Identification of microorganisms in wet coffee fermentation *Coffea arabica* Var Catimor and Castillo in Jardín, Antioquia-Colombia, using culture-dependent methods

Identificación de microorganismos en fermentación húmeda de café *Coffea arabica* Var Catimor y Castillo en Jardín, Antioquia-Colombia, usando métodos cultivo-dependientes

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Abstract

Background: Mild Colombian coffees are recognized worldwide for their high-quality coffee cup. However, there have been some failures in post-harvest practices, such as coffee grain fermentation. These failures could occasionally lead to defects and inconsistencies in quality products and economic losses for coffee farmers. In Colombia, one of the fermentation methods most used by coffee growers is wet fermentation, conducted by submerging the de-pulped coffee beans for enough time in water tanks to remove the mucilage.

Objectives: We evaluated the effect of the water (g)/de-pulped coffee (g) ratio (I: 0/25, II: 10/25, III: 20/25) and final fermentation time (24, 48, and 72 hours) on the total number of microbial groups. We also identified microorganisms of interest as starter cultures.

Methods: We used a completely randomized experimental design with two factors; the effect of the water (g)/de-pulped coffee (g) ratio (I: 0/25, II: 10/25, III: 20/25) and final fermentation time (24, 48, and 72 hours), for 9 treatments with two replicates. During the coffee fermentation (1,950 g), the pH and °Brix were monitored. Total counts of different microbial groups (mesophiles, coliforms, lactic-acid bacteria, acetic-acid bacteria, and yeasts) were performed. Various isolates of microorganisms of interest as starter cultures (lactic-acid bacteria and yeasts) were identified using molecular sequencing techniques.

Results: 21 lactic-acid bacteria (LAB) isolates and 22 yeasts were obtained from the different mini-batch fermentation systems. The most abundant lactic-acid bacteria species found were *Lactiplantibacillus plantarum* (46%) and *Levilactobacillus brevis* (31%). *Pichia kluivery* (39%) and *Torulaspora delbrueckii* (22%) were the most abundant yeast species.

Conclusion: The studied factors did not have effect over the microorganism's development. The identified bacterial and yeasts species have potential as starter cultures for better-quality coffees and in fermentation-related applications.

Resumen

Antecedentes: Los cafés suaves lavados colombianos son reconocidos a nivel mundial por su buena puntuación sensorial; sin embargo, se han detectado fallas en las prácticas de postcosecha, como lo es la fermentación de los granos de café. Dichas fallas pueden causar defectos y carecer de consistencia en la calidad del producto, ocasionando pérdidas económicas para los caficultores. En Colombia, uno de los métodos más usados por los caficultores es la fermentación húmeda, la cual consiste en sumergir los granos de café despulpado en tanques con agua por un período de tiempo que permita la remoción del mucílago.

Objetivos: La presente investigación evaluó la incidencia que tienen la proporción agua/granos despulpados de café (I: 0/25, II: 10/25, III: 20/25) y el tiempo final de fermentación (24, 48 y 72 horas) en el recuento final de grupos microbianos. Por otra parte, se identificaron taxonómicamente microorganismos de interés para su uso como cultivos iniciadores.

Métodos: Mini-lotes consistieron en café despulpado (1950 g) puesto en recipientes de plástico abiertos y sumergidos en agua. Se aplicó un diseño experimental completamente aleatorizado de dos factores (proporción agua/ granos de café despulpado y tiempo) a tres niveles, para un total de nueve tratamientos con dos replicas. Durante las fermentaciones de café (1,950 g), el pH y los grados ºBrix, fueron monitoreados. Se realizaron los recuentos totales de los diferentes grupos microbianos: mesófilos, coliformes, bacterias ácido-lácticas, bacterias ácido-acéticas y levaduras. Se identificaron molecularmente diferentes aislados con potencial para ser usados como cultivos iniciadores (bacterias ácido-lácticas y levaduras).

Resultados: Los resultados obtenidos mostraron que no hubo diferencia estádisticamente significativa entre los tratamientos aplicados y el recuento final de microorganismos. Un total de 21 aislados de bacterias ácido-lácticas (BAL) y 22 levaduras lograron obtenerse a partir de los diferentes sistemas de fermentación en mini-lote. Las especies de bacterias ácido-lácticas con mayor porcentaje acorde a su identificación taxonómica, corresponden a *Lactiplantibacillus plantarum* (46%), *Levilactobacillus brevis* (31%). Las especies de levaduras con mayor porcentaje acorde a su identificación taxonómica corresponden a *Pichia kluivery (39%)* y *Torulaspora delbrueckii (22%)*.

Conclusión Los factores estudiados no afectaron el crecimiento de ninguno de los grupos microbianos presentes en la fermentacion del café. Las especies de microorganismos identificados tienen potencial para se usados como cultivos *starter* o en aplicaciones dentro de las ciencias de fermentación.

Introduction

Coffee fermentation is a crucial step in the production of high-quality coffee, and the microorganisms involved in the process play a significant role in determining the flavor and aroma of the final product [1,2]. The unique growing conditions and fermentation methods used in Colombia coffee production have contributed to the country's reputation establishing it as the third major coffee-producing country [3,4]. However, there have been some challenges in the post-harvest practices, particularly in the fermentation process, leading to occasional defects and inconsistencies in quality. These issues not only affect the coffee's overall quality but also result in economic losses for the farmers [5].

The fermentation of coffee fruits aims to degrade the mucilage by indigenous bacteria that feed on various components of the pulp and mucilage. Microbial diversity depends on many factors, including the agricultural area, coffee variety, harvesting, and coffee cherry storage [6,7]. Producers and some researchers only indicate fermentation for mucilage removal but do not evaluate the impact on aroma and flavor. In fermented green coffee, mucilage removal must be effective and contribute to the quality due to the biochemical changes resulting from their degradation. Besides the impact on the beverage quality, there are fermentation benefits such as decreased fungi contamination [8]. During fermentation, there are physicochemical changes in the grains, such as areduction in the water and simple sugars content and production of organic acids, alcohols, enzymes(poly-galacturanase, pectinlyase), and other metabolites. All these compounds are precursors of the coffee bean's aroma and flavor, influencing the beverage's final sensory characteristics [5,9]. Wet fermentation is one of Colombia's most widely used methods for coffee fermentation. In this method, the skin and pulp are removed mechanically, leaving some of the mucilage adhered to the beans. These de-pulped coffees are then transferred to water tanks, where they ferment for 6 to 72 hours, depending on the environmental temperature. The remaining mucilage is degraded and solubilized. The beans are then removed from the tanks and dried [9].For over a century, the wet method has been a water-intensive process, as the name indicates, and there was little concern for water consumption and contamination [10]. The constant market demand for high-quality coffees and sustainable production processes has prompted the exploration of the environment and microbiological interactions in wet coffee fermentation. This research aimed to study cultivable and viable microbial communities in the coffee from the wet fermentation process of *Coffea arabica* varieties Catimor and Castillo found at Finca La Antigua (Jardín, Antioquia, Colombia). Results generated from this study will allow knowing the diversity of viable microorganisms present in coffee fermentation under natural environmental conditions and the variables studied. These microorganisms can be" starter cultures" used in controlled fermentation processes to produce *Specialty Coffees* in Colombia.

Materials and methods

Location and coffee treatment

Coffee cherries from "La Antigua" farm (5.608263510940495, -75.81934403607546) located at 1,850 m above sea level in Jardín, Antioquia, Colombia, were hand-picked at

the mature stage (cherries) and then were classified (red cherries) comparing the cherries with a color chart (color codes: #fc4f59, #ef2b2d, #d62828, #af2626, #ef2b2d, #cc2d30, #a03033, #ce1126, #af1e2d) according to reference 11. They were de-pulped in a traditional pulper (Vencedora Estrella no. 3 1/2) to obtain beans with mucilage. The pH (UdeA device, Colombia), were measured from the fresh de-pulped coffee beans and ºBrix (Ultechnovo, USA) were measured from the cherries, by squeezing a drop of juice from cherries into a hand refractometer.

Coffee fermentation conditions

Our experimental unit was a mini-batch (plastic containers without lids (13x30x20 cm)). We used nine mini-batches with 18 Kg of de-pulped coffee cherries (a mixture of C. arabica var. Catimor and Castillo). Each one had a different water (g)/de-pulped coffee (g) ratio (I: 0/25, II: 10/25, III: 20/25) and a final time of fermentation (A: 24h, B:48h, C:72h). The pH was registered through the fermentation process of the pulped coffee beans. ºBrix was measured at the end of each fermentation experiment. The experimental design consisted of a three-by-two factorial design, being the factors the water (g)/pulped coffee (g) ratio and the fermentation time. Each experiment was done by duplicate. Each experiment was done by duplicate.

Microbial count and isolation of lactic-acid bacterial and yeast

300 g of samples from each mini-batch were collected in sterile plastic bags in three moments of fermentation: t0 (beginning), t2 (half-time:12h, 24h, 36h), and t3 (final time: 24h, 48, 72h). Afterward, each sample was stored at 4ºC and then transported in ice boxes to the Food Microbiology laboratory at Universidad de Antioquia, Medellín, Colombia. 10 g of each sample was added to a bottle containing 90 ml of saline-peptone water (0.1% bacteriological peptone, Merck, USA). After mixing, six-fold dilutions were prepared. Microorganisms count was carried out using five different culture media and plating in duplicate using 50 μl of dilutions 10^{-4} and 10^{-6} as follows: mesophiles microorganisms in Plate count agar (PCA, Microkit, Spain) and incubated at 32 ºC for 72 h; acetic-acid bacteria (AAB) in Wallerstein Lab nutrient agar (WL, Scharlau, Spain) and incubated at 37 ºC for 24 h; coliforms in Chromocult (Merck, USA) and incubated at 37 ºC for 24 h; lactic-acid bacteria (LAB) in Man, Rogosa y Sharpe agar (MRS, Merck, USA) supplemented with Fluconazole 0.2 % p/v (La santé, Colombia); yeasts and filamentous fungi in Oxytetracycline-Glucose Yeast Extract agar (OGYE, Oxoid, UK) supplemented with 80mg gentamycin (Genfar, Colombia) in 500ml and incubated at 25 ºC for 72 h.

All colonies were counted and results were expressed in log CFU/g (Colony Forming Units per gram) and described by shape, color, height, and edge of each isolate. Gram staining and catalase tests were made. Yeasts and lactic-acid bacteria colonies with different macroscopic and microscopic characteristics were isolated, purified, and stored in vials at -80 ºC in 20 % glycerol.

Molecular identification of acid-lactic bacteria and yeast.

Molecular identification of lactic-acid bacteria (LAB) and yeasts colonies isolates was performed by MACROGEN (Korea) [12]. The samples were sent according to MACROGEN's (Korea) preparation guidelines [12]. The amplification and sequencing of the 16S ribosomal gene were done using the universal primers: 785F (3' GGA TTA GAT CCC TGG TA 5') and 907R (5' CCG TCA ATT CCT TTR AGT TT 3'). Yeast molecular identification was performed by amplifying and sequencing of 26S rDNA region using the universal primers ITS4 (TCC TCC GCT TAT TGA TAT GC) and ITS5 (GGA AGT AAA AGT CGT AAC AA GG) for sequencing of 18S rDNA region. The microbial sequencing chromatograms were depurated using Benchling [13]. The sequences were compared with yeasts and LAB references from the NCBI GenBank using the Basic Local Alignment Search Tool (BLAST) algorithms [14]. Finally, phylogenetic trees were made using MEGA X software [15].

Data analysis

The experiment design was completely randomized in a factorial arrangement of three different fermentation times and three water(g)/de-pulped coffee(g) ratios for 9 treatments with 2 replicates. Analysis of variance (ANOVA) and Pearson correlation were conducted using R software [16]. P-values less than 0.05 were considered statistically significant.

Results

pH and ºBrix in the coffee fermentation process.

pH and ºBrix in de-pulped coffee during the fermentation process are reported in Figure 1 and Table 1.
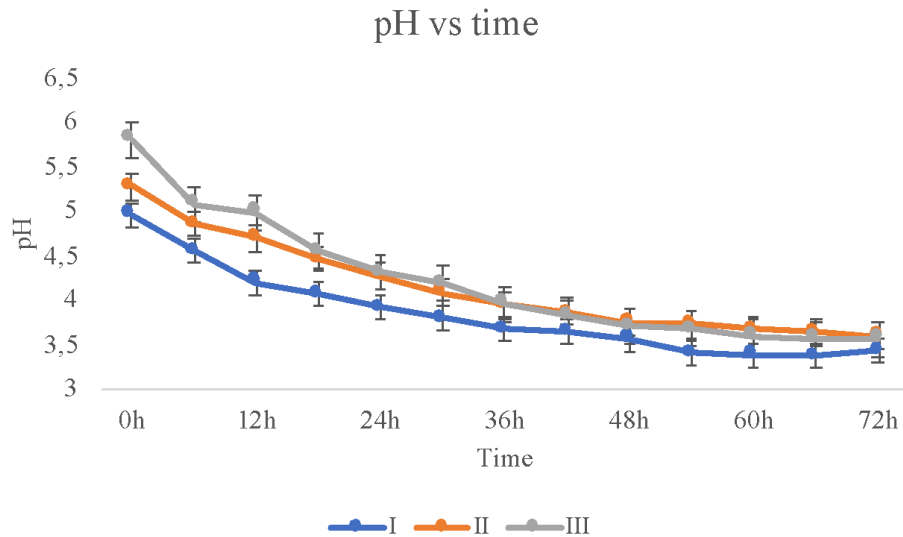
Figure 1. pH during fermentation of different water/pulped coffee ratios (I: 0/25, II: 10/25, III: 20/25) and final time of fermentation (A: 24h, B:48h, C:72h).

The water(g)/de-pulped coffee(g) ratio influenced the initial pH values of the mini-batch systems. A low positive correlation of 0.30 (data not shown) suggests a basic state in rich water media (mini-batch III: 5.82, mini-batch II: 5.28, and mini-batch I: 4.96). Additionally, the pH values in each system gradually decreased during fermentation and converged at the end of each process (mini-batch III: 3.56, mini-batch II: 3.56, and mini-batch I: 3.52).

The value of °Brix for all mini-batch treatments is presented in table 1. °Brix of mature coffee cherries were in the optimal range of 15 and 15.8, corresponding to the optimum maturity of the coffee fruit Colombia variety [17]. Mini-batches A and C started with slightly higher values but in general, the data shows a decreasing behavior of the concentration of mucilage sugars in all treatments.

Table 1. °Brix media values of coffee processed with different water/de-pulped coffee ratios and final time of fermentation (A: 24h, B:48h, C:72h).

	A	B	C	SD
Mature coffee cherry	15.8	15.0	15.6	0.4
I (0/25)	9.8	6.5	10.0	2.0
II (10/25)	5.0	2.8	3.5	1.1
III (20/25)	1.8	1.5	1.8	0.1

Microbiological counts and isolation of lactic-acid bacteria and yeasts.

Bacterial and yeast populations in mini-batch fermentations were quantified by plating on selective media. Microbial counts of mesophilic aerobic bacteria, coliforms, AAB, LAB, and yeast found during coffee processing are illustrated in Figure 2 to Figure 6.
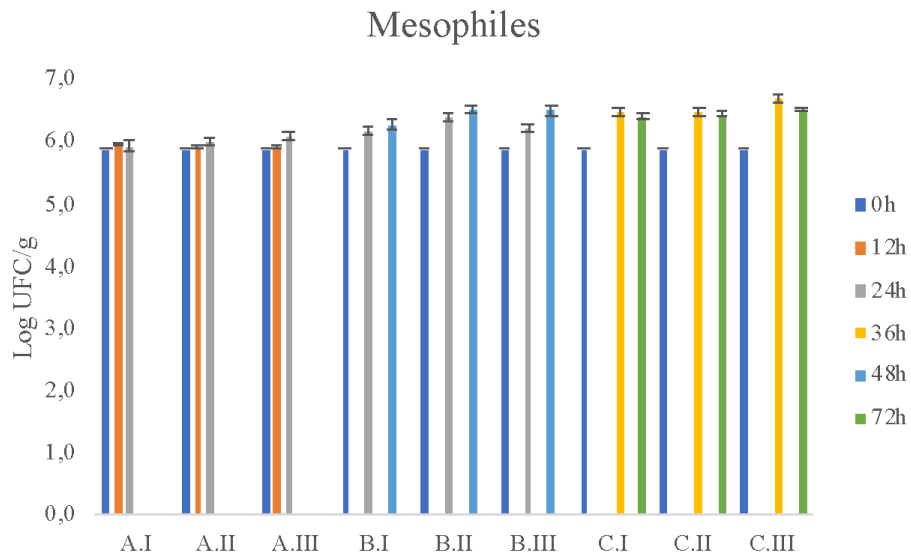
Mesophiles

Figure 2. Total count (CFU / g) of mesophilic microorganisms in Plate count agar (PCA) medium during coffee fermentation (final time A: 24h, B:48h, C:72h) and different water(g)/pulped coffee (g) ratios (I: 0/25, II: 10/25, III: 20/25).
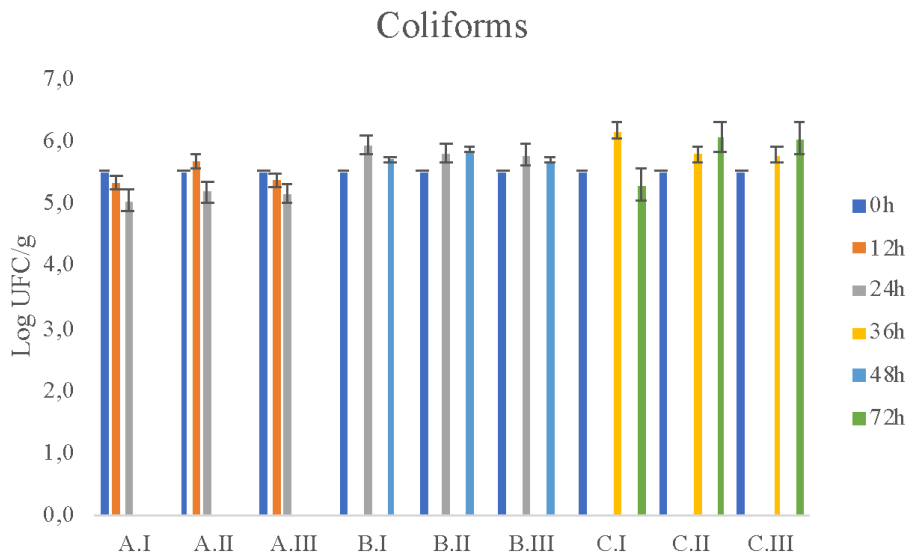
Coliforms

Figure 3. Coliforms total count (CFU / g) in Chromocult medium (CHC) during coffee fermentation (final time A: 24h, B:48h, C:72h) and different water(g)/pulped coffee(g) ratios (I: 0/25, II: 10/25, III: 20/25)
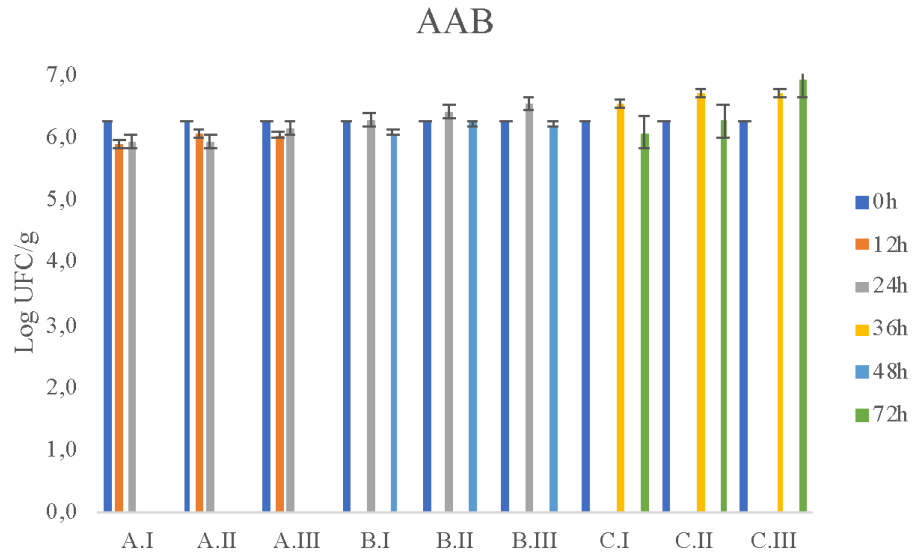
AAB

Figure 4. Acetic-acid bacteria total count (CFU / g) of in WL medium during coffee fermentation (final time A: 24h, B:48h, C:72h), and different water/pulped coffee ratios (I: 0/25, II: 10/25, III: 20/25).
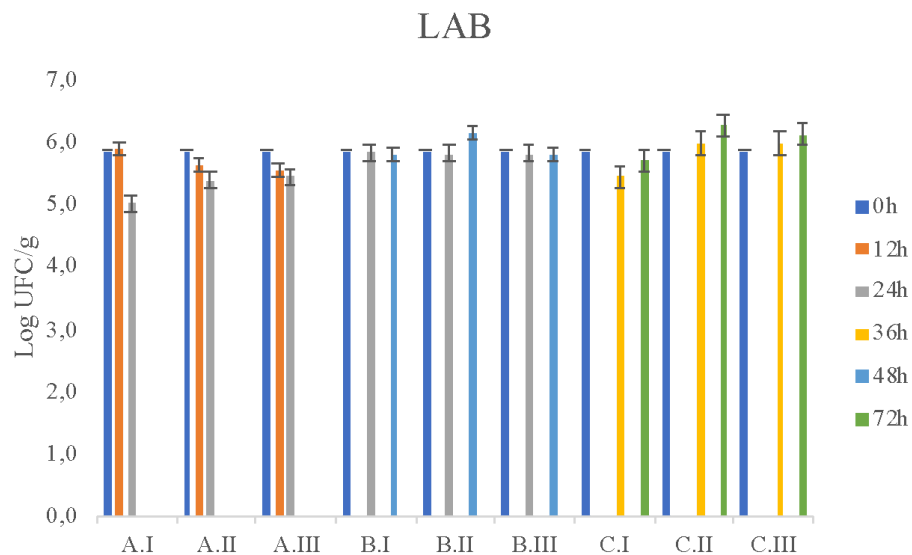
LAB

Figure 5. LAB total count (CFU / g) in MRS medium during coffee fermentation (final time A: 24h, B:48h, C:72h), and different water/pulped coffee ratios (I: 0/25, II: 10/25, III: 20/25).
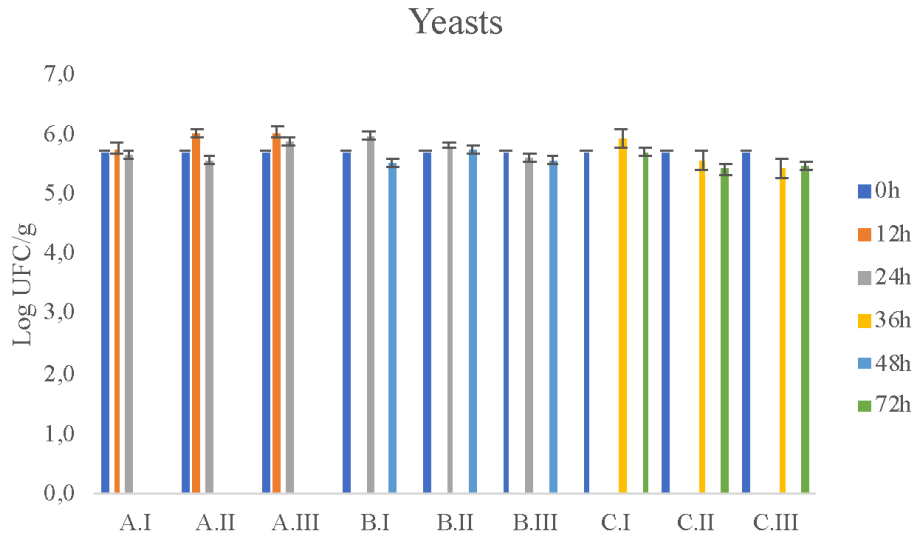
Figure 6. Yeasts total count (CFU/ g) in OGYE medium during coffee fermentation (final time A: 24h, B:48h, C:72h), and different water/pulped coffee ratios (I: 0/25, II: 10/25, III: 20/25).

Table 2 presents the statistical analysis data (P-values) of the effect of each factor and both factors combined (water/de-pulped coffee ratio and fermentation final time) over the counts in each microbial group.

Table 2. P- values obtained of statistical evaluation of water/de-pulped coffee ratio and final time fermentation factors over microbiological counts p- values.

	Water/de-pulped coffee	**Time**	**Combined effect of factors***
Mesophiles	0.7084	0.1245	0.9809
Coliforms	0.415	0.308	0.744
Yeasts	0.821	0.128	0.513
LAB	0.1084	0.0213 [a]	0.3712
AAB	0.0538	0.0176 [a]	0.0933

[a] p-value significance level >= 0.05
* Effect of Water/de-pulped coffee and Time on microbial counts

All bacterial and yeast isolates were identified according to 16S rRNA and ITS gene sequencing, respectively. Taxonomic identification of the isolates was determined by aligning LAB and yeast sequences and the species with a 99-100% identity percentage [18] were selected. The amount of species of lactic-acid bacteria and yeast isolates present in coffee fermentation are shown in Figure 7 and Figure 8. The percentages are the quantity of successfully recovered isolates, meaning viable and cultivable microorganisms.

The isolation of different lactic-acid bacteria (LAB) species corresponds to the first 48 hours of fermentation mini-batches A and B and water/pulped coffee ratios of I: 0/25 and II: 10/25. The most abundant LAB isolates correspond to *Lactiplantibacillus plantarum (*46%), and *Levilactobacillus brevis (*31%). *Lactiplantibacillus pentosus, Lacticaseibacillus rhamnosus* and *Lactobacillus sp.* had approximately the same recuperation; 8% for the first two species and 7% for *Lactobacillus*
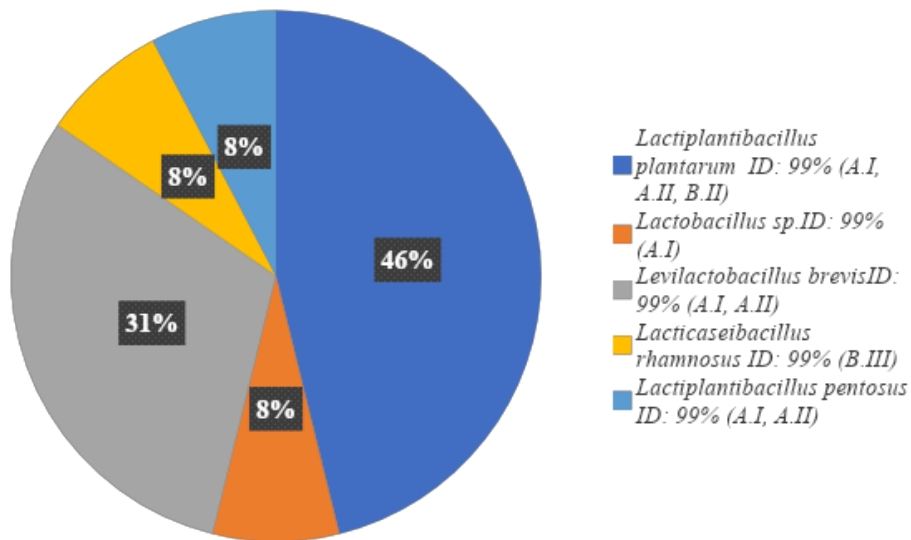
LAB species percentage

Figure 7. Species of lactic-acid bacteria present in coffee fermentation at final time (A: 24h, B:48h, C:72h), and different water/de-pulped coffee ratios (I: 0/25, II: 10/25, III: 20/25).
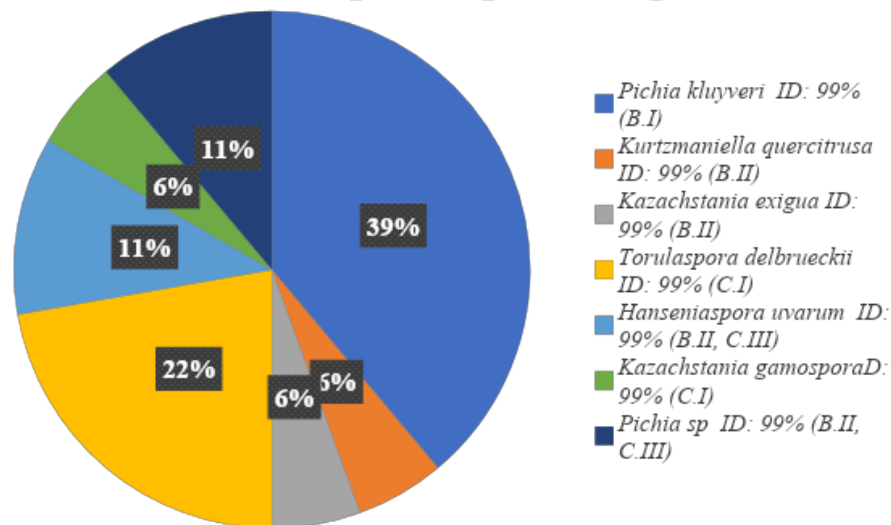
Yeast species percentage

Figure 8. Species of yeast present in coffee fermentation at final time (A: 24h, B:48h, C:72h), and different water/de-pulped coffee ratios (I: 0/25, II: 10/25, III: 20/25).

Genetic relationships between representative isolates of lactic-acid bacteria and yeasts in coffee fermentation are represented in phylogenetic trees (Figures 9 and 10). The phylogenetic trees were built to establish the evolutionary distance within each group of LAB and yeasts identified in this study and primally assess if they could be safe to be used as starter cultures in other fermentations or if they have been reported as species generally recognized as safe (GRAS) in the National Center for Biotechnology Information (NCBI) database.
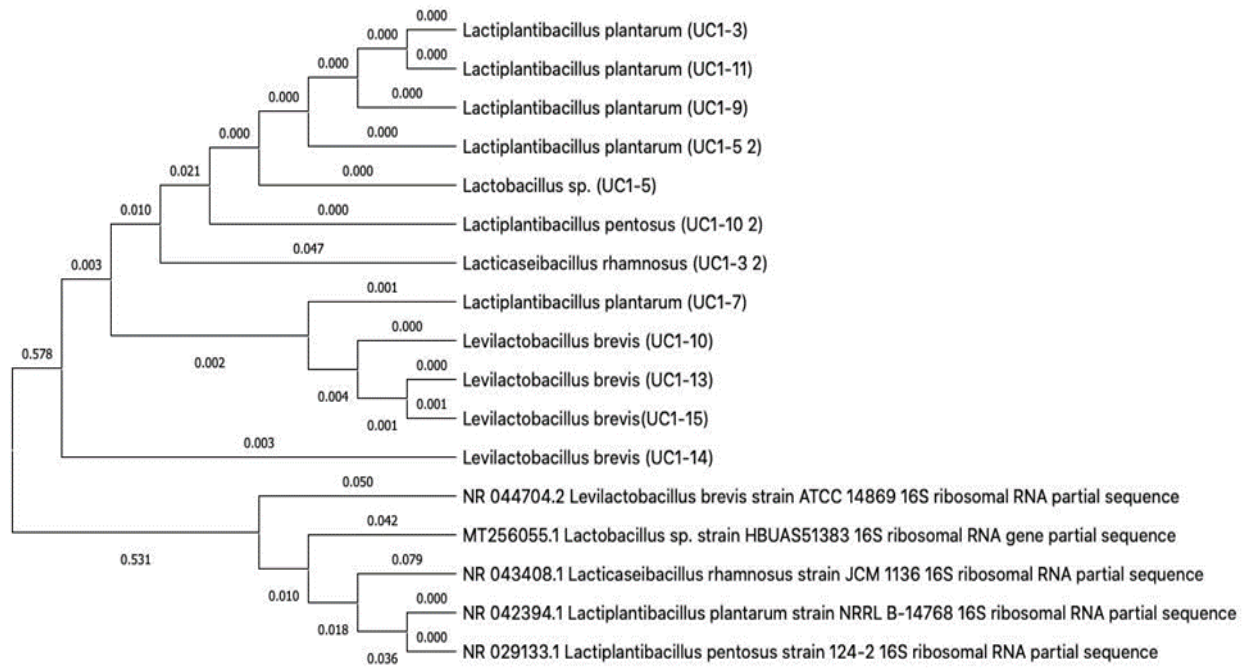
Figure 9. Phylogenetic relationship between the identified lactic acid bacteria from wet coffee fermentation and other 16S rRNA sequences of published strains.
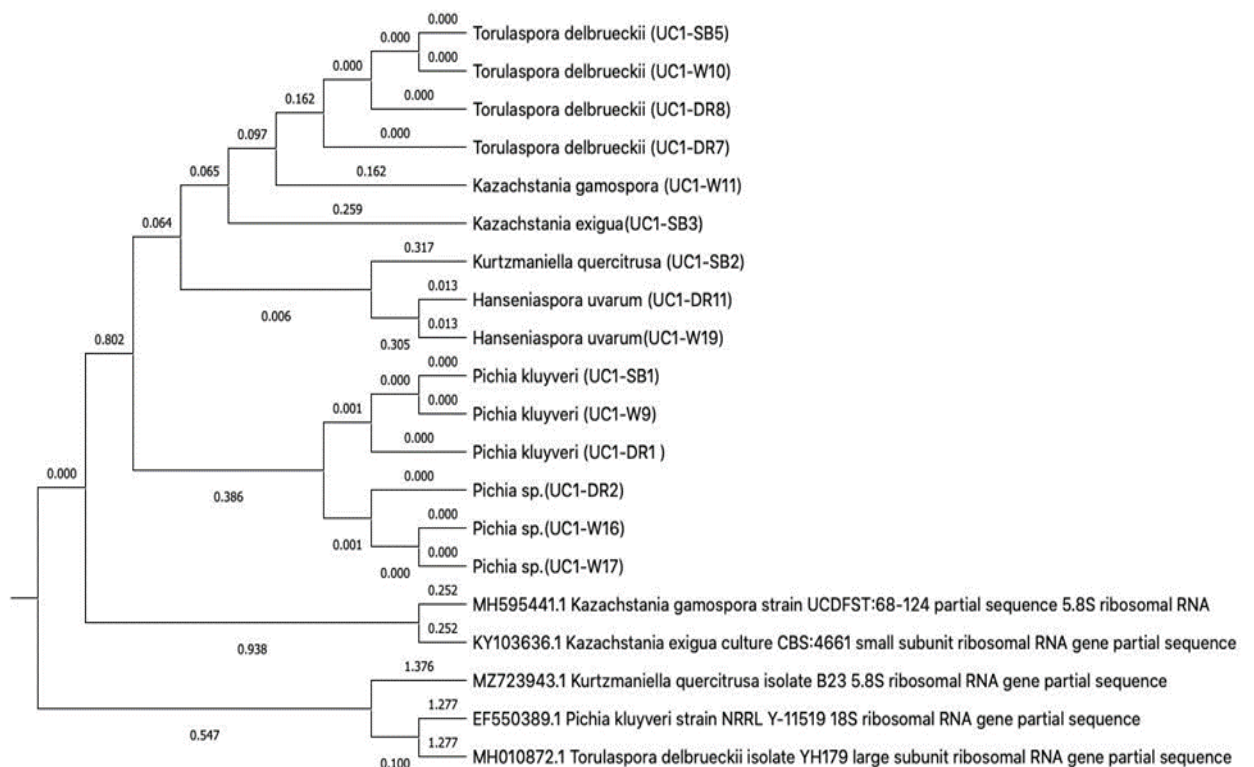
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Figure 10. Phylogenetic relationship between the identified yeasts from wet coffee fermentation and other ITS sequences of published strains.

Discussion

pH in coffee fermentation process.

According to the results shown in figure 1, the water(g)/de-pulped coffee(g) ratio influenced the initial pH values of the mini-batch systems. The decrease in the pH during coffee bean fermentation has been reported as the degradation of mucilage's components (e.g., pectic substances, complex sugars, amino acids, and proteins) [19] into simpler sugars due to the action of microorganisms [20].

°Brix in the coffee fermentation process.

The standard deviation (SD) shows a low variability of the °Brix throughout time (Table 1). Regardless of the water (g)/de-pulped coffee (g) ratio used in the mini-batch fermentation, the °Brix decreased at the end of all the experiments since microorganisms consumed sugars to develop their metabolic processes. The aforementioned is in agreement with other studies [8]. For example, Pantoja López.[21] reported a °Brix exponential decline in a study conducted in Huila, Colombia, and Betancur Henao.[22] obtained the same results in southwest Antioquia, Colombia.

Microbiological counts and isolates of lactic-acid bacteria and yeasts.

In table 2 is observed that the fermentation time factor had influence over the LAB and AAB counts, but the combination of the factors; time and water/de-pulped coffee level ratio, did not show there was a significant amount difference in microbial counts (P-value > 0.05).
The viable and cultivable counts of the different microbial groups analyzed in the wet fermentation were between 10^5 and 10^6 log CFU/g of fermented coffee (Figure 2 to Figure 6). As no treatment exceeds at least 3 logarithms above or below the counts it is concluded that there are no statistically significant differences LAB has been recognized as an integral component of coffee processing in most coffee-producing countries [23–25]. The LAB growth is because of adaptability to the environment and stress factors of coffee processing, such as pH variation, sugar availability, and competition with other microorganisms [23,26,27]. LAB species can catabolize pentoses and hexoses in coffee pulp into a vast range of end-metabolites, including lactate, acetate, CO_2, and ethanol, via the phosphoketolase or pentose phosphate pathway [28]. The results revealed that the most prevalent yeast species were *Pichia kluivery*, accounting for 39% of the isolates, followed by *Torulaspora delbrueckii* at 22%. *Hanseniaspora uvarum*, *Kurtzmaniella quercistrusa*, and *Kazachstania gamospora* were also detected, although at lower amounts of 11%, 5% and 6%, respectively.
The presence of *Pichia kluivery*, *Torulaspora delbrueckii* and *Hanseniaspora uvarum*, as the dominant yeast isolates suggests their potential role in driving the fermentation process and influencing the final coffee product. The use of these yeast species as starter

cultures has been studied due to their association with desirable sensory characteristics and are recognized for their ability to contribute to the development of distinct flavors and aromas, thereby playing a crucial role in the production of specialty coffee [2,29,30].

Lactic acid bacteria were isolated from the first 48 hours while yeasts were isolated after 48 and up to 72 hours. LAB are well-adapted to the pre-existing environmental conditions; therefore, it is common to find LAB species instead of yeasts in the initial hours of coffee fermentation[25,31]. The microbial community is known to vary in composition and initial abundance during this process, and the succession of bacterial and fungal species is complex because their behavior depends on the time and environmental factors [32–34].

A phylogenetic tree analysis was conducted to examine the genetic relationships among representative isolates of lactic acid bacteria (LAB) species belonging to the *Lacticaseibacillus, Lactiplantibacillus,* and *Levilactobacillus* genera (Figure 9)

The phylogenetic tree revealed distinct clusters representing each genus. The *Lacticaseibacillus* and *Lactiplantibacillus* cluster comprised species within the *plantarum* group lactobacilli. These clusters showed a close evolutionary relationship to other lactobacillus groups commonly used as starter cultures in fermentations [35], antimicrobials [36,37], and the next generation of probiotics [38,39]. This suggests a shared genetic heritage and functional similarities among these lactobacilli.

The *Levilactobacillus* cluster represented a group of lactobacilli known for their leavening potential [40]. Multiple species within this genus have been identified in type sourdoughs used as leavening agents. The presence of *Levilactobacillus* highlights the diversity and adaptability of these lactobacilli in different fermentation environments [40]. Understanding the genetic relationships among these species contributes to our knowledge of their diversity, evolutionary history, metabolic capabilities, and potential applications. It provides a framework for further investigation into the functional properties, ecological roles, and their impact on fermentation and product quality.

The yeast isolates identified in this research belong to five different genera (Figures 8 and 10), namely *Torulaspora, Kazachstania, Kurtzmaniella, Hanseniaspora* and *Pichia*. Of particular interest is the isolation of *Torulaspora delbrueckii,* which has recently been reported in metagenomic studies conducted in Colombia [41] and has also been investigated in Latin America [42]. This highlights the presence and potential significance of *T. delbrueckii* in coffee fermentation processes. Yeast species from the genus *Kazachstania*, including *Kazachstania gamospora*, have been documented in both Colombia [43] and Rwanda [44]. This suggests that these yeasts are not limited to a specific geographic region and may have a broader distribution in coffee-growing areas. Coffee processing steps have been found to harbor a diverse range of yeast species from various genera such as *Pichia, Candida, Saccharomyces, and Torulaspora* [45]. The presence of these yeast species broadens the understanding of the diversity and distribution of yeast species in coffee processing.

The analysis of LAB and yeasts in coffee fermentation provides valuable insights into the diversity and functional attributes of these microorganisms; including the production of flavor-active compounds and the establishment of desirable fermentation profiles. Further investigations into the metabolic pathways and interactions of these microorganisms will contribute to optimizing coffee fermentation processes and enhancing the quality of the final coffee product.

Conclusions

This research study aimed to know the influence of fermentation time and water on the development of viable microorganisms. Firstly, the study revealed that no treatment had an effect on the different microbial groups without controlling other environmental factors such as temperature and anaerobiosis. The identification of diverse bacterial and fungal species, including *Pichia, Torulaspora*, and *Lactiplantibacillus,* emphasizes their potential as starter cultures in controlled coffee fermentations and their relevance in fermentation technology and other biotechnological applications. The findings in this study are important for the future production of sustainable high-quality coffees and the development of starter cultures that have the potential of developing distinctive flavor profiles and aromatic compounds.

Conflicts of interest: The authors declare that they have no conflict of interest.

Authors´ contributions.

Karina E. Motato-Rocha: Conception and design of the work, data collection, data analysis and interpretation, drafting and critical revision of the article, and final approval of the version to be published.
Valentina González-Montero: Conception and design of the work, data collection, data analysis, and interpretation, drafting the article.
María O Román-Morales: Conception and design of the work, critical revision of the article, final approval of the version to be published.

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