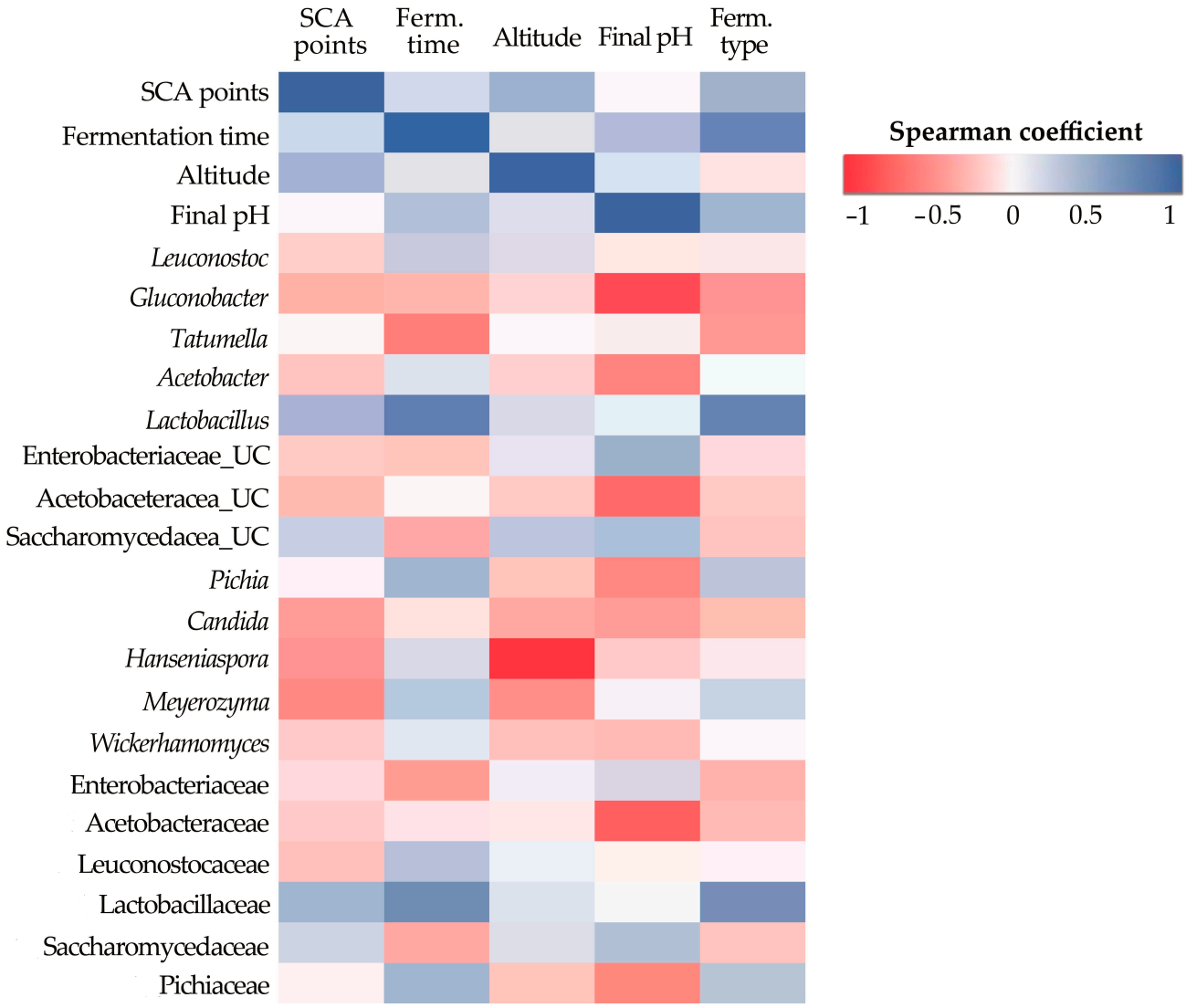


Figure 7. Heatmap of Spearman's correlation values (R^2) for fermentation process characteristics and most abundant bacteria and yeast/fungi genera and families.

4. Discussion

This research on metataxonomic coffee fermentation provided a snapshot of microbial communities associated with this process in coffee-producing farms in the Department of Quindío, Colombia. Diversity was described based on the conjugation between richness, composition, and abundance [19], since it varies with changes in the environment, generated by the different methods for performing this process. Our research is relevant, considering that coffee fermentation by the wet method, which is the most traditional way to remove coffee mucilage, not only in Colombia but also in numerous countries, is performed naturally by bacteria and yeasts, mainly. These microbial populations and their interactions along the process influence coffee bean chemical composition and final beverage quality.

Coffee fermentations are characterized by high richness, abundance, and diversity index values, which can vary depending on the sampled ecosystem and conditions during the fruit (berry) formation [5,50], so it is possible to obtain similar richness indexes between samples with different composition. This is evident for different coffee production areas in Colombia. The indexes estimated here for samples from the Department of Quindío showed the aforementioned observations, and they presented greater bacterial than fungal diversity richness and abundance at the beginning of fermentation (Tables S3 and S4), similar to the

results obtained by De Oliveria Junqueira et al. [3] in the southern coffee region of Colombia but with lower values for these indexes. By contrast, Cruz-O'Byrne et al. [20], studied fermentations in the northern coffee zone of Colombia and found that values for these indexes were higher, and the richness and diversity for fungi were also greater than for bacteria. It is important to highlight that values obtained in Quindío samples were obtained from samples collected in several fermentation processes, with variability in conditions. High species richness and diversity present in fermentation have been indicated due to the wide range of substrates present in coffee mucilage and pH and temperature conditions in which this process occurs [3,51]. At the end of fermentation, there was a general decrease in both richness and diversity, a behavior that has been commonly related to a decrease in pH in particular, as well as changes in environmental conditions, as oxygen and substrate availability are restricted, allowing fewer microbial species to remain active at the end of this process [20,27–29].

Taxonomic identification using high-throughput sequencing (HTS) is one of the most widely used techniques to generate information on microorganisms and genera involved in coffee fermentation, mainly in *C. arabica* varieties [3,20,24,27–29,52], in which a few genera are dominant. In addition, it is possible to effectively assess microbial diversity with the indexes used here [19]. In this research, the correlation of nucleotide sequences with the SILVA database corresponded to 1627 OTUs of microbial genera, much higher than what was reported in previous studies, due to the greater number of samples collected here. In particular, each fermentation had an average of 252 bacterial genera (range 86–438) at the beginning of fermentation and an average of 158, ranging between 83 and 262, at the end of this process. In the same way, there were 57 fungal genera, on average (range 27–106), at the beginning of fermentation and 39 (range 16–87) at the end. For most types of fermentation samples, predominant genera were common, such as lactic acid bacteria (LAB), including *Leuconostoc* and *Lactobacillus*, acetic acid bacteria (AAB), such as *Gluconobacter* and *Acetobacter*, and OTUs from the family Enterobacteriaceae, in which *Tatumella* and *Pantoea* were also found. Other genera present at proportions less than 3% included *Pseudomonas*, *Rosenbergiella*, *Frateuria*, *Lactococcus*, *Zymomonas*, and *Weisella*, some of which have been reported as natural coffee microbiota or in fermentation processes of coffee and other products [9,13,14,22,27,53,54]. Similarly, predominant and common fungal genera between fermentations were represented by the Saccharomycedaceae family, specifically *Pichia* and *Candida* genera. Most of the microbial genera that varied between fermentation types, and that allowed for the identification and taxonomic classification of this large number of reported OTUs, were related to populations that participated, to a lesser extent (Others), in each fermentation and that also decreased significantly in samples at the end of the fermentation. Therefore, their participation does not seem to have a direct effect on the process.

It is important to consider that the dominance of a few genera may be related to the "terroir" microbiota for the sampling site, since native microorganisms have a great impact on fermentation development, and, therefore, they can influence the coffee quality, as has also been considered by other authors [3,29,52]. The identification of other predominant bacterial and fungal predominant genera at the beginning of fermentation, in addition to those reported in this study, could lead to this differentiation—for example, *Lactococcus* and *Dipodascus* in southern Colombia [3], *Kazaschastania* in northern Colombia [20], and *Erwinia* in southern Brazil [26,28].

In this study, the assigned OTUs to bacterial genera of the Enterobacteriaceae_UC family decreased in abundance for all collected samples at the end of fermentation, regardless of the type of process performed (Figure 4). Microorganisms belonging to this family have been associated with other plant structures and with tools or utensils used or in contact with coffee before its processing [5,9,13,21,50]. Abundance at the beginning of fermentation is related to harvest and postharvest practices on each farm at the time of processing. Increases in the abundance of the Saccharomycedaceae family and the *Leuconostoc*, *Lactobacillus*, *Tatumella*, *Gluconobacter*, and *Acetobacter* genera may be related to

changes in environmental and other conditions during fermentation. However, only the last two genera were related to pH decreases, since they were present at high proportions in lower pH fermentations. This did not happen when *Lactobacillus* was the governing genus in the fermentation (79.5%), where pH was 4.06 (Figure 7 and Table S1). It has recently been proposed that pH values higher than 4.0 at the end of fermentation have a positive relationship with coffee quality [5,14].

Our research suggested that there is no relationship between coffee quality and pH (Figure 7). pH values above 4.5 at the end of fermentation occurred with Enterobacteriaceae_UC proportions higher than 40%, which occurred in traditional fermentations, those lasting between 12, 16, and 22 h, and those with SCA cup scores between 80 and 81 points, which are standard good-quality scores for Colombian coffee. From these cases, it can be inferred that these fermentations did not advance sufficiently to allow these bacterial genera to decrease, and increases in AAB and LAB occurred; consequently, pH decreased due to AAB activity, and the desired compounds developed due to LAB, as previously proposed by Pothakos et al. [13]. These authors found that Enterobacteriaceae (*Tatumella, Pectobacterium, Klebsiella, Pantoea, Enterobacter*, and *Rahnella*) and AAB (*Acetobacter* and *Gluconobacter*) genera had the highest relative abundance (%) at the beginning of fermentation (0 h), but it was progressively reduced during the first 8 h of fermentation, with pH changing from 5.2 to 4.5. In our research, *Leuconostoc*, Enterobacteriaceae family, *Gluconobacter,* and *Tatumella* also had high relative abundance at the beginning (0 h), with pH values between 5.9 and 4.3. Subsequently, for the second phase from 8 to 24 h [13], the most important change was an increase in *Leuconostoc* and *Lactobacillus* species and a decrease in some Enterobacteriaceae, with a stable pH of 4.0. Similarly, on most of our studied farms, for traditional fermentation ending from 12 to 22 h, an increase in relative abundance of *Leuconostoc* and *Gluconobacter* was observed as well as a decrease in Enterobacteriaceae OTUs, with a pH between 4.5 and 3.0. By contrast, Cruz-O'Byrne et al. [20] observed a progressive increase in *Leuconostoc*, *Acetobacter*, *Lactobacillus,* and *Weissella*, among others, in the first 18 to 24 h of fermentation, with no decrease in the first phase (0 to 8 h).

Among LAB metabolites, bacteriocins are mentioned, related to the growth inhibition of microorganisms that generate compounds involved with coffee's undesirable flavors [13,29]. One of the samples from these fermentations had a defect called "earthy", characterized by humidity and a soil-like smell and taste, related to erroneous practices during coffee drying, which can generate filamentous fungal growth. Although these fungi were not identified here, as they have been reported at low concentrations in depulped coffee fermentations using wet methods [5], it could be confirmed that a pH decrease is not only an indicator of fermentation progress but also indicates an increase in populations of genera that are necessary to produce protective compounds of coffee quality, even at later postharvest stages.

Another finding of this research was the relative abundance of more than 80% of *Gluconobacter* and *Acetobacter* in a traditional fermentation type lasting 15 h, which presented a pH of 3.51 and a "fermented" defect in the coffee beverage. Accumulated production of acetic acid, typical of these bacterial genera, has been related to the presence of undesirable flavors and overfermentation [21,55]. The presence of this defect was not related to fermentation time, since, in prolonged fermentations lasting between 39 and 72 h, the "fermented" defect did not appear, but neither did it occur when these bacteria were present at a proportion below 56.6%, this time with the participation of *Leuconostoc* and *Lactobacillus*, at more than 30% and 1%, respectively. High proportions of *Lactobacillus* were obtained with induced anaerobic fermentation in the coffee fruit for 72 h, generating one of the highest SCA cup scores, in agreement with the results obtained by Da silva et al. [28] and Pereira et al. [51]. However, a similar beverage quality with high SCA scores was obtained when *Leuconostoc*, *Gluconobacter*, *Tatumella*, and *Lactobacillus* had proportional populations, with a final pH of 3.73, and traditional fermentation lasted for 16 h.

It is inferred from our studies that much of the variability in classification between microbial groups cannot be explained by the relative abundance of genera related to

coffee quality (Figure 6A). It has been widely cited that, during fermentation, a complex succession of bacterial and fungal species occurs, varying not only in composition and initial proportion but also greatly influencing the ecosystem in which they live [5,9,13,50]. Therefore, this variability influences both the initial populations and their development, leading to the observed high richness, abundance, and variability in composition in the region under study.

Yeasts play an important role in coffee fermentation, although their contribution to final quality has not been fully identified [5,6]. An increase in the OTU populations assigned to the Saccharomycetaceae family was commonly observed in all fermentations, ranging between 4.7% and 39.1% (Figure 5), making this family one of the most important in fermentation processes [56]. Genera, such as *Hanseniaspora* [22,23,57], *Kluveromyces*, *Kazachstania* [20,58], *Sacharomyces* [5,26], *Torulaspora* [23], and *Candida* [21], have been significantly associated with coffee fermentation. Their role in aromatic compound development, enzyme production for mucilage degradation, regulation of fermentation time, and protection of the product from filamentous fungal growth, which are considered some of the basic functions for yeasts in food fermentative processes, has been considered [56].

Alcohol and ester production by yeasts is perhaps one of the most studied aspects. They were also the main chemical groups identified in coffee samples from the farms we studied in Quindío (Figure 2). Compounds, such as phenylethyl alcohol, identified as a yeast metabolite that generates floral and fruity aromas [59–61], and methyl anthranilate, related to grape fermentation for wine production [62], were detected in all samples and have been previously reported in coffee [9,28,59,63,64] and cocoa [65] fermentations, respectively. Other alcohols and esters, such as 2,3-butanediol, ethyl acetate, and methyl salicylate, were only identified in samples in which *Lactobacillus* dominated the process; therefore, their development could be related to this bacterial genus. These compounds, present in assisted fermentations using yeast strains, LAB, or with induced anaerobic fermentations, have been related to beverage flavor and aroma improvement [9,25,66–68]. Other alcohols, such as 1-octen-3-ol, 1-pentanol, and benzyl alcohol, found in this study have been reported in fermentations dominated by LAB and yeasts as well [3,69,70].

Most of the compounds identified in our study came from the metabolic processes of microorganisms during fermentation, and it has been proven that this process modifies chemical composition of beans, since these chemical groups are not detected or are detected in very low concentrations in coffee that did not undergo fermentation as a method to remove mucilage [5,7,71].

5. Conclusions

The microbial diversity in fermented coffee samples collected in the Department of Quindío, Colombia, showed high richness, diversity, and abundance levels (indexes), which were higher for bacteria than for fungi or yeasts. Their variability was related to different methods of performing these processes. Enterobacteriaceae and Saccharomyceaceae family populations generally dominated the beginning of fermentations. The initial populations of these microorganisms are important since they determine the progress of fermentation. It was identified that genera, such as *Gluconobacter* and *Acetobacter*, are necessary to achieve pH acidification. However, populations with an abundance greater than 80% can be related to the "fermented" defect. High *Lactobacillus* populations were related to the type and time of fermentation, high SCA grades in beverage cups, and the presence of volatile compounds, such as 2,3-butanediol, ethyl acetate, and methyl salicylate, which are important for aroma profile, highlighting coffee beverage attributes. Interestingly, there was no relationship between pH and coffee beverage quality in the SCA score rating. There was not a unique or specific method to obtain higher-quality coffee or outstanding scores, and the conventional recommended fermentations usually lasting less than 16 h are appropriate (Table S1). There are several myths and practices that do not add much to coffee quality. By contrast, there were some specific practices, beyond the recommended ones or problems that happened in steps after traditional fermentation, that could affect coffee quality, generating defects, such

as fermented, phenol, and earthy. These results could help to direct research efforts towards mechanisms driving fermentation to improve coffee quality. There is significant potential for farmers and industry to use or formulate products based in native and engineered microorganisms, enzymes, and metabolites in coffee fermentation, to accelerate or modify it, or to improve final product quality and its added value.

Since it was not possible to determine taxonomic classification at the genus level within the Saccharomyceaceae family, deeper sequencing studies should be conducted using metagenomic techniques, which will allow us to identify associated genera within the families that were not classified and even determine species within the *Leuconostoc* and *Lactobacillus* genera, which had an important role in these fermentations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9040343/s1, Figure S1: Boxplot of diversity indexes, namely Observed, Chao1, Shannon, and inverse Simpson, for bacterial communities (A), as well as for yeast/fungal microbiota (B), from sampled and studied farms; Table S1: Information on fermentation type performed in each studied and sampled farm, pH and temperature of the coffee bean mass and sensory quality; Table S2: Volatile organic compounds (GC-MS peak areas $\times 10^4$) found in green coffee beans from studied and sampled farms; Table S3: Results of Illumina MiSeq amplicon sequencing of bacterial 16S and microbial community richness and diversity; Table S4: Results of Illumina MiSeq amplicon sequencing of fungal ITS region and microbial community richness and diversity, Table S5: Relative abundance of bacterial and fungal family level obtained by metataxonomic analysis from 16S and ITS rRNA gene sequences, for samples of coffee at the beginning of fermentation (CD) and at the end of fermentation (CF), Table S6: Relative abundance of bacterial and fungal genera obtained by metataxonomic analysis from 16S and ITS rRNA gene sequences, for samples of coffee at the beginning of fermentation (CD) and at the end of fermentation (CF).

Author Contributions: Conceptualization, project administration, investigation, formal analysis, data curation, writing—original draft preparation, A.E.P.-M.; methodology, formal analysis, data curation, writing—review and editing, A.V.V.-E.; methodology, review and editing C.A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was developed under the project: Experimental Development for the Competitiveness of the Coffee Sector of the Department of Quindío, code 2017000100099, financed by the General System of Royalties, Gobernación de Quindío in agreements signed with the National Colombian Coffee Growers Federation (Cenicafé—Crossref Funder ID 100019597). No. 002-of-2020.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article or Supplementary Material. High-throughput sequencing (HTS) data are available in repository NCBI SRA BioProject, accession no: PRJNA940080.

Acknowledgments: Authors are grateful to the Department of Quindío Committee of the Colombian Coffee Growers Federation (FNC) and Cenicafé; to the farmers that supported and allowed sample collection in coffee wet processing to take place; to faculty and staff of the Centro Nacional de Secuenciación Genómica for their support and advice.

Conflicts of Interest: The authors declare no conflict of interest.

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