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Phenotypic and Molecular Characterization of Yeast Diversity Associated to Postharvest Fermentation Process of Coffee Fruits in Southern Ecuador

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Abstract: Coffee (*Coffea arabica*), produced and marketed in Ecuador and worldwide, can be organoleptically improved by means of microorganisms such as well-characterized yeasts. This study aimed to isolate and characterize yeasts from three postharvest fermentation processes (i.e., Natural aerobic at room temperature; Carbonic maceration with a CO_2 atmosphere at room temperature; and Carbonic refrigerated maceration with a CO_2 atmosphere to 10 °C) of coffee fruits in Ecuador. Phenotypic and molecular analyses were conducted on 329 yeast isolates obtained from coffee farms in Loja, Olmedo, and Gonzanamá. Three universal media were used for yeast isolation diversity, and phenotypic characterization included morphology, sugar fermentation, salt tolerance, and ethanol resistance. Molecular characterization involved DNA analysis. The isolated diversity was classified into 12 morphotypes, nine distinct biochemical groups and nine genetic species. Only six species (i.e., *Kurtzmaniella quercitrusa*, *Hanseniaspora opuntiae*, *Pichia. kluyveri*, *Torulaspora delbrueckii*, *T. quercuum*, and *Wickerhamomyces anomalus*) identified phylogenetically corresponded to the designated morphotypes. But surprisingly, nine genetic species matched with the nine biochemical groups determined phenotypically analyzed using principal component analysis (PCA). Most of this diversity was found in the coffee plantation located in Gonzanamá, in contrast to Olmedo and Loja, without statistical significance (p value: 0.08295). On the other hand, the richness is not similar statistically (p value: 0.02991) between postharvest fermentation treatments. The findings suggest that the application of biochemical tests is useful for species determination, although morphological data may be ambiguous. Notably, *Pichia kluyveri*, detected in this study, holds potential for biotechnological evaluation in coffee fermentation processes.

Keywords: coffee fruit; fermentation; isolation; characterization

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1. Introduction

Coffee is currently one of the most traded products worldwide, as it is one of the main agro-export products in Ecuador [1]. In total, 20 of the country's 24 provinces cultivate commercial coffee species: 135,466.2 ha are occupied by arabica varieties (*Coffea arabica* L.) and 63,748.8 ha are occupied by robusta (*Coffea canephora* Pierre.) [2]. The Ecuadorian region produces quality coffee, but its chemical and organoleptic improvements are sought through fermentative processes (beneficiation) prior to drying and milling [3].

In beneficiation processes, microorganisms such as yeasts play important roles as facilitators in speed and conversion of organic compounds, helping coffee beans to improve

their organoleptic properties (taste and aroma) [4]. Fermentation involves several catabolic processes where organic substances are oxidized, transforming sugars into energy and other simple compounds such as ethanol, acetic acid, lactic acid, and butyric acid [5]. Usually, the farmers apply a typical or natural fermentation method after the harvest where the coffee fruits are dried after being cleaned [4], and other farmers employ carbonic maceration, an analogous technique to the wine fermentation process, where the coffee grains undergo fermentation within a medium saturated with carbon dioxide (CO_2), generally maintained at room temperature or below 10 °C [5,6].

Microorganisms such as yeasts, a group of unicellular fungi with or without the presence of hyphae or pseudohyphae, act strongly in that fermentation processes [7]. Ecologically, the yeasts are present in a diverse range of habitats, including soil, aquatic environments, plant surfaces, foods, and skin and mucosal surfaces of animal hosts, but the soil environments represent the major ecological niche for fungi including yeast [8], helping in metabolism and facilitating carbon assimilation of fruits [9].

However, the diversity of microorganisms, especially yeasts, has been reported using molecular techniques [10], especially next-generation sequencing methods (i.e., metagenomics), useful mainly for diversity estimates using operational taxonomic units [11]. Likewise, the nuclear ribosomal DNA region ITS-5.8S is one of the most widely used regions as a DNA barcode [12], as well as the D1/D2 region of the 26S gene [13]. Also, yeast species can be determined by biochemical tests (mandatory to describe new species) through their fermentative capacity, as well as resistance to ethanol, NaCl, or germ tube generation tests [9]. The diversity of yeasts in coffee still requires exploration and adequate taxonomic characterization, as well as their ecological or biotechnological functionality in fermentative processes of fruits such as coffee [1,14,15].

Worldwide, the use of yeasts to improve organoleptic properties is gaining popularity, modifying the flavor and potential of the coffee bean with the use of species such as *Saccharomyces cerevisiae* and *Pichia kluyveri* [16,17]. However, in Ecuador and in the province of Loja renowned for its quality coffee [18], studies on improvements in coffee varieties and coffee pathogenic microorganisms are recorded [19]. However, in other localities, biotransformation of coffee flavor is proposed with the use of yeasts such as *Saccharomyces cerevisiae* and *Pichia kluyveri* through inoculations [6].

Now, very little is known about the biodiversity of yeasts associated with coffee fermentation processes on Ecuador, with only the study by Jumbo and Martínez [20], which evaluates the capacity of the *Saccharomyces cerevisiae* species to improve the chemical and organoleptic properties of coffee.

Due to the lack of information on the culturable diversity of yeasts from coffee in this region, the present research sought to isolate, phenotypically and molecularly characterize yeasts from three fermentation processes of coffee fruits (i.e., Natural aerobic at room temperature; Carbonic maceration with CO_2 atmosphere at room temperature; and Carbonic refrigerated maceration with CO_2 atmosphere to 10 °C) in three different farms in the province of Loja applied by local farmers to improve the sensory properties of coffee. In addition, the conservation of pure strains can be evaluated in the future in controlled fermentation processes of coffee, and the effect on the chemical and sensory properties of the final beverage can be determined.

2. Materials and Methods

2.1. Sampling

Nine samples of coffee beans were randomly collected from coffee farms located in the cantons (Figure 1, Table 1): Loja, Olmedo and Gonzanamá, collecting beans of the Geisha variety of *Coffea arabica*, characterized mainly by its slender trunk, elongated leaves and branches pointing towards the sky [21]. It is a variety of coffee growing around the Andes Mountain range known as shade coffee, and it is considered to be the best-quality coffee in the region [18].

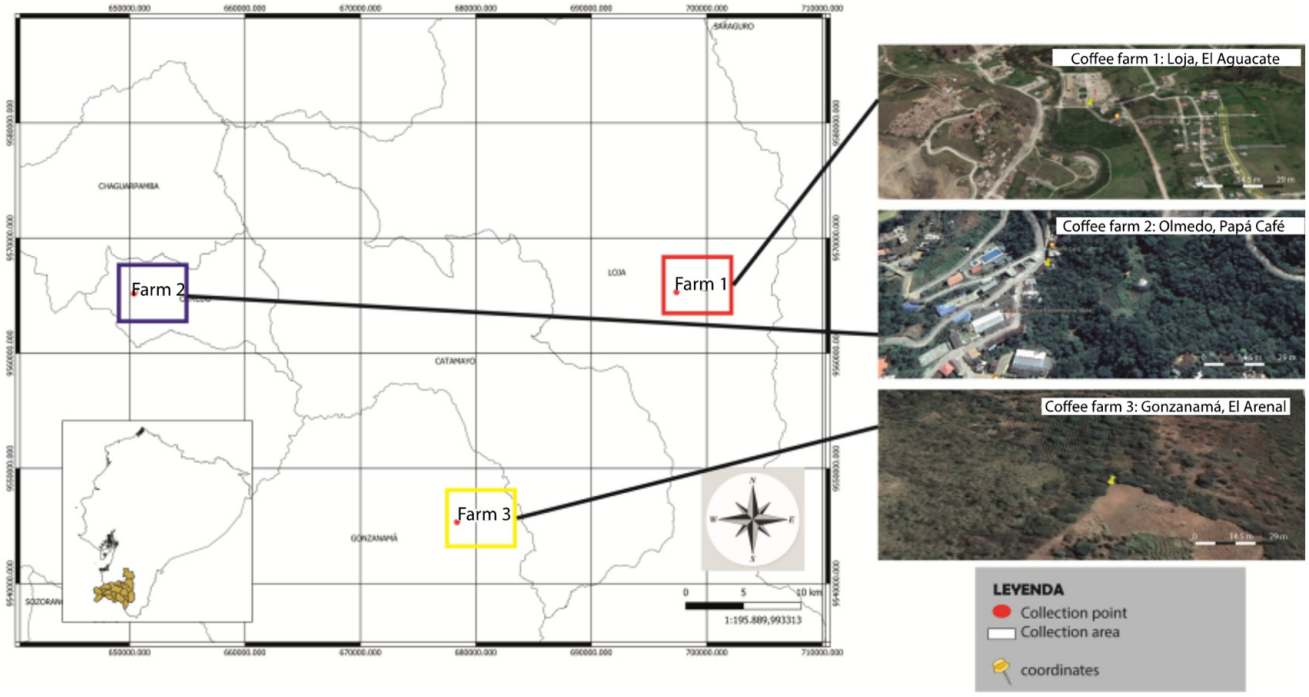

Figure 1. Geographical location of the coffee farms sampled in the province of Loja.

Table 1. Reference information of the samples evaluated for each treatment and farm.

Canton	Farms by Sector	Latitude and Longitude	Altitude (m.a.s.l.)	No Sample Code	Postharvest Fermentation Process
Loja	El Aguacate	3°55.848′ S 79°13.340′ W	2010	A	Natural
				AC	Carbonic maceration with CO_2 atmosphere at room temperature
				AF	Carbonic refrigerated maceration with CO_2 atmosphere up to 10 °C
Olmedo	Papá Café	3°55.960′ S 79°38.729′ W	1230	B	Natural
				BMF	Carbonic maceration with CO_2 atmosphere at room temperature
				BMC	Carbonic refrigerated maceration with CO_2 atmosphere up to 10 °C
Gonzanamá	El Arenal	4°06′02.0″ S 79°23′40.4″ W	1500 m	M	Natural
				MC	Carbonic maceration with CO_2 atmosphere at room temperature
				MF	Carbonic refrigerated maceration with CO_2 atmosphere up to 10 °C

2.2. Postharvest Fermentation Process

Coffee fruits (*Coffea arabica* L.) of the Geisha variety were collected in three farms at different altitudes (1230, 1500, 2010 m a.s.l) located in Loja province of Ecuador. The harvest was conducted by hand, and only ripe fruits were used. The samples were cleaned under continuous flow of tap water [3]. Then, the coffee fruits were placed in 4 L polyethylene bottles and fermented for 144 h under three different conditions: (a) Natural or "blank sample" aerobic at room temperature (N); (b) Carbonic maceration with a CO_2 atmosphere at room temperature (CM); and (c) Carbonic refrigerated maceration with a CO_2 atmosphere up to 10 °C (CRM). The atmospheric air in the bottles was constantly monitored by a gas

analyzer (Oxybaby M+, Dortmund, Germany). Immediately after the fermentation process was completed, the microbiological analyses were carried out [5].

2.3. Yeast Isolation

In total, 10 g of each sample was inoculated into a sterilized stock solution (200 mL) of Yeast Peptone Dextrose Broth (YPD-DIFCO) and incubated at 27 °C for 24 h with continuous motion at 50 rpm [22].

After the incubation, a volume of 30 µL of the stock culture was seeded by a depletion technique on three solid media: Yeast Mold Agar (YMA-DIFCO), Sabouroad Dextrose Agar (SDA-DIFCO) and Yeast Extract Glucose agar (YGC) using Floran (FAVETEX) as a broad-spectrum antibiotic. The cultures were also incubated at 27 °C for 1–2 days. Subsequently, a random number (between 10 and 20 colonies) was selected for replication and purification on Potato dextrose Agar (PDA-DIFCO) solid medium plus Floran antibiotic at a final concentration of 1%. This process was repeated at 48 and 72 h [23].

2.4. Phenotypic Characterization of Pure Strain Sampling

Morphology: Strains were classified by morphotypes (MT) according to their growth form based on outline, size, coloration (British Standard Specification for colors) and aroma [20]. In addition, yeast shape and size were microscopically checked using an Olympus CX41 optical microscope and a 100× magnification. Preparations were performed under direct staining with 1% Phloxine B and 10% KOH.

Biochemistry: Biochemical tests correspond to (a) Sugar fermentation (differential with positive red and negative yellow staining) on a YPD-DIFCO culture medium (5 mL of medium plus 1.6 mL of methyl red prepared at 100 ppm) [21]; (b) Tolerance to NaCl at 10 and 15% (positive when there is colony growth) in a YMA medium under depletion stress and incubation at 30 °C (overnight); (c) Resistance to ethanol in a YPD medium, two tubes for each strain, one with 4.5 mL plus 0.5 mL of 96% ethanol and another 4.25 mL plus 0.75 mL of 96% ethanol, incubated at 30 °C with continuous movement at 60 rpm [24].

Additionally, a yeast germ tube test was performed to determine whether any yeast species corresponded to *Candida albicans*. All pure strains were tested by inoculating them in human serum and incubating at 37 °C for 2 h [25].

2.5. Statistical Analysis of Ordination and Analysis of Isolates by Farms

To determine differences between yeast species richness by location and treatment, we used one-way analysis of variance (ANOVA). We tested the normality of distributions of richness using the Shapiro–Wilk test (p value > 0.05).

Principal component analysis was performed based on the presence of each yeast population recovered to visualize the grouping of biochemical characteristics by species according to the characteristics of fermentation tests based on the Jaccard similarity index. Principal component analysis was performed using freely available statistical analysis PAST software version 4.10 [26]. We also computed a non-metric MDS (multidimensional scaling) ordination from the species to reveal the degree of similarity among treatments. We used the Euclidean distance as a metric for species similarity.

2.6. DNA Extraction, Amplification and Sequencing

A total of 36 strains were selected to be molecularly worked up due to similar characteristics found in the phenotypic analysis of the strains. Three strains from the same morphotypes, MT, were chosen. One colony for each selected strain was used for DNA extraction and amplification using the Phire Plant Direct PCR Master Mix commercial kit (Thermo Scientific, Vilna, Lithuania) according to the manufacturer's specifications. The ITS-5.8S region of DNArn and partial LSU (D1/D2) was amplified with the following primers: ITS1F 5′ CTGGTCATTTAGAGGAAGTAA 3′ [27] and NL4 5′ GGTCCGTGTTTCAAGACGG 3′ [28]. PCR conditions were as follows: an initial denaturation at

98 °C for 5 min, followed by 45 cycles of denaturation at 98 °C for 10 s; banding at 55 °C for 10 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min.

PCR results were evaluated by 1% agarose gel electrophoresis and 1X GelRED staining. Positive products were purified using the PureLinkTM Quick PCR Purification Kit (Invitrogen, Vilna, Lithuania) and sequenced with the same set of PCR primers at Macrogen (Seoul, Republic of Korea).

2.7. Phylogenetic Analysis

The sequences obtained were visualized and edited in CodonCode Aligner 9.0.2 software (CodonCode Corporation, Centerville, MA, USA). The concatenated sequences (forward and reverse) were compared in GenBank Blast (https://www.ncbi.nlm.nih.gov/genbank/ accessed on 22 June 2023) to download the most similar sequences, preferably with taxonomic value (assigned species names). All sequences (37 new and 35 from GenBank) were aligned in MAFFT Version 7.489 using the G-INS-i strategy [29].

Two phylogenetic trees were performed under Maximum Likelihood algorithms [30], Kimura-2 parameter model and G + I nucleotide substitution rate model, followed by 1000 Bootstrap replicates employing MEGA 11 software [31].

2.8. Strain Preservation

The pure strains obtained were inoculated in a solution of 1.64 mL YPD medium plus 20% glycerol (permeable cryoprotectant) and allowed to grow for 1 h at 27 °C. Subsequently, they were frozen at −80 °C in cryovials. Additionally, yeast suspensions were made in 2 mL of triple-sterilized distilled water and 2 mL of 100% sterile mineral oil, which were kept refrigerated at 4 °C [32,33].

3. Results

3.1. Phenotyping and Diversity

A total of 329 pure strains were obtained and classified into 12 morphotypes (MT) (Figure 2). Several MT correlated by shape: rounded (MT1, MT3 and MT5) differentiated by attachments such as protrusions (MT5), visualization of vacuoles (MT1) and visualization of cell walls and membranes typical of the genera *Torulaspora* and *Wickerhamomyces*. On the other hand, oval-type morphology was found in MT2, MT6 and MT10, typical of the genera *Kurztmaniella*, *Hanseniaspora* and *Pichia*, respectively. Furthermore, elongated morphotypes (MT4, MT7, MT8, MT9 and MT11) were classified as recurrent in the genus *Pichia*; finally, protrusions attached to a rectangular oval MT (MT12) were found. Sporulation of MT2 strain BMF4L2 was recorded (Figure 2), and four internal segments were differentiated in the yeast. Most of the strains isolated and macroscopically checked showed similar characteristics in terms of colony shape and odor.

The morphotype designated for *Kurtzmaniella quercitrusa* clustered with similar biochemical properties to those of *Pichia fermentans*, *Hanseniaspora opuntiae* and *Torulaspora delbrueckii* (Figure 3), standing out from the others by the presence of a germ tube, as well as growth in lactose and 15% NaCl. *P. kluyveri*, *H. uvarum* and *Wickerhamomyces anomalus* showed fermentation affinity in glucose and maintained a higher resistance in 10% NaCl.

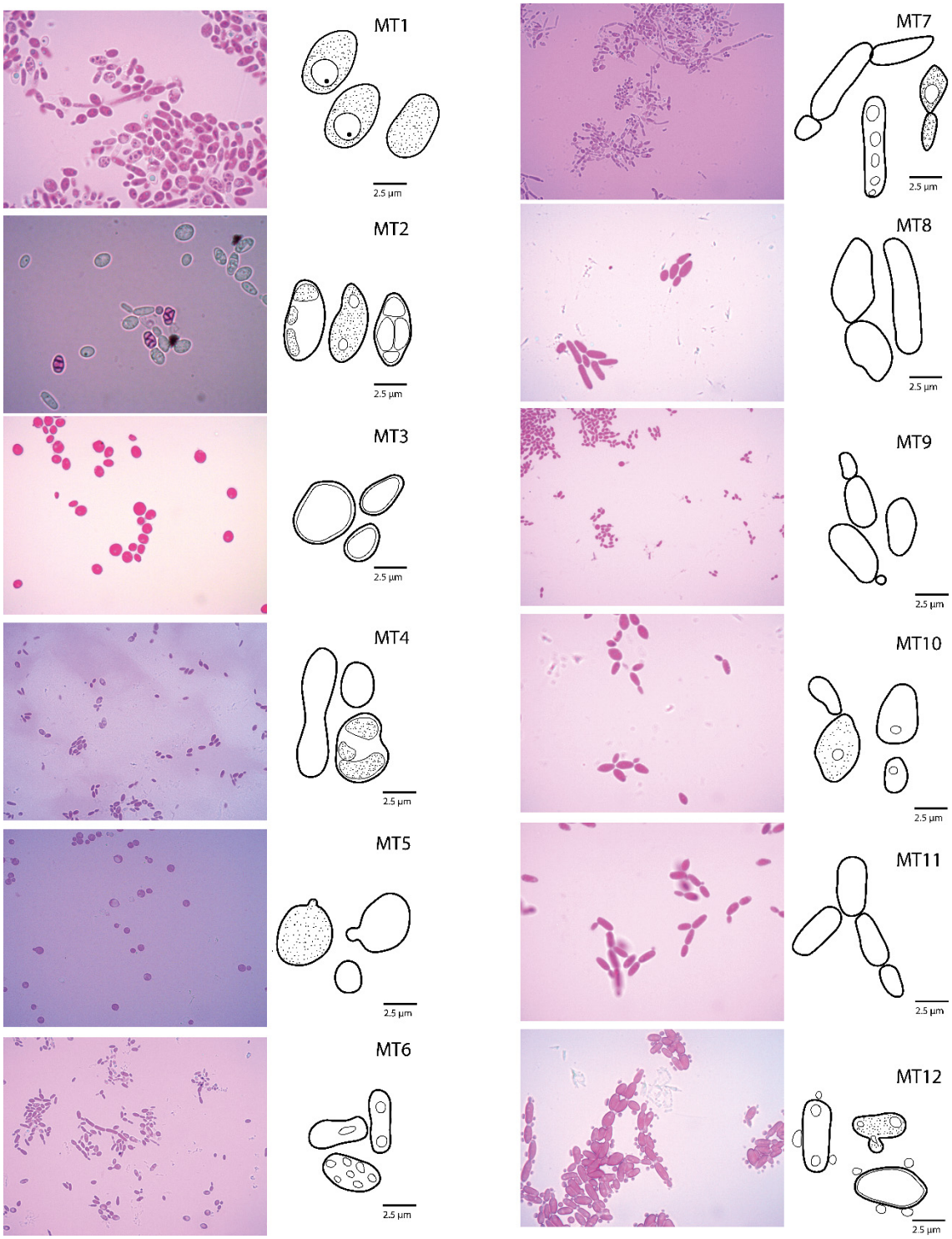

Figure 2. Morphotypes determined for the yeast isolates in this study. Photographs indicate reddish staining by Phloxine 1%. Illustrations represent in detail the yeast forms.

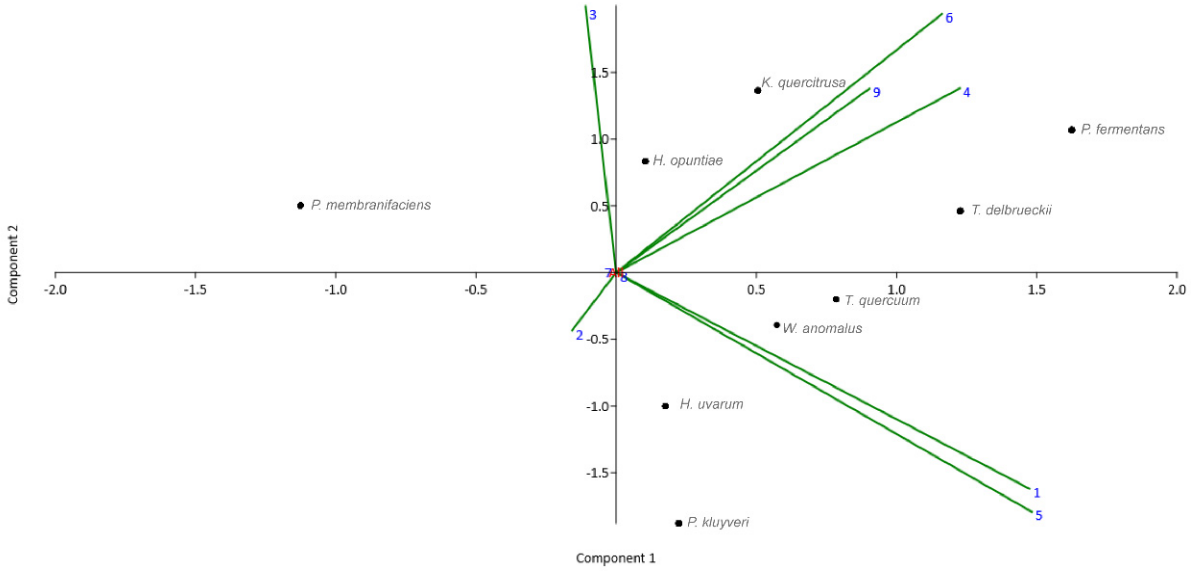

Figure 3. PCA for the nine species determined biochemically, according to the fermentation tests on (1) malt, (2) sucrose, (3) glucose, (4) lactose. Tolerance to (5) 10% NaCl, (6) 15% NaCl, (7) 10% ethanol, (8) 15% ethanol. (9) Germ Tube Test.

Most species diversity was found in the coffee plantation located in Gonzanamá (Figure 4), in contrast to Olmedo and Loja, but statistically not significant (*p* value: 0.08295) (Table 2, Figure 4). In the isolates from Olmedo and Loja, the species *Hanseniaspora opontuiae* was not identified (Table 3). On the other hand, the isolates from Loja "El aguacate" (Table 1) presented a lower number of species that did not include *Kurtzmaniella quercitrusa* and *Torulaspora delbrueckii*, but presented a higher abundance for the genus *Pichia* (Table 2, Figure 4).

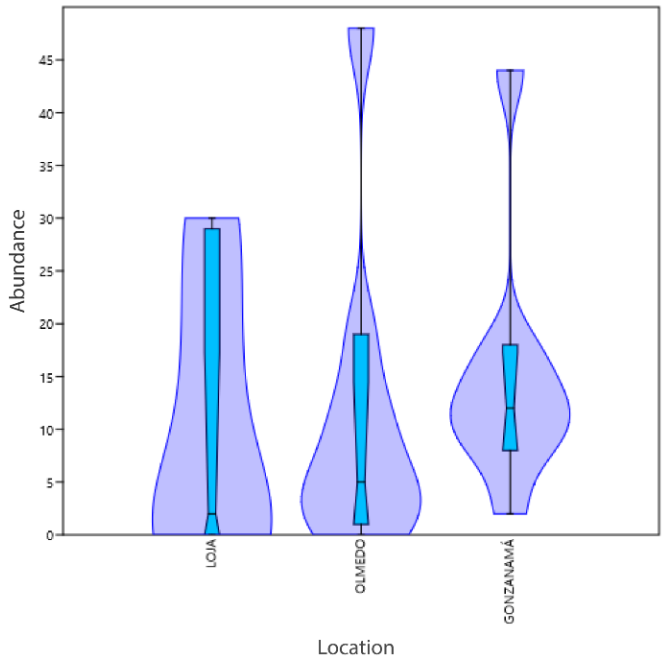

Figure 4. Diversity analysis by sampling site. The numbers on abundance axis represents isolates by each of the nine species.

Table 2. ANOVA of the richness of yeasts found according to the fermentation treatment used and the origin of the sample.

		Sum of Squares	Df	Mean Square F	F	*p* Value
Richness according to the type of fermentation	Between groups	15.7407	2	7.87037	4.077	0.02991
	Within groups	46.3333	24	1.93056		
	Total	62.0741	26	0.3307		
Richness by location	Between groups	11.6296	2	5.81481	2.767	0.08295
	Within groups	50.4444	24	2.10185		
	Total	62.0741	26	0.08731		

Table 3. Strains obtained and their BLAST reference information.

Species	Collection Number	Morphotype Referential	GenBank Accession Number	Country	Literature Reference
Kurztmaniella quercitrusa	CM1L1	MT7	OR145791	Ecuador	This study
Candida quercitrusa	HA 1669	-	AM160627	Austria	Xiao [34]
Kurztmaniella quercitrusa	BMF2L3	MT7	OR145790	Ecuador	This study
Hannseniaspora opuntiae	MF3L2	MT8	OR145783	Ecuador	This study
Hannseniaspora opuntiae	JEY258	-	KC111445	Tunisia	Eddouzi et al. [35]
Hannseniaspora opuntiae	KOT1	-	KY849378	Tunisia	Ben Taheur [36]
Hannseniaspora opuntiae	SM10UFAM	-	MN268780	Brazil	Silva et al. [37]
Hanseniaspora uvarum	AC3L1	MT9	OR145784	Ecuador	This study
Hanseniaspora uvarum	P43C012	-	JX188164	USA	Bourret et al. [38]
Hanseniaspora uvarum	Mer383	-	MT734682	Slovenia	Zabukovec et al. [39]
Krutzmaniella quercitrusa	CBS 4412	-	MK394107	Netherlands	Stavrou et al. [40]
Krutzmaniella quercitrusa	LHCT1	-	KX961193.	China	Li et al. [41]
Nuerospora crassa	AR14R	-	OK148118	India	Direct submission
Nuerospora crassa	I1-3	-	MH507014	China	Direct submission
Nuerospora crassa	I1-3	-	MK064500	China	Direct submission
Pichia fermentans	AF2L7	MT4	OR145756	Ecuador	This study
Pichia fermentans	CM1L6	MT4	OR145758	Ecuador	This study
Pichia fermentans	AC3L10	MT4	OR145755	Ecuador	This study
Pichia fermentans	AC4L2	MT4	OR145757	Ecuador	This study
Pichia fermentans	HR-1 DS1308	-	KM029994	USA	Direct submission
Pichia fermentans	HA-1	-	KU820951	Republic of Korea	Direct submission
Pichia fermentans	ATCC 10651	-	GQ458040	Canadá	Arteau et al. [42]
Pichia fermentans	CBS 187	-	MK394169	Netherlands	Stavrou et al. [40]
Pichia kluyveri	BMC4L9	MT1	OR145759	Ecuador	This study
Pichia kluyveri	AF2L3	MT10	OR145761	Ecuador	This study
Pichia kluyveri	BMF4L1	MT10	OR145762	Ecuador	This study
Pichia kluyveri	AF2L1	MT10	OR145760	Ecuador	This study
Pichia kluyveri	P25B004	-	JX188197	USA	Bourret et al. [38]
Pichia kluyveri	H12	-	MN266784	Japón	Vasques et al. [43]
Pichia kluyveri	KBP:Y-4954	-	MG367291	Moscow	Direct Submission
Pichia kluyveri	PMM10-1033666L	-	KP132502	Australia	Irinyi et al. [44]
Pichia kluyveri	CBS 188	-	MK394165	Netherlands	Stavrou et al. [40]

Table 3. *Cont.*

Species	Collection Number	Morphotype Referential	GenBank Accession Number	Country	Literature Reference
Pichia membranifaciens	M2L7	MT6	OR145770	Ecuador	This study
Pichia membranifaciens	MF4L8	MT6	OR145772	Ecuador	This study
Pichia membranifaciens	BMF1L6	MT6	OR145774	Ecuador	This study
Pichia membranifaciens	AC1L5	MT10	OR145767	Ecuador	This study
Pichia membranifaciens	BMF1L2	MT6	OR145769	Ecuador	This study
Pichia membranifaciens	BMF1L5	MT6	OR145775	Ecuador	This study
Pichia membranifaciens	BMF4L9	MT12	OR145773	Ecuador	This study
Pichia membranifaciens	AC3L3	MT6	OR145766	Ecuador	This study
Pichia membranifaciens	MF1L3	MT6	OR145776	Ecuador	This study
Pichia membranifaciens	MF1L7	MT6	OR145777	Ecuador	This study
Pichia membranifaciens	AC2L2	MT11	OR145764	Ecuador	This study
Pichia membranifaciens	AF2L6	MT6	OR145765	Ecuador	This study
Pichia membranifaciens	P43C010	-	JX188208	USA	Bourret et al. [38]
Pichia membranifaciens	CBS 82	-	DQ198951	China	Wu et al. [45]
Pichia membranifaciens	CBS 107	-	MK394163	Netherlands	Stavrou et al. [40]
Pichia membranifaciens	MC1L9	MT6	OR145771	Ecuador	This study
Pichia membranifaciens	BMF1L9	MT6	OR145768	Ecuador	This study
Pichia membranifaciens	MF4L5	MT12	OR145763	Ecuador	This study
Torulaspora delbrueckii	MC4L2	MT2	OR145788	Ecuador	This study
Torulaspora delbrueckii	MC1L1	MT2	OR145787	Ecuador	This study
Torulaspora delbrueckii	MC2L1	MT2	OR145789	Ecuador	This study
Torulaspora delbrueckii	B2/III/17	-	HE799671	Slovenia	Golic et al. [46]
Torulaspora delbrueckii	CBS 1146	-	MK394138	Netherlands	Stavrou et al. [40]
Torulaspora quercuum	BMF4L7	MT3	OR145786	Ecuador	This study
Torulaspora quercuum	AF2L8	MT3	OR145785	Ecuador	This study
Torulaspora quercuum	CBS:11403	-	KY105673	Netherlands	Vu et al. [47]
Torulaspora quercuum	CGMCC AS 2	-	NR137029	China	Wang et al. [48]
Torulaspora quercuum	XZ-46A	-	FJ888525	China	Wang et al. [48]
Torulaspora quercuum	XZ19100-5	-	MW710147	China	Direct Submission
Wickerhamomyces anomalus	BMC2L7	MT5	OR145778	Ecuador	This study
Wickerhamomyces anomalus	AC4L9	MT5	OR145782	Ecuador	This study
Wickerhamomyces anomalus	BMF2L7	MT5	OR145779	Ecuador	This study
Wickerhamomyces anomalus	MF1L9	MT5	OR145780	Ecuador	This study
Wickerhamomyces anomalus	MC3L1	MT5	OR145781	Ecuador	This study
Wickerhamomyces anomalus	CBS 5759	-	MK394130	Netherlands	Stavrou et al. [40]
Wickerhamomyces anomalus	I 29	-	HF952836	Netherlands	Zha et al. [49]
Wickerhamomyces anomalus	VIT-ASN01	-	KX253664	India	Direct Submission
Wickerhamomyces anomalus	DBL01s1	-	LC120363	Japan	Tanahashi and Hawes [50]
Wickerhamomyces anomalus	iL-51-2	-	FN868149	Germany	Glushakova et al. [51]
Wickerhamomyces anomalus	CBS 5759	-	MH545921	Netherlands	Stavrou et al. [40]

The richness was not similar between treatments (p value: 0.02991), which was statistically different from the natural fermentation, with a dispersion like the mean F: 4.077 (Figure 5A,B).

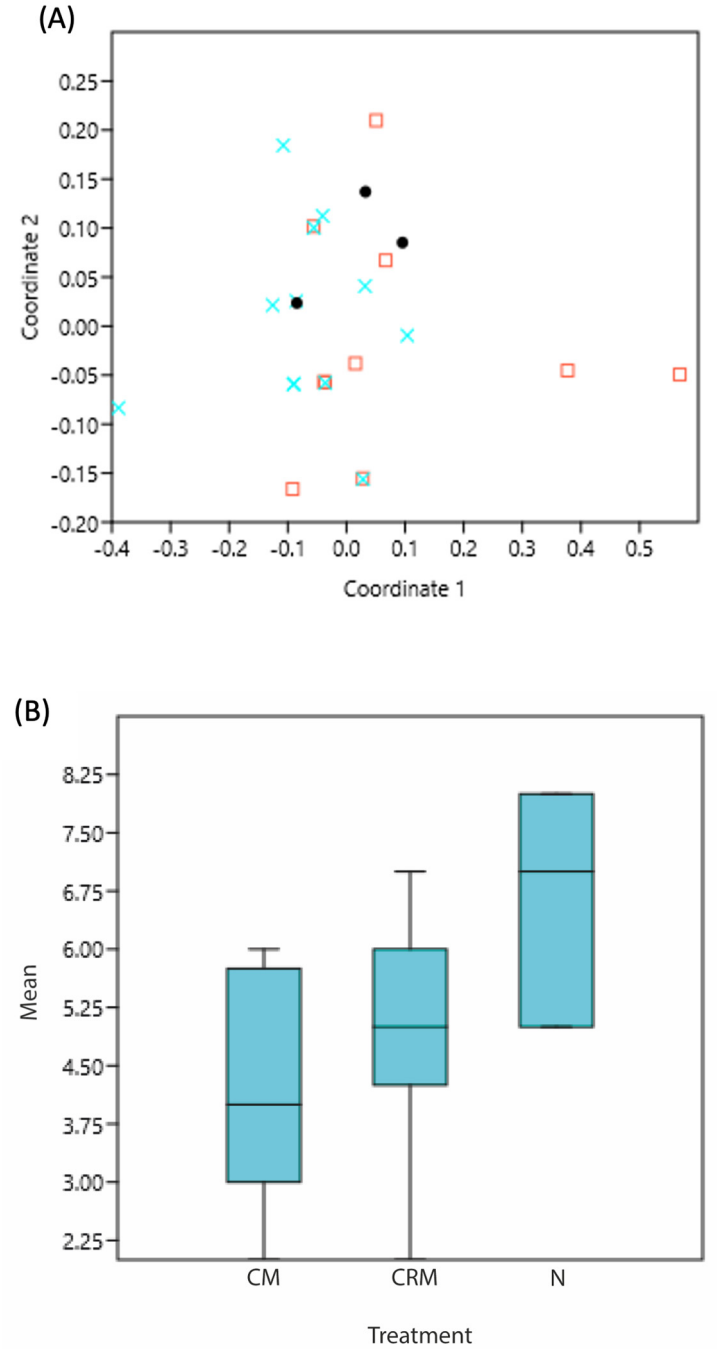

Figure 5. (**A**) NMDS of the species found according to the treatment: x = Natural or "blank sample" aerobic at room temperature (N); □ = Carbonic maceration with CO_2 atmosphere at room temperature (CM); and • = Carbonic refrigerated maceration with CO_2 atmosphere to 10 °C (CRM), (**B**) boxplot of the species according to the treatments.

Biochemically from 329 strains, nine groups corresponding to the species Hanseniaspora opuntiae, *H. uvarum*, *Kurtzmaniella quercitrusa*, *Pichia fermentans*, *P. kluyveri*, *P. membranifaciens*, *Torulaspora delbrueckii*, *T. quercuum* and *Wickerhamomyces anomalus* were determined based on the description of Kurtzman et al. (2011).

Kurtzmaniella quercitrusa, *Hamniospora opuntiae*, *P. fermentans* and *Torulaspora delbrueckii* showed higher fermentation in glucose and lactose. In addition, they grew on media with NaCl up to 15% and 15% ethanol; this differed from *P. kluyveri*, which maintained similar tolerance characteristics up to 10% of each reagent.

3.2. Molecular Species Definition

From 36 molecularly worked-up strains, 60 (ITS-5.8S plus D1/D2 partial LSU) sequences (forward and reverse) were obtained. Twelve sequences were discarded due to multiple peaks in the chromatograms. All concatenated sequences (37 in total, Table 3) corresponded to the Ascomycota division (Table 3). Nine genetic species were determined (Table 3, Figure 6). The species *Pichia membranifaciens* was considered the most recurrent with 15 sequences and strains clustered in one clade (Figure 6).

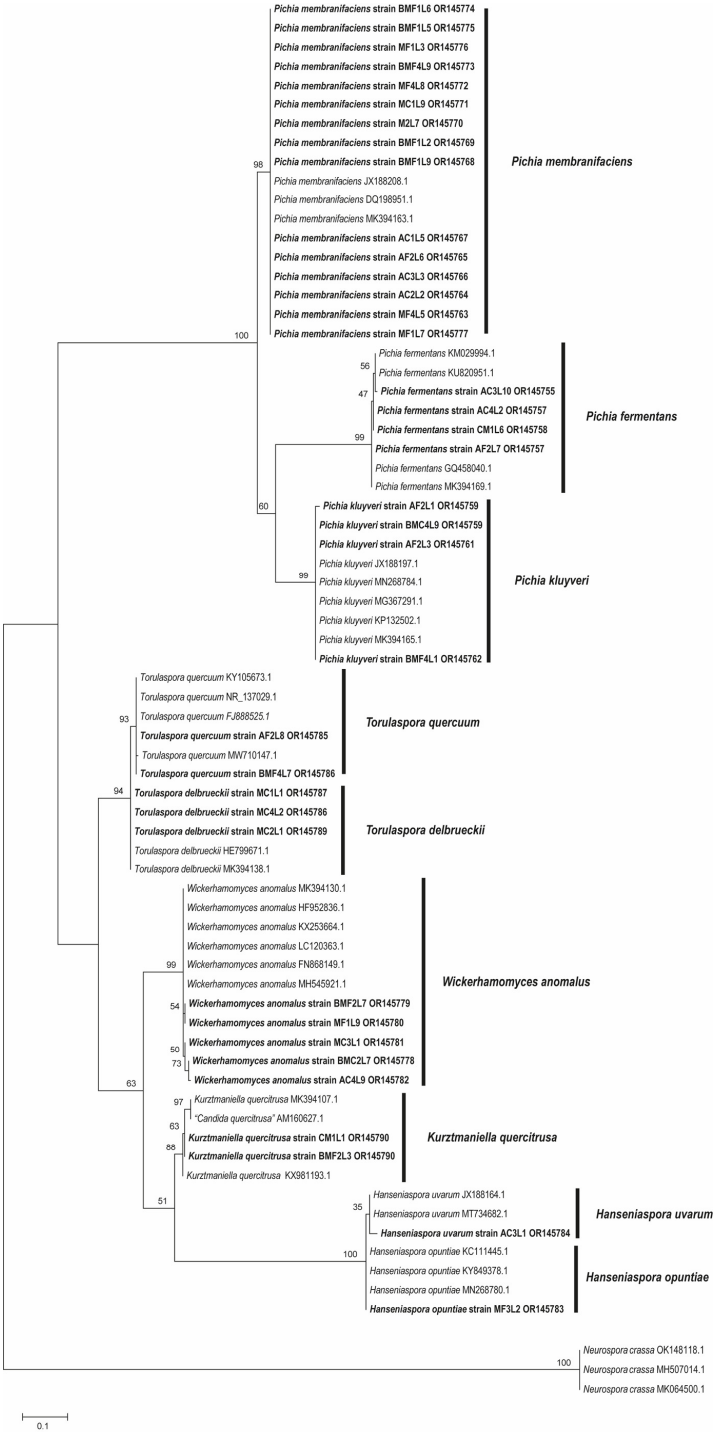

Figure 6. Phylogenetic placement of ITS-5.8S plus D1/D2 LSU partial of yeast sequences. Maximum likelihood phylogenetic analysis, with Bootstrap values greater than 50, are presented above the nodes.

The nine genetic species (Figure 6) corresponded to the nine biochemically determined phenotypes (i.e., *Hanseniaspora opuntiae*, *H. uvarum*, *Kurtzmaniella quercitrusa*, *Pichia fermentans*, *P. kluyveri*, *P. membranifaciens*, *Torulaspora delbrueckii*, *T. quercuum* and *Wickerhamomyces anomalus*) (Figures 2 and 3). On the other hand, the nine genetic species only corresponded to six MTs where MT1 matched with *Torulaspora delbrueckii*; MT2 to *Kurtzmaniela quercitrusa*; MT3 to *Wickerhamomyces anomalus*; MT5 to *Torulaspora quercuum*; MT9 to *Hanseniaspora opuntiae* and MT10 matched with *Pichia kluyveri*.

4. Discussion

The diversity of microorganisms in different ecosystems is high and still unknown [1]. Also, not all microorganisms are culturable. However, 329 strains were isolated in this study from coffee fruits corresponding to 12 morphotypes, suggesting 12 morphospecies. Moreover, nine biochemical groups corresponding to the species *Hanseniaspora opuntiae*, *H. uvarum*, *Kurtzmaniella quercitrusa*, *Pichia fermentans*, *P. kluyveri*, *P. membranifaciens*, *Torulaspora delbrueckii*, *T. quercuum*, and *Wickerhamomyces anomalus* (Figures 3 and 5) were determined.

Surprisingly, all these species identified biochemically are of the same genetic species determined phylogenetically. Nonetheless, only six of the designated morphospecies (*Kurtzmaniella quercitrusa*, *Hanseniaspora opuntiae*, *Pichia. kluyveri*, *Torulaspora delbrueckii*, *T. quercuum*, and *Wickerhamomyces anomalus*) corresponded to the genetic species. This is an indication that morphological characters are not entirely conclusive for defining yeast species [48] or other fungal species [24]. This is due to the variability that microorganisms can generate depending on the environment in which they develop [50]. In this regard, rapid and accurate identification of yeasts can be more effective using molecular tools [11], such as metagenomics [10].

On the other hand, it is well known that biochemical tests are an essential tool for the description of microorganisms [51] and are widely used in the description of yeast species such as within the genus *Candida* [52]. Likewise, De Melo Pereira et al. [53] indicate that fermentative capacity are unique characteristics to define species within different genera like *Saccharomyces* into *Saccharomycotina*. Biochemically and morphologically, the MT2 was determined as the species *Candida quercitrusa*, actually synonymized with *Krutzmaniella quercitrusa* [34]. The genetic species determined as *Pichia membranifaciens* (98% Bootstrap) contains several sequences from strains M2L7, MF4L8, BMF1L6, AC1L5, BMF1L2, BMF1L5, BMF4L9, AC3L3, MF1L3, MF1L7, AC2L2, AF2L6, MC1L9, BMF1L9 and MF4L5, which were preliminarily classified into two morphotypes, MT7 and MT12, respectively (Figure 2). It is likely that the morphological variation corresponds to an adaptation of each strain to the culture medium, as suggested for other yeasts [54].

Of the species isolated in this study, the species *Torulaspora delbruekii* and *T. quercuum* have been previously reported in studies of fungal diversity associated with coffee in the species *Coffea arabica* L. in different locations of the planet such as USA, Europe and Asia, but no records of these species were found in Ecuador [54,55]. These species are considered to be sisters, as they are grouped within the same phylogenetic clade [15], accompanied by morphological similarity as indicated in this study (Figure 2 (MT1 and MT5)). Additionally, similar results in NaCl, ethanol resistance, malt and lactose fermentation were found for *Torulaspora delbruekii* and *T. quercuum*, contributing to the evidence of their taxonomic closeness.

Similarly, the species *Krutzmaniella quercitrusa*, *Hanseniaspora opuntiae*, *Pichia kluyveri*, and *Torulaspora delbrueckii* [56,57] have been reported from coffee fruits in nearby countries such as Brazil, Colombia and Chile. In addition, isolation of yeasts from coffee (*Coffea arabica*) belonging to the genera *Pichia*, *Candida* and *Saccharomycopsis* has been reported [58,59]. For the southern region of Ecuador, the nine genetic species described in this study and species such as *Candida albicans* and *Saccharomyces cerevisiae* have been reported in a study on the microbiome in the beverage called colada morada [60]. It is known that the diversity

of microorganisms can vary due to environmental factors [55] such as fermentation process in closed tanks with airlocks used by the farmers to generate new sensory profiles.

Local farmers apply similar fermentation processes as evaluated here: natural fermentation or controlled atmosphere with a CO_2-rich environment at room temperature and refrigeration to improve sensory profiles mainly conducted by development of different microorganisms like yeasts [56,57]. Biotechnologically, yeasts such as those determined in this study, for example, *Pichia membranifaciens*, are applicable in fermentation processes of fruits such as coffee [58,59,61], or as biocontrollers of fungal pathogens (e.g., *Botrytis cinerea*) [62]. Likewise, species such as *Pichia fermentans* and *P. Kluyveri* have been used in the wine and brewing industry including various fruits such as coffee [61]; likewise, *Hanseniaspora uvarum* and *Wickerhamomyces anomalus* have been used to date in the wine industry, increasing their organoleptic properties such as aroma and flavors [60].

On the other hand, the species *Torulaspora delbrueckii*, a yeast with remarkable resistance to osmotic and freezing stress [53], possesses flavor- and aroma-enhancing properties in wine, beer, or bread dough fermentation processes [58]. This yeast is considered a biotechnological model that can be used in food industries [53]. *Kurtzmaniella quercitrusa* and *Hanseniaspora opuntiae* species have been used and reported in the fermentation of cocoa beans in Malaysia [14]. In the case of *Torulaspora quercuum*, it has been reported in cider fermentation in association with other yeasts described in this study [15]. However, its metabolic potential is currently being studied, and it is defined as a potential biotechnological model in the production of ethanol associated with microalgae [63].

Future studies are required to determine the capabilities and behavior of these yeasts in coffee fermentation processes and the effect on the chemical and sensory properties of the final beverage.

5. Conclusions

The diversity of yeast can vary due to environmental factors such as fermentation process, as is evaluated here (i.e., Natural aerobic at room temperature; Carbonic maceration with CO_2 atmosphere at room temperature; and Carbonic refrigerated maceration with CO_2 atmosphere to 10 °C), allowing the obtention of culturable yeast. This diversity after biochemical and molecular test is represented by nine species, *Hanseniaspora opuntiae*, *H. uvarum*, *Kurtzmaniella quercitrusa*, *Pichia fermentans*, *P. kluyveri*, *P. membranifaciens*, *Torulaspora delbrueckii*, *T. quercuum*, and *Wickerhamomyces anomalus*. These nine species determined biochemically and molecularly have congruent correspondence, contrary to morphological characters that can be ambiguous due to overlapping between species as, for example, within the genus *Pichia*.

The nine species determined here are considered new Saccharomycotina records for southern Ecuador according to the revised literature.

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