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Preliminary Assessment of Cultivable Bacterial Diversity During Coffee Post-Harvest Fermentation in Southern Ecuador via 16S rRNA Gene Barcoding

Darío Cruz [1,*], Valeria Hidalgo [1], José Miguel Fernández [2], Débora Masache [3], Paola Duque-Sarango [4], David Apolo [5], Andrea Jaramillo-Riofrío [5,6] and Ángel Benítez [7]

1 Microbial Systems Ecology and Evolution Research Group, Department of Biological and Agricultural Sciences, Biology School, Universidad Técnica Particular de Loja, San Cayetano Alto s/n, Loja 1101608, Ecuador; vmhidalgo2@utpl.edu.ec
2 Innovative Processes for the Development and Quality of Healthy Products and Foods Research Group, Department of Chemistry, Universidad Técnica Particular de Loja, San Cayetano Alto s/n, Loja 1101608, Ecuador; jmfernandez@utpl.edu.ec
3 Faculty of Agricultural Sciences, Aquaculture School, Universidad Tecnica de Machala, Machala 070150, Ecuador; drmasache@utpl.edu.ec
4 Water Resources Research Group, Universidad Politécnica Salesiana, El Vecino Campus, Calle Vieja 12–30 y Elia Liut, Cuenca 010102, Ecuador; pduque@ups.edu.ec
5 Microbial Systems Ecology and Evolution Research Group, Department of Health Sciences, Universidad Técnica Particular de Loja, San Cayetano Alto s/n, Loja 1101608, Ecuador; djapolo@utpl.edu.ec (D.A.); acjaramillo4@utpl.edu.ec (A.J.-R.)
6 Département de Biologie, Ecologie et Evolution, Université de Liège, Quai Van Beneden, BE-4000 Liège, Belgium
7 Biodiversity of Tropical Ecosystems Research Group, Department of Biological Sciences, Biology School, Universidad Técnica Particular de Loja, San Cayetano Alto s/n, Loja 1101608, Ecuador; arbenitez@utpl.edu.ec
* Correspondence: djcruz@utpl.edu.ec

Abstract

The organoleptic profile of coffee is modulated by microbially mediated fermentation processes; thus, characterizing the coffee microbiome is essential for the specialty coffee industry. This research investigated three coffee farms in Loja, Ecuador, to determine the cultivable bacterial diversity in coffee cherries (*Coffea arabica* var. Geisha) subjected to different post-harvest processing methods. Bacteria were isolated on Trypticase Soy Agar (TSA), Blood Agar Base, and MacConkey Agar after a broth enrichment step. Isolates were initially grouped into 14 morphotypes (MT) based on colony morphology and Gram staining. Molecular identification was performed by 16S rRNA gene sequencing, followed by phylogenetic analysis and Operational Taxonomic Unit (OTU) delimitation at a 99% identity threshold. Although 45 purified strains (19 Gram-positive and 26 Gram-negative) were initially clustered, morphological characterization showed significant taxonomic limitations compared with molecular tools. Molecular analysis identified nine genera and 19 bacterial genotypes, with *Lactococcus lactis* and *Enterobacter* spp. being the most frequently recovered and resilient taxa across all treatments. Statistical analyses (PERMANOVA and PCA) revealed no significant differences in bacterial community composition among post-harvest processes (Natural or Carbonic Maceration) or among farms, indicating that the predominant cultivable isolates remained consistent under the conditions evaluated, as presented here. According to the literature, several of the recovered species have functional roles in coffee fermentation, providing a baseline for the future development of starter cultures to optimize and standardize coffee sensory quality in southern Ecuador.

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1. Introduction

Coffee is one of the world's second most traded commodities and one of the most widely consumed beverages globally [1]. The species *Coffea arabica* L. (1753) stands out as one of the most cultivated crops in the tropical and subtropical regions of Africa, Asia, and Latin America, covering approximately 10.2 million hectares [2]. In addition to its social relevance, coffee is of critical economic importance [2], with an estimated annual demand of 10.5 million tons [1] and a global retail coffee market valued at over USD 200 billion annually [3]. In Ecuador, *C. arabica* cultivation accounts for 68% of the national coffee-growing area, with the province of Manabí being the most productive region, followed by Sucumbíos and Orellana [4]. In contrast, in the Loja province of southern Ecuador, coffee production is driven by smallholder farming families operating across distinct altitudinal gradients and unique microclimates, conditions that consistently yield internationally recognized specialty coffee [5].

Coffee quality is determined by sensory parameters, such as unique aromas and distinctive flavors, which are attributable to chemical compounds including phenols, alkaloids, amino acids, lipids, and organic acids [6,7]. The accumulation and transformation of these bioactive and aromatic precursor compounds—such as chlorogenic acids, caffeine, and volatile profiles—are strongly driven by a continuum of factors including agricultural origin, growing region, post-harvest processing methods [8], but especially the final roasting dynamics [9,10]. Indeed, processing methods (e.g., natural, washed, honey, controlled fermentation, or maceration) directly alter the chemical and biological composition of the bean, laying the foundation for specialty coffee [11,12]. These attributes could be modulated by microorganisms (MOs), primarily bacteria and yeasts, which carry out metabolic processes during the fermentation of the pulp and mucilage. This biochemical activity induces changes in the bean's composition and modifies the presence of compounds responsible for the final organoleptic qualities [13,14]. Furthermore, while native microbial activity dictates initial flavor precursor generation, downstream processing—particularly the roasting level—plays a pivotal role in determining final coffee quality, including volatile profiles, bioactive capacity, and human health-promoting properties [15]. Among the main bacterial groups reported in these processes, *Lactic acid bacteria* (LAB) are prominent as producers of enzymes of significant interest to the food industry [16]. Generally, these microorganisms degrade sugars, lipids, and proteins, converting them into alcohols and other metabolites that transform odor, color, and chemical parameters such as pH [17].

Consequently, the isolation of bacterial strains for bioprospecting purposes requires an integrative characterization that combines cultural, biochemical, and molecular methods [18]. While phenotypic characterization allows for the observation of morphology growth, and biochemical reactions, it does not guarantee precise identification because traits overlap among species and taxonomic groups [19]. Therefore, it is essential to integrate molecular information; for instance, 16S rRNA gene analysis enables the detection of bacterial communities with high resolution, discriminating even at the species level [20] and providing robust data for evolutionary studies [21]. This gene combines conserved regions, which allow the design of universal primers, with hypervariable regions that provide taxon-specific signatures for species discrimination [20].

Globally, numerous studies have addressed the microbiology of coffee fermentation [22–24]. Species such as *Lacticaseibacillus plantarum*, *Levilactobacillus brevis*, *Lactococcus lactis*, and *Leuconostoc mesenteroides* have demonstrated potential for enhancing beverage quality and increasing economic benefits for producers [25]. In Ecuador, although documentation of fungal biodiversity in Loja's coffee plantations has begun—identifying yeast genera such as *Saccharomyces*, *Pichia*, and *Hanseniaspora* [26]—studies on bacterial biodiversity in the coffee fruit remain scarce, often limited to general analyses of fermented foods [27].

In this context, the isolation and characterization of local bacterial strains is fundamental to understanding their potential application in optimizing fermentation processes [28]. Regional agronomic management, microclimatic conditions, and processing methods interact closely with microbial ecosystems to shape bean functionality [8,17]. Therefore, evaluating native microbiota from high-altitude environments in Ecuador becomes crucial. This research aims to isolate, characterize phenotypically and molecularly, and compare the diversity of potential functional bacteria from coffee fruits collected in three farms in Loja province.

2. Materials and Methods

2.1. Study Area and Sampling

Coffee cherries (*Coffea arabica* L. var. Geisha) were manually harvested at optimal maturity R5 as suggested by Li et al. [29]. The sampling design and post-harvest process details were identical to those described by Apolo et al. [26]. Samples were gathered from three representative smallholder specialty coffee farms located in the province of Loja, Ecuador, based on their accessibility, same variety (Geisha), and application of the same post-harvest processes, with the owners' permission to sample. The farms are located in three different cantons and at three different altitudes—Loja Canton (L) (3°55′0.01″ S, 79°13′0.01″ W) 2010 m.a.s.l., Gonzanamá (G) (4°6′0.01″ S, 79°23′0.01″ W) 1500 m.a.s.l., and Olmedo (O) (3°55′0.16″ S, 79°38′0.01″ W) 1230 m.a.s.l. (Table 1)—with similar cultivar conditions, such as the coffee plants being mostly cultivated within agroforestry systems integrated with fruit crops, primarily banana (*Musa* × *paradisiaca* L.), orange (*Citrus* × *aurantium* var. *sinensis* L.), guaba (*Inga edulis* Mart.), and avocado (*Persea americana* Mill.). Additionally, specific shade tree species vary by farm: (*Ceiba trischistandra* Bakh.) and [*Vachellia macracantha* (Humb. & Bonpl. ex Willd.) Seigler & Ebinger] predominate in Gonzanamá and Olmedo, whereas (*Jacaranda mimosifolia* D.Don) and [*Leucaena leucocephala* (Lam.)] de Wit were recorded for Loja.

Three post-harvest processing methods were evaluated per farm, totaling 27 samples, all belonging to the Geisha variety (Table 1). Due to strict material availability constraints and the limited volume of uniform coffee cherries provided by the smallholder farms, experimental replication was strategically allocated. The traditional 'Natural' process (i.e., ripe cherries washed with tap water and sun-dried) was restricted to a single sample per farm, serving as an in-situ baseline to evaluate the initial microbial diversity of each specific terroir. In contrast, the innovative treatments—Carbonic Maceration at room temperature and Refrigerated Carbonic Maceration at 10 °C (both treatments utilized sterile water, distinct from the water employed in wet processing)—were prioritized for biological replication, utilizing four distinct replicates (n = 4) per farm to strengthen comparisons within the novel fermentation configurations.

Table 1. Number of samples evaluated per farm and processing method.

Canton	Farms by Sector	Treatment	Sample Code	No. of Samples
Loja	El Aguacate	Natural	A	1
		Carbonic Maceration at room temperature	AC	4
		Refrigerated Carbonic Maceration at 10 °C	AF	4
Olmedo	Papá Café	Natural	B	1
		Carbonic Maceration at room temperature	BMF	4
		Refrigerated Carbonic Maceration at 10 °C	BMC	4
Gonzanamá	El Arenal	Natural	M	1
		Carbonic Maceration at room temperature	MC	4
		Refrigerated Carbonic Maceration at 10 °C	MF	4
	TOTAL			27

2.2. Isolation and Cryopreservation of Bacteria

For bacterial isolation, a targeted broth enrichment step was implemented to recover a conservative representation of the low-abundance cultivable bacterial diversity across treatments, rather than to track real-time community dynamics. Briefly, 10 g of unwashed coffee cherries from each sample (Table 1) were inoculated directly into 200 mL of Trypticase Soy Broth (TSB, DIFCO, Detroit, MI, USA). This stock solution was incubated at 37 °C for 24 h with constant agitation (50 rpm). The pH was recorded before and after inoculation. Subsequently, 30 µL aliquots were spread onto three solid media: Trypticase Soy Agar (TSA, DIFCO, Detroit, MI, USA), Blood Agar Base (DIFCO), and the selective MacConkey Agar (DIFCO, Detroit, MI, USA).

Plates were incubated at 37 °C for 24 to 72 h. To translate plate recovery into quantifiable data for ecological and statistical analyses, a structured colony selection protocol was applied: Colonies were selected based on macroscopic differences and purified by streak plating on TSA supplemented with an antifungal agent (Fluconazole 0.5%). Pure strains were coded and cryopreserved at −80 °C in a mixture solution of Luria–Bertani (LB) broth and Thioglycollate medium supplemented with 20% glycerol [30,31] in the UTPL Culture Collection (Ceparo UTPL).

2.3. Phenotypic Characterization of Bacterial Isolates

Pure strains were phenotypically characterized using both macro- and microscopic evaluations. Macroscopic traits included colony morphology, margin, elevation, and shape. Microscopic analyses determined cellular morphology (cocci or bacilli) and Gram-staining reactions. Additionally, basic biochemical assays were performed: catalase activity was evaluated using 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA), and oxidase activity was determined using OxiStrips (Hardy Diagnostics, Santa Maria, CA, USA). Binary data (presence/absence) of these phenotypic traits were processed through hierarchical agglomerative clustering analysis to construct a dendrogram using the *stats* package in R Software version 4.3.3 [32], allowing the identification of specific 'morphotypes (MT)' across the different farms. This systematic morphotype classification established discrete pheno-

typic operational units, providing both a standardized matrix for downstream diversity indices and a practical baseline tool for coffee producers.

2.4. DNA Extraction, Amplification, and Sequencing

All 45 purified isolates, representing the 14 MT, were subjected to genomic DNA extraction using the Invitrogen™ PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA). Bacterial colonies were suspended in sterile water and adjusted to a cell density equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL), verified by spectrophotometry using a DEN-1B densitometer (Grant Instruments, Cambridge, UK). The 16S rRNA gene was amplified by PCR using the universal primers 27F and 1492R. The reaction mixture was prepared using the 2X Phusion High-Fidelity PCR Master Mix (Thermo Scientific, Waltham, MA, USA). Thermal cycling conditions consisted of an initial denaturation at 98 °C for 5 min, followed by 35 cycles of 98 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. PCR products were verified by 1% agarose gel electrophoresis stained with 1X GelRed® Nucleic Acid Stain (Biotium, Fremont, CA, USA). Amplicons were subsequently purified using the UltraClean® PCR Clean-Up Kit (Qiagen, Hilden, Germany) prior to Sanger sequencing at Macrogen (Seoul, Republic of Korea).

2.5. Phylogenetic Analysis and Bacterial OTUs Determination

Raw chromatograms were visually inspected and edited using CodonCode Aligner v5.1.4 [33], resulting in 45 partial 16S rRNA gene sequences, one per isolate, with lengths of approximately [950–1050] bp after quality trimming. To construct the alignment dataset, these sequences were queried against the NCBI GenBank database (https://www.ncbi.nlm.nih.gov). Priority was strictly given to reference type strains; however, when type strains were unavailable, well-authenticated reference sequences displaying 97–100% sequence identity were selected. Multiple sequence alignments were subsequently performed in MAFFT v7.4 [34] using the accurate G-INS-i iterative refinement strategy.

Phylogenetic reconstructions were inferred using both Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. The ML analysis was performed in MEGA X using the Kimura 2-parameter model with Gamma distribution and invariant sites (K2P + G + I), and node support was evaluated with 1000 bootstrap replicates. The BI analysis was conducted in MrBayes v3.2 using the GTR + I + G substitution model [35]. For the Bayesian Posterior Probability (BPP) estimation, two independent runs of four Markov chains each were executed for 4 million generations with random starting trees. Trees were sampled every 100 generations, discarding the initial burn-in phase to generate a 50% majority-rule consensus tree from the final 24,000 trees. Convergence and effective sample sizes (ESS > 200) were verified using Tracer v1.5 [36].

The phylogenetic trees presented represent the best-scoring topologies inferred from the ML analysis, with node support values indicated as ML bootstrap percentages (BS) and BPP from the MrBayes analysis. The Gram-positive tree was rooted with *Klebsiella oxytoca* (strain CM1B5 and reference sequence MN621850.1), and the Gram-negative tree with *Enterococcus gallinarum* (strain CM1B7 and reference sequence OK602691.1), each being a phylogenetically distant member of the opposite Gram group. Finally, Operational Taxonomic Units (OTUs) were defined using OPTSIL 1.5 software, implementing a strict 99% identity threshold and a 0.5 clustering fraction. All 45 sequences generated in this study were deposited in GenBank under accession numbers PZ531582–PZ531626 (Supplementary, Table S1).

2.6. Diversity Indices and Multivariate Community Analysis

To evaluate the structural properties of the cultivable bacterial pool recovered after post-harvest fermentation and standardized enrichment, bacterial richness and the Shannon–Weaver index were analyzed across processing treatments and localities. Because the Shapiro–Wilk test indicated that these variables were not normally distributed, the non-parametric Kruskal–Wallis test was applied. Differences in species composition among treatments were visualized using Principal Component Analysis (PCA). To test for significant treatment effects on bacterial community structure, a permutational multivariate analysis of variance (PERMANOVA) was performed based on Bray–Curtis distances with 9999 permutations using the vegan 2.7-6 package [37]. All statistical analyses and community ecology visualizations were conducted using R Software version 4.3.3 [32].

3. Results

3.1. Identification and Characterization of Microorganisms

The study of the bacterial microbiota associated with coffee farms in Loja initially yielded 99 pure strains. Following subculturing and purity verification, 45 isolates (45.5%) were selected for detailed analysis. The remaining strains were excluded due to the persistence of complex bacterial consortia or recurrent cross-contamination, phenomena commonly observed in environmental isolates with high metabolic competition.

Preliminary phenotypic characterization revealed a heterogeneous structural diversity comprising 14 MT (Figure 1), among which the MT5 clade was the most homogeneous, corresponding predominantly to the molecular species *Lactococcus lactis*. However, this clade also included strain CM1B7, genetically identified genetically as *Enterococcus gallinarum*, highlighting the limitations of phenotypic traits in accurately determining bacterial groups or species.

3.2. Molecular Identification and Bacterial Phylogeny

A total of 45 partial 16S rRNA gene sequences were obtained and compared with reference type sequences available in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov). The isolates were genetically assigned to 19 taxa (Figures 2 and 3). The Gram-positive group included: *Enterococcus gallinarum* (1), *Enterococcus* sp. (3), *Enterococcus faecium* (1), *Lactococcus lactis* (12), and *Leuconostoc pseudomesenteroides* (2). The Gram-negative group consisted of: *Enterobacter bugandensis* (1), *Enterobacter* sp1 (3), *Enterobacter* sp2 (3), *Enterobacter* sp3 (1), *Escherichia* sp. (1), *Escherichia coli* (1), *Escherichia fergusonii* (2), *Hafnia* sp. (2), *Hafnia alvei* (1), *Klebsiella oxytoca* (3), *Klebsiella pneumoniae* (3), *Klebsiella* sp. (2), *Providencia rettgeri* (1), and *Serratia liquefaciens* (2).

From a phylogenetic perspective, Gram-positive bacteria represented 42.22% of the total (19 of 45 sequences), while Gram-negative bacteria accounted for 57.78% (26 of 45 sequences). Clades corresponding to Gram-positive bacteria showed a well-supported topology, with values exceeding the 50% threshold for both Bayesian posterior probabilities (BPP) and maximum likelihood (ML) bootstrap analyses. (Figures 2 and 3). When genotypes were contrasted with phenotypic profiles (Figure 1), no direct correspondence was observed between morphotypes and genetic species, except for the *Lactococcus lactis* clade, which largely, but not entirely, matched the MT5 morphotype clade.

In terms of recovery frequency and genetic diversity, *L. lactis* (12 sequences) was the predominant taxon among Gram-positive bacteria, while *Enterobacter* spp. (8 sequences) were the most frequently recovered among Gram-negative bacteria. After applying a 99% identity threshold (1% difference), five Operational Taxonomic Units (OTUs) were defined for Gram-positive bacteria across the genera *Enterococcus*, *Lactococcus*, and *Leuconostoc*. Conversely, Gram-negative bacteria showed significantly higher genetic richness, with

14 OTUs distributed among the genera *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Providencia*, and *Serratia*.

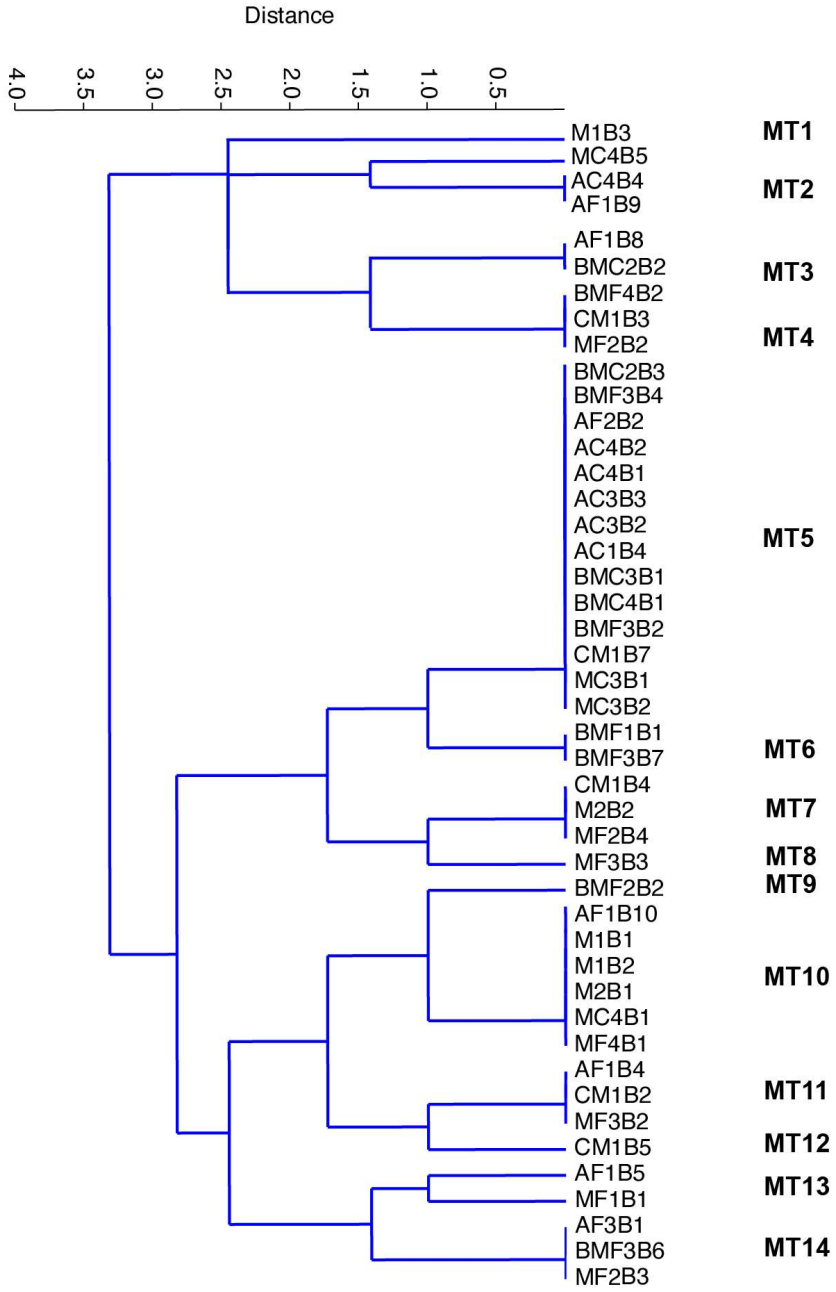


Figure 1. Hierarchical agglomerative clustering dendrogram of the 45 bacterial isolates obtained from different coffee-producing farms. Labels on the right indicate the assigned morphotypes (MT1 to MT14). Sample codes correspond to the post-harvest processing methods described in Table 1, followed by specific numerical identifiers for each bacterial isolate.

Overall, the isolated Gram-positive and Gram-negative bacteria represent a cultivable community associated with the coffee beans across the sampled farms. Based on the literature evidence, these isolates encompass potential ecological roles ranging from plant nutrition and growth promotion to plant and human pathogenicity, as well as promising metabolic traits for coffee post-harvest processing (Table 2).

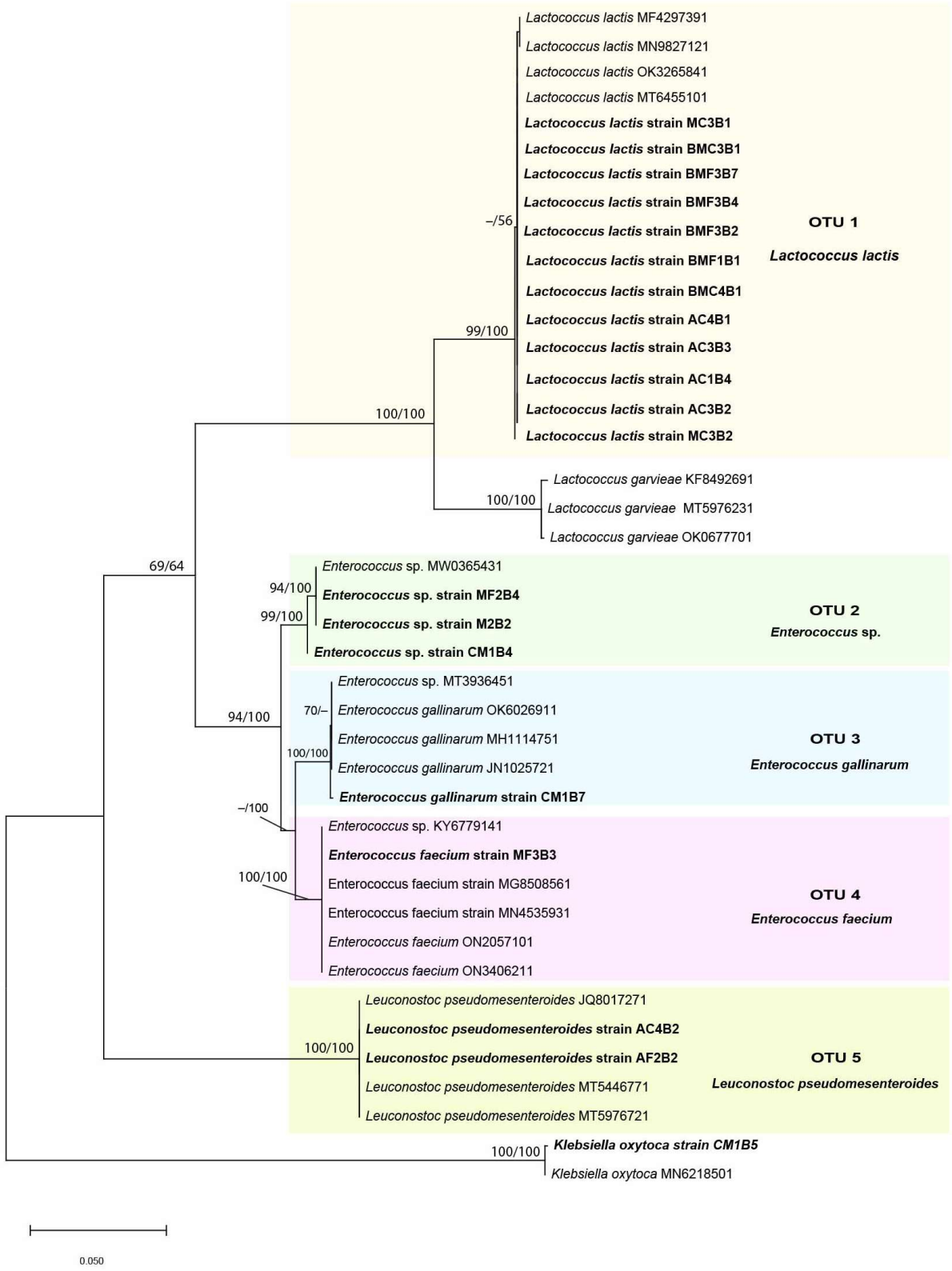


Figure 2. Phylogenetic positioning of 16S rRNA sequences from Gram-positive bacteria and clustering according to OTU determination at a 1% threshold (highlighted in color). Values at the nodes correspond to Maximum Likelihood (ML) bootstrap support and Bayesian posterior probabilities (BPP), both expressed as percentages, with only values higher than 50% displayed. The tree was rooted with *Klebsiella oxytoca* (CM1B5; MN621850.1). Accession numbers for newly generated sequences (PZ531582–PZ531600) and reference sequences are detailed in Supplementary Material Table S1.

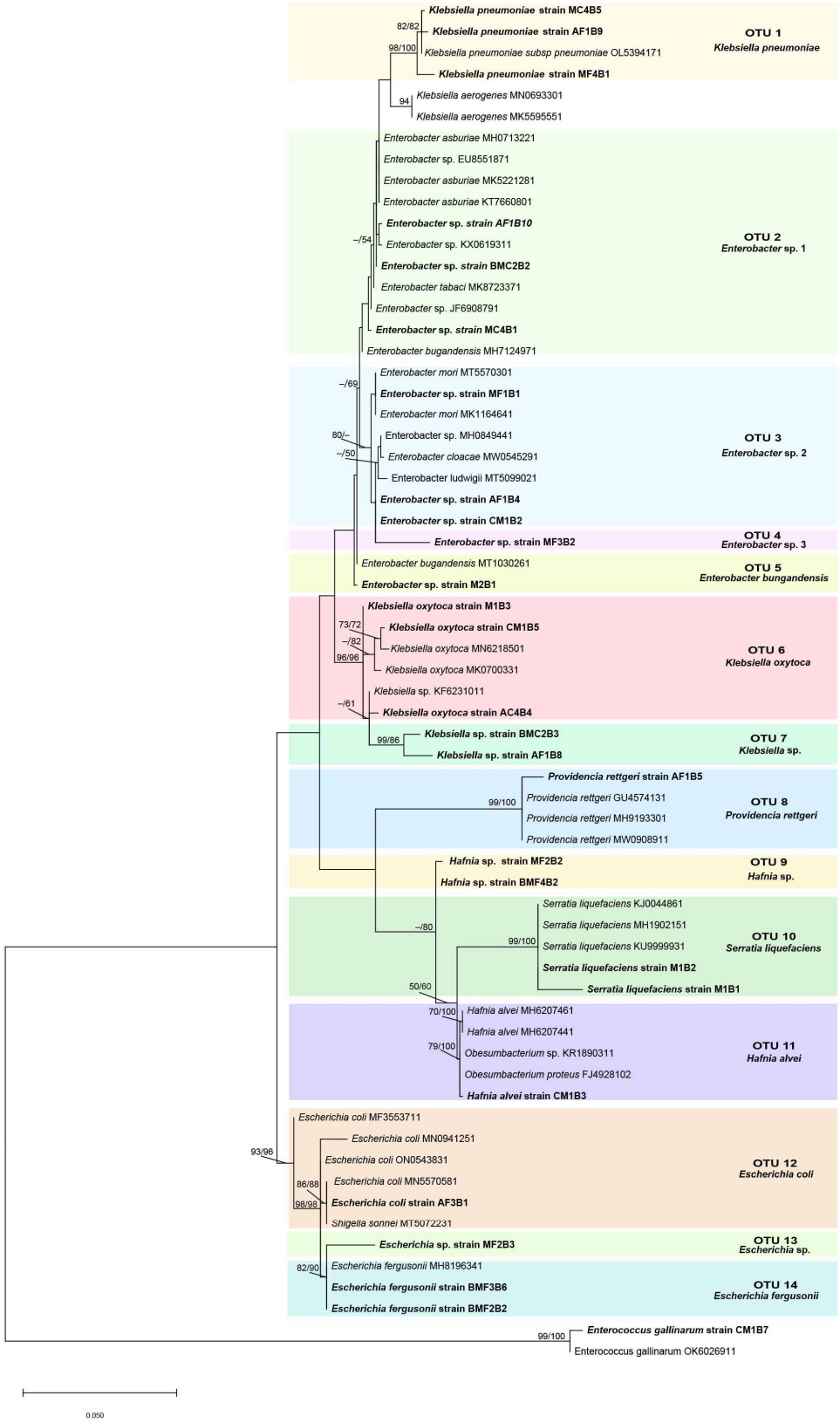


Figure 3. Phylogenetic positioning of 16S rRNA sequences from Gram-negative bacteria and clustering according to OTUs at a 1% difference threshold. Values at the nodes correspond to Maximum Likelihood (ML) bootstrap support and Bayesian posterior probabilities (BPP), both expressed as percentages, with only values higher than 50% displayed. The tree was rooted with *Enterococcus gallinarum* (CM1B7; OK602691.1). Accession numbers for newly generated sequences (PZ531601–PZ531626) and reference sequences are detailed in Supplementary Material Table S1.

Table 2. Functional traits reported in the literature for the bacterial species and OTUs identified in this study.

Bacterial Species/OTU	Strain Code	Ecological Niche	Potential in Fermentation Processes	Pathogenicity
GRAM-POSITIVE BACTERIA				
Leuconostoc pseudomesenteroides	AC4B2, AF2B2	Plant surfaces, fermented foods, cane juice, sourdough, kimchi, apple dumpster, caecum, and human adenoid [38].	Key heterofermentative bacterium; secretes pectinases and aroma volatiles (esters, pyrazines, diacetyl), enhancing coffee mucilage degradation and flavor profiles [39].	Non-pathogenic (GRAS status); beneficial in food bioprocesses and starter cultures, though rare cases of bacteremia have been reported in immunocompromised patients [40].
Lactococcus lactis	MC3B1, BMC3B1, BMF3B7, BMF3B4, BMF3B2, BMF1B1, BMC4B1, AC4B1, AC3B3, AC1B4, AC3B2, MC3B2	Diverse ecosystems like plant surfaces, dairy products, fermented plant materials, and soil [41].	Major homofermentative lactic acid producer; lowers pH during coffee wet fermentation and synthesizes aroma precursors (diacetyl (2,3-butanedione), acetoin) [42].	Generally Recognized as Safe (GRAS) and non-pathogenic, though rare opportunistic clinical cases have been reported for *Lactococcus lactis* subsp. *cremoris* [43].
Enterococcus sp.	MF2B4, M2B2, CM1B4	Primarily the gastrointestinal tract of animals, as well as environmental matrices including soil, surface water, and vegetation [44].	Active lactic fermenter of simple/complex sugars (cellobiose, xylan). Exhibits esterolytic, proteolytic, lipolytic, and citrate-metabolizing traits for flavor precursor formation, while producing enterocins and organic acids against spoilage microbes [45–47].	Potential opportunistic pathogens depending on strain virulence factors [48].
Enterococcus gallinarum	CM1B7	Intestines of animals, environmental soil, and plant surfaces; contributes to organic matter decomposition in soil ecosystems [49].		Uncommon opportunistic pathogen; intrinsically resistant to vancomycin [50].
Enterococcus faecium	MF3B3	Animal digestive tract, soil, water, and fermented food matrices; involved in organic nutrient breakdown [49].		Opportunistic and especially nosocomial pathogen, with multidrug-resistant strains [51].
GRAM-NEGATIVE BACTERIA				
Serratia liquefaciens	M1B2, M1B1	Soil, plant roots (rhizosphere), water, and decaying matter [52].	Produces proteolytic enzymes (pectinases, proteases) and can break down mucilage cell walls [53].	Opportunistic nosocomial pathogen in vulnerable humas [54].
Providencia rettgeri	AF1B5	Soil, water, sewage, and insect guts [55].	Participates in folate (vitamin B9) synthesis and exhibits urease activity, with potential function as a redox mediator [56,57].	Opportunistic nosocomial human pathogen (urinary tract and wound infections) [58].
Klebsiella sp.	BMC2B3, AF1B8	Soil, water, plant rhizospheres, phyllosphere, and organic residues; some strains capable of phosphate solubilization and siderophore production. Also present in intestines of a wide range of animals [59].	Pectolytic bacteria commonly found in the fermentation of *Coffea arabica* producing pectate lyase and secreting ethanol and low level of organic acids [13].	Variable potential opportunistic pathogen. Nosocomial and opportunistic pathogen in humans and animals [60]
Klebsiella pneumoniae	MC4B5, AF1B9, MF4B1			
Klebsiella oxytoca	M1B3, CM1B5, AC4B4			
Hafnia sp.	MF2B2, BMF4B2	Environment soil, water, plant surfaces, and gastrointestinal tract and food products [61].	Performs facultative anaerobic fermentation and produces N-acyl homoserine lactones (AHLs) for quorum sensing signaling [62].	Rare opportunistic pathogen in immunocompromised patients [23]. Opportunistic or rare pathogen causing intestinal and extraintestinal infections [63].
Hafnia alvei	CM1B3			

Table 2. *Cont.*

Bacterial Species/OTU	Strain Code	Ecological Niche	Potential in Fermentation Processes	Pathogenicity
Escherichia sp.	MF2B3	Widespread across animal gastrointestinal tracts, soil, water bodies, and agricultural environments [64].	Drives rapid mixed-acid sugar fermentation, yielding organic acids (acetate, formate, lactate, succinate), ethanol, and gases (H_2, CO_2) mainly present in no non-controlled fermentations [65,66]	Encompasses commensal and pathogenic strains (enteric/extraintestinal), acting as opportunistic human pathogens and emerging threats in livestock and poultry [67,68].
Escherichia fergusonii	BMF3B6, BMF2B2			
Escherichia coli	AF3B1			
Enterobacter sp. 1	AF1B10, BMC2B2, MC4B1	Widespread in soil, aquatic systems, plant surfaces, and endosphere/phyllosphere niches [69]	Drives active mixed-acid and 2,3-butanediol sugar fermentations, generating ethanol, organic acids (acetic, formic), gases (CO_2, H_2), and volatile precursors (acetoin, short-chain volatiles) that aid early pectin degradation [40,70,71].	Opportunistic human pathogens in vulnerable hosts, driven by strain-specific genes, including emerging strains with high multidrug resistance profiles [69,72].
Enterobacter sp. 2	MF1B1, AF1B4, CM1B2			
Enterobacter sp. 3	MF3B2			
Enterobacter bugandensis	M2B1			

3.3. Diversity Richness, and Community Composition

Bacterial alpha diversity was evaluated across the post-harvest processing methods and localities using species richness and the Shannon–Weaver index (Figure 4). The non-parametric Kruskal–Wallis test revealed no statistically significant differences among treatments for either species richness ($\chi^2 = 5.63$, $p = 0.059$) or the Shannon–Weaver index ($\chi^2 = 1.69$, $p = 0.428$); similarly, no significant differences were found among localities for species richness ($\chi^2 = 0.07$, $p = 0.965$) or the Shannon–Weaver index ($\chi^2 = 0.45$, $p = 0.796$). Despite the lack of significance, a visual trend towards higher bacterial richness and diversity was observed in both innovative treatments—Carbonic Maceration (CM) and Refrigerated Carbonic Maceration (CRM)—compared to the traditional Natural baseline control, which exhibited the lowest median values and a narrower distribution profile. Among localities, richness and diversity tended to be higher in Loja (2010 m a.s.l.) and Gonzanamá (1500 m a.s.l.) than in Olmedo (1230 m a.s.l.), the lowest-altitude site (Figure 4).

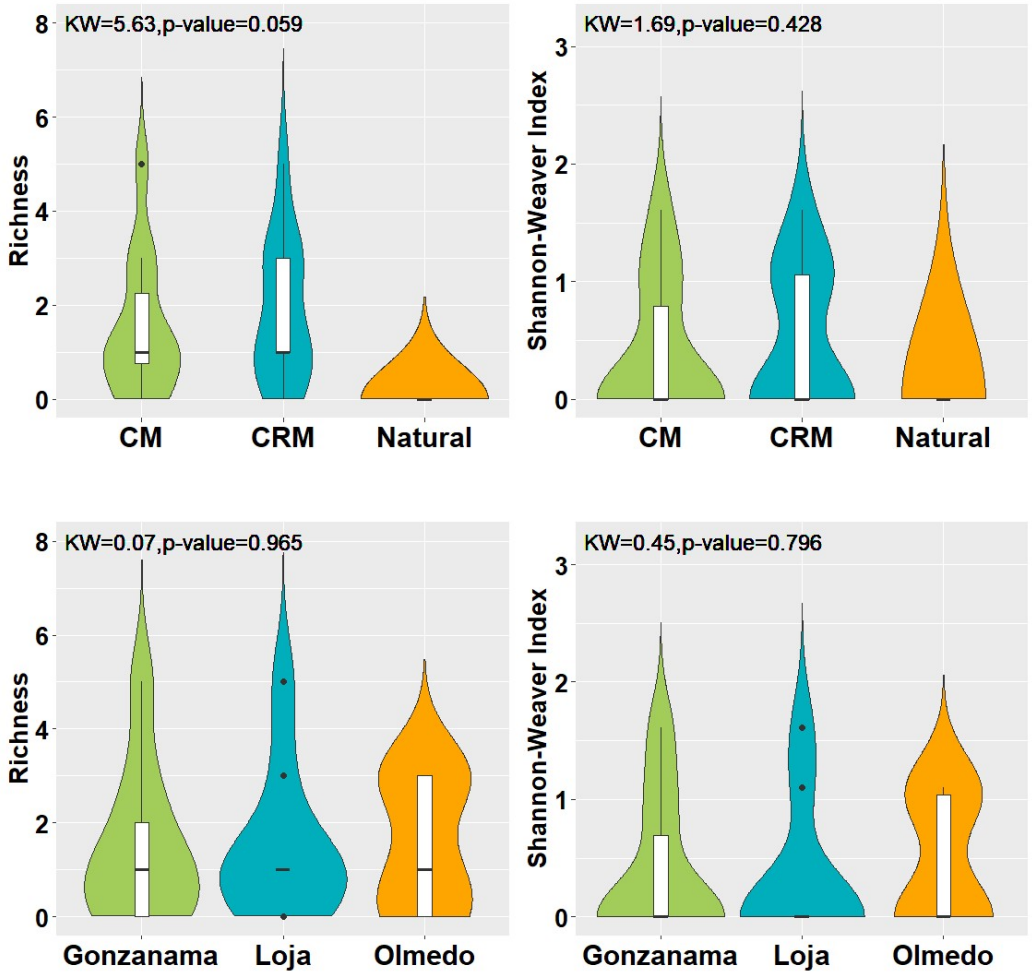


Figure 4. Violin plots showing bacterial richness and the Shannon–Weaver index across treatments and localities.

Bacterial community structure was further examined via Principal Component Analysis (PCA), where the first two dimensions captured 35.1% of the compositional variance (PC1, 18.0%; PC2, 17.1%; Figure 5). Samples from Carbonic Maceration (CM), Refrigerated Carbonic Maceration (CRM), and Natural treatments overlapped extensively, and their centroids were located near the origin of the ordination. In agreement with this overlap, the PERMANOVA confirmed that bacterial composition did not differ significantly among processing types ($F = 0.703$, $p = 0.775$). Taxa with short vectors near the origin, such as *Ser-*

ratia liquefaciens, *Escherichia coli*, and *Leuconostoc pseudomesenteroides*, contributed little to the separation of samples on the first two components, which is consistent with their recovery across treatments. The dispersion observed was driven mainly by a few individual CM and CRM samples rather than by treatment groups. Along the positive side of PC1, *Providencia rettgeri*, *Klebsiella pneumoniae*, *Klebsiella* sp., *Enterobacter* sp. 1, and *Enterobacter bugandensis* were associated with a small number of CM and CRM samples, while *Enterobacter* sp. 2 was oriented toward the upper-right quadrant. The upper-left quadrant (positive PC2) was associated with *Hafnia alvei*, *Enterococcus gallinarum*, *Enterococcus* sp., and *Klebsiella oxytoca*, together with one CM sample with a high PC2 score. In contrast, the lower-left quadrant (negative PC2) was associated with *Lactococcus lactis*, *Escherichia fergusonii*, *Enterobacter* sp. 3, and *Enterococcus faecium*. The Natural samples remained close to the origin, although this treatment included only one sample per farm. Overall, the cultivable bacterial composition was similar across post-harvest processing methods, and the variability observed reflected differences in the recovery of specific taxa in individual samples rather than a consistent effect of ambient or refrigerated carbonic maceration.

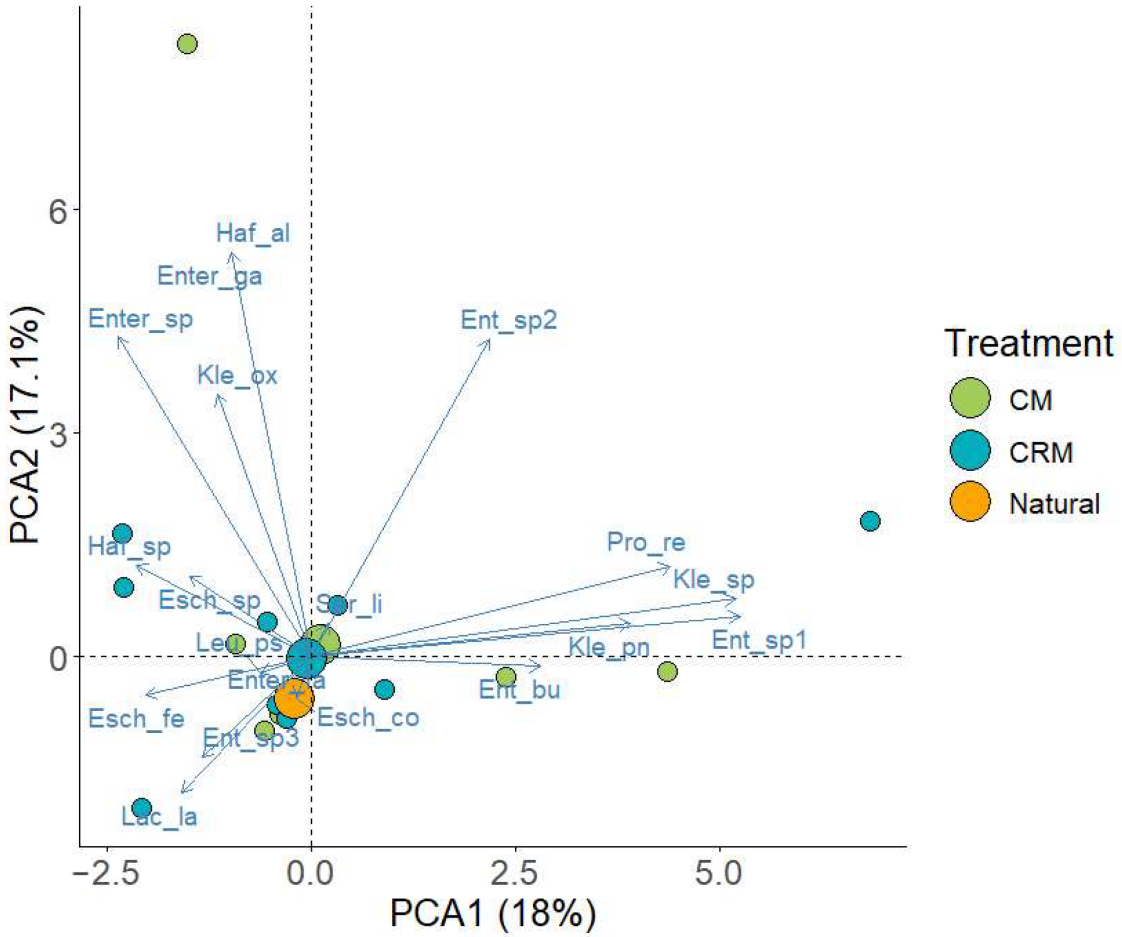


Figure 5. Principal Component Analysis (PCA) showing bacterial community composition across treatments. Circles represent samples coloured by treatment (CM, CRM and Natural); circle size is proportional to the frequency of isolates. Arrows indicate the contribution of each bacterial taxon to the principal components. Dashed lines mark the zero values of PCA1 and PCA2.

4. Discussion

The characterization of the coffee-associated microbiome in Loja revealed a taxonomic complexity that far exceeds initial estimates derived from conventional culture-dependent methods. Although 99 bacterial strains were preliminarily isolated, only 45 could be

successfully purified and clustered into 14 distinct morphotypes (Figure 1). Subsequent molecular identification revealed a significant phenotypic overlap between species. This limited taxonomic resolution of morphological and biochemical traits aligns with previous reports [73,74], reinforcing the premise that phenotypic characterization—while useful as a preliminary screening—must be integrated with high-precision molecular tools such as 16S rRNA gene barcoding [75].

Notably, this baseline inventory reflects the cultivable bacterial fraction selected via TSB enrichment (37 °C) to isolate viable strains from low-concentration matrices, rather than depicting unculturable microbial succession or real-time fermentation dynamics. Because enrichment and colony selection favor fast-growing, mesophilic, and facultatively anaerobic taxa, such as Enterobacteriaceae, the recovery frequency of each taxon cannot be interpreted as its abundance in the original fermentation, and the diversity reported here corresponds only to the cultivable fraction.

Despite the effectiveness of 16S rRNA for genus-level discrimination, intrinsic phylogenetic limitations were observed when resolving closely related species [76]. Cases such as the *Escherichia coli* clade (OTU 12), which included sequences of *Shigella sonnei*, or OTU 11 (*Hafnia alvei*), which grouped sequences of *Obesumbacterium*, suggest a high phylogenetic proximity that challenges delimitation through a single marker. For these complex groups, transitioning toward housekeeping genes (e.g., *rpoB*) or implementing MALDI-TOF MS protein profiling is highly recommended to achieve species-level resolution in future taxonomic updates [77,78].

A pivotal finding of this research is the disparity in genotypic richness between microbial domains within the same study area. While the fungal component previously reported for these specific farms in Loja comprised only nine yeast species [26], our findings reveal a substantially higher bacterial diversity, with 19 distinct genotypes identified (14 Gram-negative and 5 Gram-positive OTUs). This asymmetry suggests that bacteria—particularly Gram-negative species, which comprised 57.78% of the 45 isolates—may possess a greater colonization capacity or a more diversified niche response to post-harvest processing in southern Ecuador. As reported in the literature (Table 2), this predominant bacterial pool likely influences coffee processing by secreting functional enzymes and metabolites during fermentation processes that may also shape final coffee quality.

Interestingly, this dominant Gram-negative fraction, represented mostly by the family Enterobacteriaceae, showed strong congruence between genetic identity and phenotypic traits. Their frequent recovery across processing methods may be linked to specific environmental conditions or to the intrinsic dynamics of the post-harvest processing stations [79]. This Gram-negative pool encompasses pectolytic and fermentative taxa, including *Klebsiella* spp., *Serratia* spp., *Enterobacter* spp., and *Hafnia* spp. (Table 2), which could degrade mucilage and transform multiple compounds, generating aroma precursors through mixed-acid and 2,3-butanediol fermentation [13,45–47,53,62]. However, uncontrolled activity by these strains risks overproducing volatile fatty acids or biogenic amines, leading to sour, oniony, or rancid taints [80].

The structural consistency of the bacterial community is further supported by the analysis of alpha and beta diversity. The violin plots (Figure 4), representing bacterial richness and the Shannon–Weaver index, revealed no significant differences across treatments or localities, indicating that the overall complexity of the cultivable bacterial community remains inherently stable. This is corroborated by the PCA ordination (Figure 5) and the subsequent PERMANOVA ($F = 0.703$, $p = 0.775$), which demonstrate that post-harvest processing—whether Natural, CM, or CRM—does not induce significant shifts in the recoverable bacterial composition under the tested conditions. This consistency is likely explained by the fact that the three farms cultivated the same variety (Geisha) and applied

the same post-harvest processes. Nevertheless, richness and diversity were slightly lower in Olmedo (Figure 4), the lowest-altitude site (1230 m a.s.l.), which could be related to altitude and to the diversity of crops associated with coffee in each agroforestry system (see methodology of Study Area and Sampling); this hypothesis requires a future study with balanced replication across farms. In addition, because the Natural treatment was represented by a single sample per farm, the absence of differences involving this treatment should be interpreted with caution.

The convergence of these results suggests that the coffee-associated cultivable bacteria in Loja are characterized by a high degree of compositional resilience and spatial homogeneity across the region. This stability implies that the predominant cultivable isolates (including *Lactococcus* and *Enterobacter*) are well-adapted to the coffee fruit environment, maintaining their predominance regardless of the metabolic pressures or microenvironmental changes introduced by different fermentation configurations.

Within the Lactic Acid Bacteria (LAB) group, the identification of *Lactococcus lactis* and *Leuconostoc pseudomesenteroides* is of significant biotechnological interest. These LAB are specialized to metabolize pulp sugars and degrade mucilage pectin, lowering the pH through lactic acid production [81]. Additionally, *L. lactis* and *L. pseudomesenteroides* yield aroma volatiles (diacetyl, acetoin, esters) and pectinases that facilitate mucilage solubilization [39,42], while *Enterococcus* species contribute to sugar fermentation, aroma precursor formation, and enterocin secretion [45–47]. This metabolic activity not only inhibits undesirable microbiota [82] but also enriches the organoleptic profile with aromatic precursors, such as esters and ketones, which contribute to the floral and fruity notes characteristic of specialty coffee [42]. In this sense, controlled acidification by microorganisms could promote a clean acidity, whereas uncontrolled lactic acid production could result in an over-acidified and astringent cup.

The functional profiling, according to the cited literature, of these 19 genotypes (Table 2) reveals a biosafety dichotomy in the fermentation processes evaluated in this study. While most bacteria contribute to sugar degradation and flavor precursor formation, several Enterobacteriaceae and *Enterococcus* taxa can act as opportunistic human pathogens or hygiene indicators. Specifically, *Klebsiella pneumoniae*, *K. oxytoca*, *Escherichia coli*, *Enterobacter* spp., *Providencia rettgeri*, *Serratia liquefaciens*, *Enterococcus faecium*, and *E. gallinarum* include strains reported as opportunistic or nosocomial pathogens (Table 2). Their recovery likely reflects contact of the cherries with soil, water, and handling during harvest and processing [79], and may have been favored by the enrichment step at 37 °C. Therefore, these taxa should not be considered candidate starter cultures; any future selection should focus on taxa with a history of safe use, such as *Lactococcus lactis* and *Leuconostoc pseudomesenteroides* (GRAS status), and should include strain-level safety screening (e.g., hemolytic activity, antibiotic resistance, and virulence genes) before application in coffee fermentation [43]. Consequently, monitoring fermentation processes is crucial to prevent unpredictable sensory spoilage and microbiological hazards.

From a bioeconomy perspective, the high stability of these regional cultivable bacteria provides a consistent and predictable baseline for the development of standardized starter cultures. Although this study did not track real-time metabolite dynamics or final cupping scores, establishing this cultivable diversity is an indispensable first step. While inoculation studies traditionally rely on a narrow set of commercial strains [14,83], the bacterial diversity recovered in Loja provides a broader matrix of functional candidates to modulate sensory-active compounds [84]. Screening these native strains for beneficial traits (e.g., rapid mucilage degradation, volatile synthesis) while excluding pathogenic or spoilage-associated isolates allows for the design of tailored starters to consistently optimize coffee quality. Consequently, the 19 distinct genotypes isolated here have been

cryopreserved in the UTPL Culture Collection (Cepario UTPL), establishing a biobank for upcoming controlled inoculation trials and sensory evaluations [3,85] that could improve the quality of Ecuadorian coffee.

5. Conclusions

This study reveals a taxonomically complex, cultivable bacterial community (45 isolates and 19 genotypes) associated with coffee post-harvest processing in Loja. While 16S rRNA barcoding effectively resolved most genera, the observed phenotypic overlap underscores the need for multi-marker integration to achieve precise species-level delimitation. Multivariate analyses demonstrated high compositional resilience, as the recoverable bacterial profile remained stable across post-harvest processing methods and across three farms located at different altitudes, all with the same coffee variety and processes. Importantly, these findings represent the cultivable bacterial fraction selected via a prior liquid enrichment step (TSB) rather than real-time total microbial succession. Nevertheless, these predominant cultivable isolates—including biotechnologically relevant taxa such as *Lactococcus lactis*—provide a predictable baseline for regional starter cultures, provided that potentially pathogenic taxa are excluded. The slightly lower diversity observed in Olmedo warrants future studies on the influence of altitude and associated crops. All isolated genotypes have been cryopreserved in the UTPL Culture Collection, securing a native genetic reservoir for future controlled fermentations to evaluate functional traits, metabolite dynamics, and sensory cup quality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation12100479/s1, Table S1: Taxonomic assignment based on BLASTn sequence homology against the GenBank database for the bacterial strains isolated in this study alongside worldwide reference sequences. The table details internal strain codes, identified species with their respective taxonomic authorities, and official sequence identifiers.

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Abbreviations

The following abbreviations are used in this manuscript:

16S rRNA	16S ribosomal Ribonucleic Acid
BPP	Bayesian Posterior Probability
BI	Bayesian Inference
BLASTn	Basic Local Alignment Search Tool for nucleotides
BS	Bootstrap percentage
CFU	Colony-Forming Unit
ESS	Effective Sample Size
ITS	Internal Transcribed Spacer
K2P	Kimura 2-parameter model
LAB	Lactic Acid Bacteria
MAFFT	Multiple Alignment using Fast Fourier Transform
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
NCBI	National Center for Biotechnology Information
OTU	Operational Taxonomic Unit
PCA	Principal Component Analysis
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UTPL	Universidad Técnica Particular de Loja

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