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Effect of demucilagination and soaking in water with organic acids on the microbial, chemical, and sensory characteristics of coffee (*Coffea arabica*)

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ABSTRACT

There are divided opinions regarding sensory quality of mechanically demucilaginated (MD) coffee versus coffee that has undergone conventional fermentation (biological demucilagination). Fermenting and washing (wet process) requires high amounts of water that has contaminating effects upon its completion. Studies indicate that MD with soaking in organic acids could develop similar sensory quality to wet processed coffee. Organic acids are bioactive compounds that are naturally produced during conventional fermentation, which is why coffee has unique characteristics in the final cup profile. This study was conducted to assess the effects of soaking with organic acids (citric, ascorbic, and acetic) on the microbial, chemical, and sensory attributes of MD coffee. A Completely Randomized Design (CRD) was used, with a factorial arrangement (2×4+2) for a total of 10 treatments. The treatments were two soaking times (24 and 48 hours) and four soaking solutions (citric, ascorbic, acetic acid and water) in coffee with mechanical demucilagination, one treatment with mechanical demucilagination and one with fermentation and washing, both without soaking or acids. Microbiological counts of fungi, yeasts, and lactic acid bacteria (LAB) were carried out before and after soaking. Sensory characteristics were evaluated through cupping and chemical content and properties were studied by liquid chromatography and spectrophotometry. Microbial population demonstrated normal succession throughout the experiment with LAB been the most prevalent family during MD and fermentation. Soaking coffee in acid solutions maintained overall cupping scores with different attributes being detected by panelists. Phenolic compounds, caffeine and chlorogenic acids increased in soaked samples (acetic acid) but were similar to the control (fermentation). Flavonoid content ranged from 22 to 35 mg EC/g and was higher in samples soaked in acids compared to the controls. Green coffee extracts in general showed antioxidant activities greater than 80.9% comparable to other studies. Soaking time did not improve the quality characteristics of the coffee, but the type of acid used was able to modify the content and proportion of various families of chlorogenic acids in green coffee and total polyphenols, while maintaining sensory properties in comparison to fermented coffee.

Key words: Acid solution; bioactive compounds; coffee extract; wet milling.

1 INTRODUCTION

Coffee (Arabicas and Robustas) is the second most traded product in the world after crude oil with more than 5 million tonnes of green coffee exported as of June 2023 (International Coffee Organization - ICO, 2023). This industry generates more than USD 220 billion each year providing a livelihood for more than 100 million people around the world (Bozzola et al., 2021). For Central America, especially for Honduras, coffee is instrumental for the economy being the sixth exporter of this product worldwide with 6.2 million bags produced in 2021-2022 (Instituto Hondureño de café - IHCAFE, 2022).

Knowing the importance of the coffee ecosystem for many countries, it is important to assess its impact on the environment, wellbeing of producers and constant innovation in coffee processing for years to come (Bozzola et al., 2021; ICO, 2022). Throughout the coffee production chain there is need for water, especially during postharvest where up to 40 liters of water per kilogram of dry parchment coffee are required for pulping, selection, and washing (Figueroa Campos et al., 2020; Gonzalez-Ávila; Vázquez; López, 2020). Considering the limited access to water in many coffee regions, technologies have been developed to reduce this consumption overall. Over the last 50 years, the National Center for Coffee Research (CENICAFE, for its acronym in Spanish) in Colombia has developed machinery that uses less than 1 L (water)/kg (dry parchment coffee) during pulping and mechanical demucilagination, therefore reducing contamination by up to 90% (Sanz Uribe et al., 2011). However, some authors report that mechanically demucilagination limits cup quality compared to coffees that undergo spontaneous fermentation (conventional process), generating different sensory profiles and limiting certain fruity and floral attributes in coffee (González-Rios et al., 2007a; Steinhaus; Schieberle; Schieberle, 2007; Jackels; Jackels, 2005; González-Rios et al., 2007b). In contrast, other authors conclude that there are no differences in terms of sensory quality (Abubakar et al., 2019), and that mechanical demucilagination brings benefits where the quality of coffee is maintained in storage while creating good quality coffee with unique notes of flavor, taste, and aroma.

Mechanical demucilagination (semi-washed method) is an alternative that can be used to process coffee reducing the amount of time and water used to physically remove the mucilage (Sinaga; Julianti, 2021). Additionally, soaking coffee beans in organic acids can be an alternative to differentiate products. Based on the idea that coffee can improve its attributes depending on the amount of time

it remains in contact with water, Velmourougane (2011) evaluated the effect of dilution of organic acids in soaking in water after mucilagination, obtaining favorable results for mechanically demucilaginated coffee. It was highlighted that not only the sensory properties improved, but also physical aspects such as coloration were favored and the coffee came out with a lighter shade, which is well perceived by the market. The use of water for mechanical demucilagination plus soaking in organic acids could represent a significant reduction in water needed, thus maintaining the ecological and sustainable purpose by reducing consumption and contamination of water in the postharvest of coffee.

Other attempts to modify the postharvest conditions of the coffee, modifying the medium where the coffee rests after removing the mucilage have been carried out, with positive results (Penson et al., 2014; Liu et al., 2019) in the sensory properties whose assertions are supported by information from microbiological analyzes that derive in the modification of the volatile compounds of coffee (Zhang et al., 2019a).

This study focused on studying the microbiological dynamics during demucilagination and soaking in acid-rich water and to evaluate the effect of these procedures and different types of acids on the physical, chemical, and sensory characteristics of coffee.

2 MATERIAL AND METHODS

2.1 Study location

The study was carried out in the municipality of Santa Ana, Department of La Paz, located about 170 km from Tegucigalpa, the capital of Honduras. The facilities of the Cacauterique Mill were used, at an elevation of 1,535 masl and an average annual rainfall of 1,300 mm. The collection of coffee cherry samples for processing were made on the farm of the same local producer, 2 km away from the wet mill. The plantation plot that was selected for the study was the most uniform in terms of plant health, slope of the land, variety, and agronomic management in terms of fertilization, pruning, and pesticide applications. The collections were carried out in the month of February 2021, just at the peak of production and when the best quality intermediate harvests are carried out in this locality, thus ensuring the best quality of the coffee used in the research.

All microbiological, physical, and chemical analyses were conducted in Zamorano University, Valle del Yeguare, Francisco Morazán, 30 km from Tegucigalpa. Sensory evaluation was conducted with the support of IHCAFÉ in their laboratory in San Pedro Sula, Cortés, Honduras.

2.2 Experimental design

A Completely Random Design (CRD) was used, with a factorial arrangement (2×4+2) for a total of 10 treatments. The treatments were two soaking times (24 and 48 hours) and four soaking solutions (citric, ascorbic, acetic acid and water) in coffee with mechanical demucilagination, one treatment with mechanical demucilagination and one with fermentation and washing, both without soaking or acids (Table 1). Three repetitions were used for all analyses making a total of 30 experimental units.

Table 1: Treatments description.

Demucilagination	Treatment	Acid	Soaking time (h)
Mechanical	1	Citric	24
	2		48
	3	Ascorbic	24
	4		48
	5	Acetic	24
	6		48
	7	Water (no acid)	24
	8		48
	9[2]	-	-
[1]Biological	10[2]	-	-

[1]Demucilagination through fermentation (24-36 h) and washing, [2]Control treatments with no soaking.

2.3 Sample collection and postharvest management

Harvesting was carried out carefully with previously trained personnel in clean plastic containers and transported by vehicle in new nylon sacks to the wet mill. Ripened beans were selected (150 kg), avoiding the collection of green, overripe, or dry beans. Random samples were taken with a wooden board of 100 concave spaces to ensure the quality of the harvest. Up to a maximum of 5% damaged beans (broca), 2% green and 2% dry were allowed during harvest. Coffee beans were randomly assigned to each treatment (15 kg) to obtain around 3 kg of dry parchment coffee, enough to take samples and carry out the corresponding analyses. The processing equipment that was used was previously cleaned and calibrated, to avoid contamination from other batches of coffee or mechanical damage due to poor equipment calibration. A four-jet pulper with a centrifuged horizontal cylinder was used, with a pulping capacity of 1,200 kg per hour of coffee beans, moved by a 1 HP electric motor. Using an endless screw, the beans were transferred to be classified by size. The beans passed through a cylindrical screen with rotating rods with 8 mm spacing to remove the beans that were not fully pulped and had some defects, allowing only the beans that were almost completely pulped to pass.

Mechanical demucilagination was applied to treatments 1 to 9 (Table 1). Mucilage removal was performed in a vertical upward flow mechanical mucilage remover driven by a 3 HP motor with a capacity of 1,000 kg/h, calibrated with a constant water flow of 1.5 L/kg of coffee, according to the manufacturer's recommendations. Treatment 10 (control) was placed in plastic containers and a fermentation process was carried out (24-36 h), until most of the mucilage was detached from the grain and the reading pH was between 3.3 and 3.8. The washing procedure was done with clean water until all the mucilage was removed, rinsing the coffee with water repeatedly until the water did not change its crystalline hue. Control treatments (9 & 10) were immediately put to sun dried (in a structure similar to a greenhouse with a UV plastic cover that prevented direct sunlight) after mechanical demucilagination and washing, respectively.

After mechanical demucilagination of treatments 1 to 8, coffee samples were placed in square plastic containers with dimensions of 30 cm long, 30 cm wide and 15 cm deep. Acid solutions were prepared by calculating the molecular weight, concentration, and volume of the containers to reach a concentration of 0.01 M. Purified water for human consumption was used for dilutions, each container was covered with 6 L of water, totally immersing the coffee samples. The changes in pH and temperature during soaking were monitored and measurements were made every 12 h with an OHAUS brand potentiometer with a Starter 300 automatic temperature compensation extendable tester. Upon completion of soaking times according to each treatment (Table 1), coffee samples were taken for microbiological analyses and the remaining soaking water was discarded, leaving only the coffee that was immediately put to sun dry.

All samples were labeled and placed in a drying structure like a greenhouse with a UV plastic cover that prevented the incidence of direct sunlight. The samples were placed on individual wooden sieves, ensuring that the mass layer did not exceed 2 cm, and they were constantly stirred to ensure uniformity during drying. The drying was done until reaching between 10% and 11.5% humidity, constant sampling was carried out to remove it from drying when it reached optimum humidity. Dryer temperature was monitored by means of environmental thermometers so as not to exceed 45°C. The weather conditions for the month of February 2021 were favorable for drying to complete this process in approximately 5 days. Samples (parchment coffee) were stored for further analyses and hulled prior to evaluations.

2.4 Microbiological analyses

Lactic acid bacteria (LAB), yeasts and fungi were evaluated. Approximately 100 g of coffee were collected in sterile containers to avoid contamination. Samples were taken upon pulping, at the end demucilagination, and during soaking. Once collected, samples were immediately placed in cold storage (3-4°C) for their transfer to the laboratory, to later carry out the incubation and counting of microorganisms according to Pereira et al. (2012) with certain modifications. An initial dilution was made with 90 mL of 0.1% peptone water and 10 g of coffee sample reaching a 9:1 (V:W) ratio which was homogenized for 25 seconds. Further dilutions were made up to 10^{-5} for lactic acid bacteria and 10^{-3} for fungi and yeasts. Fungi and yeast counts were conducted using Petrifilm 3M Y&M, incubating at a temperature between 25-30°C for 72 hours for yeast counts and extending the time to 120 hours for fungi. Regarding the count of lactic acid bacteria, Petrifilm 3M BAL was used, incubating at 37°C for 48 hours prior to counting.

2.5 Sensory evaluation (cupping)

Evaluations were conducted under SCA cupping protocols (Specialty Coffee Association of America - SCAA, 2019). Roasting was done individually at a temperature between 160 and 180°C. Roasting time varied depending on the sample due to factors of humidity, density, uniformity, among others. Roasting profile was measured (AGTRON scale) and the values ranged between 55 and 60 and, once roasting was accomplished, samples were immediately cooled with forced air. Samples (11.5 ± 0.25 g) were ground, evaluated for aroma, and mixed with hot water (200 ± 2.5 mL at 95°C) in clean containers. Then, cupping protocol was conducted to evaluate fragrance, flavor, body, acidity, sweetness, cleanliness, and balance of the cup.

2.6 Preparation of aqueous extracts of green coffee

Green coffee extracts were obtained following the methodology reported by (Jeszka-Skowron et al., 2016) with some modifications. Green coffee beans were ground and passed through a 425 µm sieve (ASTM No. 40). One (1) gram of each sample was weighed in conical centrifuge tubes, mixed with 50 mL of distilled water (90 °C), and kept at this temperature for 10 minutes in a water bath. Extracts were centrifuged at 3,500 rpm for 5 min and filtered (Whatman #1 filter paper). Extracts were used for the determination of caffeine, chlorogenic acids, flavonoids, total polyphenols, sugars, and antioxidant capacity.

2.7 Chlorogenic acids and caffeine identification and quantification

The characterization of chlorogenic acids and caffeine was performed using high performance liquid chromatography (HPLC model 1100, Agilent Technologies, USA) together with a diode array detector system (HPLC-DAD), and according to the methodology reported by Jeon et al. (2019)

with some modifications. Prior to analysis, aqueous extracts were obtained, and 10 microliters of samples were injected onto a ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm, Agilent Technologies, USA) held at room temperature. The mobile phase used in the analysis consisted of eluent A (20 mM KH_2PO_4 buffer with 0.1% phosphoric acid) and eluent B (acetonitrile with 0.1% phosphoric acid). A gradient initially composed of a ratio of eluents A/B of 97:3 from 0 to 5 min was established, eluent B was increased to 93:7 from 5 to 15 min, and then increased slightly to 92:8 from 15 min. at 25 min, later the eluent B was increased to 75:25 from 25 to 60 min, and finally the eluent was returned to 97:3 from 60 to 70 min to equilibrate the column and wash the system prior to the next analysis. This gradient was carefully used to separate the 8 isomers of chlorogenic acids and caffeine simultaneously. The flow was kept constant throughout the analysis at 1.5 mL/min. Detection wavelengths of 272 and 324 nm were used for the analysis of caffeine and chlorogenic acids, respectively. For the quantification of each component, calibration curves were constructed with 5-caffeoylquinic acid (5-CQA) (R^2=0.9998) and caffeine (R^2=0.9980) (Sigma-Aldrich, Co., MO, USA). The identification of the isomers of chlorogenic acids was confirmed by the retention time of the analysis of the standard solutions 3-CQA, 5-CQA and 4-CQA, and based on literature (Craig et al., 2016; Jeon et al., 2019). The isomers in the samples were calculated as 5-CQA equivalents using an external standard method through calibration curves. Total chlorogenic acids were reported as the sum of the main isomers: 3-CQA, 5-CQA, 3-FQA, 4-CQA, 5-FQA, 3.4-diCQA, 3.5-diCQA and 4.5-diCQA.

2.8 Flavonoids quantification

Total flavonoids were determined following the methodology reported by Zhishen, Mengcheng and Jianming (1999), with some modifications. Briefly, 600 µL of the aqueous extract was extracted from each green coffee sample. Each sample was placed 2.58 mL of solution A, composed of: 1.8 mL of 5% $NaNO_2$ plus 24 mL of deionized water. It was left to settle for 5 min and then 180 µL of 10% $AlCl_3$ was added. It was left to rest for 1 min. Finally, 2.52 mL of solution B was added, consisting of: 12 mL of 1M NaOH plus 14.4 mL of deionized water. It was then read in a UV/VIS spectrophotometer (Cary 8454, Agilent Technologies, USA) at 415 nm and compared to a catechin (Sigma-Aldrich Inc, MO, EUA) standard curve (R^2=9998). Results were expressed in mg of catequin equivalents per gram of green coffee (mg CE/g).

2.9 Total polyphenols quantification

The total polyphenol content of the aqueous extract of green coffee was determined spectrophotometrically using the Folin-Ciocalteu method (Singleton; Orthofer; Lamuela-Raventós, 1999; Wolfe; Wu, Liu, 2003) with some modifications. Briefly, a 60 µL aliquot of the extract was mixed with 3 mL of distilled water and 250 µL of Folin-Ciocalteu's reagent (1 N) (Sigma-Aldrich, Co., MO, USA). It was allowed to equilibrate for 5 min at room temperature. 750 µL of Na_2CO_3 (20% w/v) and 950 µL of distilled water were added. Then it was allowed to equilibrate for 30 min at room temperature and the reading was carried out in a UV/VIS spectrophotometer (Cary 8454, Agilent Technologies, USA) at 765 nm. The results obtained were compared with a calibration curve (R^2=0.9995) using gallic acid (Sigma-Aldrich, Co., MO, USA). Results were expressed in mg of gallic acid equivalents per gram of green coffee (mg GAE/g).

2.10 Antioxidant capacity

Antioxidant activity of coffee extracts was measured in terms of reduction of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) following the methodology previously reported by Trandafir, Nour and Ionica (2013) and Ricci et al. (2019), with some modifications. Briefly, DPPH solution (Sigma-Aldrich, MO, USA) was prepared (0.004% w/v) and 2.50 mL were mixed with 50 µL of the aqueous coffee extract, shaken vigorously, and allowed to incubate for 30 minutes at 27ºC. It was then read on a UV/VIS spectrophotometer (Cary 8454, Agilent Technologies) at 517 nm. The antioxidant activity (By inhibition of the DPPH radical) was determined according to Equation 1 (Shen et al. 2013).

$$Inhibition(\%) = \left(\frac{Ao - As}{Ao}\right)*100$$

Where:
Ao = Initial absorbance of DPPH at the time of 0 minutes
As = Final absorbance at 30 min.

2.11 Statistical Analysis

The data were analyzed with the SAS® Version 9.4 program, through an analysis of variance and Fisher's LSD separation of means to compare the means of the factors, and LSMEANS to compare the interactions between factors, using a probability of 95%.

3 RESULTS

The experiment was conducted between February 9th and 19th 2021 and temperatures reported in Santa Ana, La Paz, Honduras ranged from 22-25 °C (maximum) and 9-10°C (minimum) (Secretaria de Agricultura y Ganadería - SAG, 2021). Initially, pH values of soaking solutions ranged from 6.6 (no acid added) to 2.9 in a 0.1 M citric acid solution (Figure 1). After 48 hours, this range was significantly reduced as the treatment in water (no acid added) presented a lower pH (4.3) while the most acidic sample (citric acid) increased its pH by 32% (3.83). Ascorbic acid and acetic acid solutions increased

their pH values slightly between 12 and 36 hours but presented no significant differences at the end of soaking (P>0.05). Regarding temperature, no significant differences were observed throughout the experiment (10 days) as all the treatments were exposed to the same conditions and initial soaking time (2:00 pm) which was programmed to avoid a differentiating effect due to temperature changes during the day.

3.1 Microbiological analyses.

Microbiota presence (fungi and yeasts) was higher prior to any type demucilagination (Table 2). Decreases of 29-31% for molds and 25-41% in yeasts were observed in samples. Mechanical demucilagination achieved similar reduction for molds (13,000 cfu/g) and greater reduction for yeasts (37,300 cfu/g) in less time compared to fermentation. LAB was the most prevalent group of microorganisms analyzed. LAB presence was significantly reduced after fermentation (48%), while no differences were observed when samples were mechanically demucilaginated. Although LAB counts showed no statistical differences, 580,000 cfu/g were lost during mechanical demucilagination which is almost 15 times greater than the yeast population lost and almost 44 times compared to molds removed during demucilagination.

After demucilagination, samples were soaked in water with acid solutions and microbiological samples (molds, yeast, and LAB) were collected (Figure 2). No differences in mold counts were observed due to types of acids applied during soaking (P>0.05). However, significant reductions in mold counts were observed over time. Yeast counts presented significant differences for acid treatments and soaking time (P<0.05). Populations in citric acid and ascorbic increased (33 and 23%, respectively) in the first 24 hours of soaking. However, yeast count in citric acid was slightly reduced to result in no differences with the initial population.

In contrast, a significant reduction was observed in ascorbic acid samples with a 35% decline in yeast population in the second day to result in an overall reduction of yeasts. Samples soaked in acetic acid and water (control) showed no difference over time. At the end of soaking, citric acid samples resulted in the highest yeast counts compared to the rest of treatments.

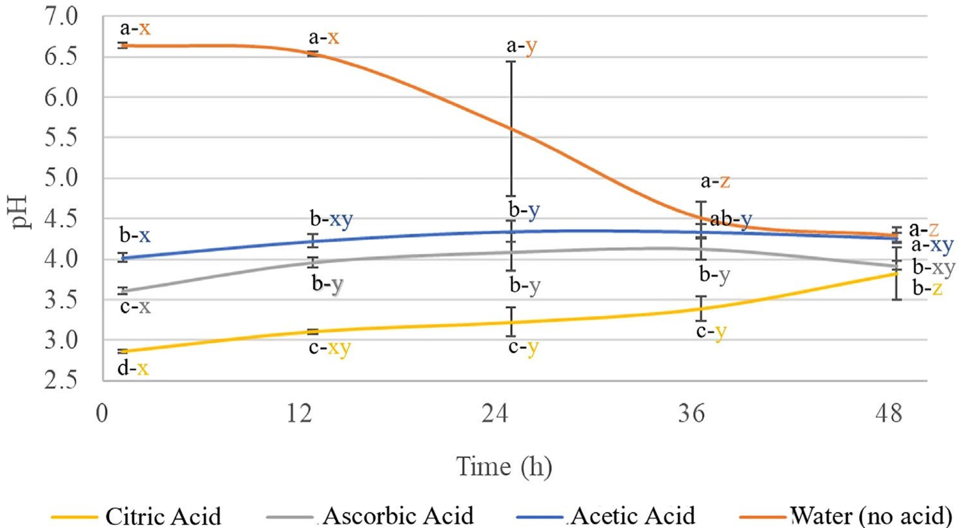

Figure 1: Water pH during soaking.

[abc]Values followed by a different letter in each time interval indicate statistical difference between treatments (P<0.05). [xyz]Values followed by a different letter in each timeline indicate statistical difference over time (P<0.05).

Table 2: Mold, yeast, and lactic acid bacteria counts before and after demucilagination.

Demucilagination		Molds $\times 10^4$ cfu[2]/g	Yeasts $\times 10^4$ cfu/g	LAB[1] $\times 10^6$ cfu/g
Before[3]		4.17 ± 0.75 [a]	9.13 ± 0.71 [a]	10.1 ± 0.85 [a]
After[4]	Mechanical	2.87 ± 0.35 [b]	5.40 ± 0.50 [c]	9.52 ± 0.08 [a]
After[5]	Biological (Fermented & washed)	2.97 ± 0.21 [b]	6.87 ± 0.64 [b]	4.93 ± 0.21 [b]
CV[6] (%)		23	22	42

[1]Lactic acid bacteria. [2]Colony forming units. [3]Sample taken before demucilagination. [4]Sample taken after mechanical demucilagination. [5]Sample taken after fermentation and washing (control). [6]Coefficient of variation. [abc]Values followed by a different letter in each time column indicate statistical difference between treatments (P<0.05).

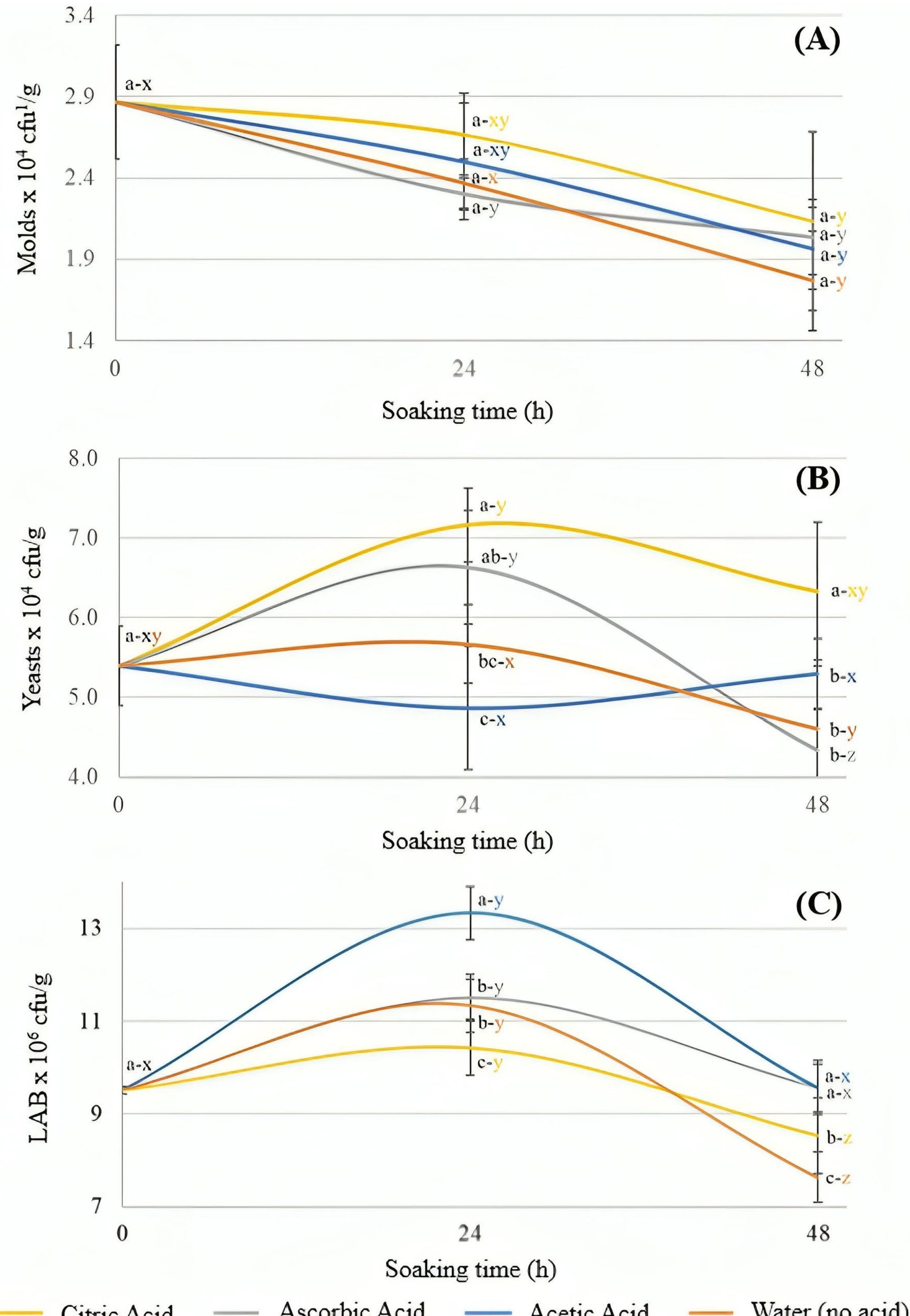

Figure 2: Mold (A), Yeast (B), and Lactic acid bacteria (LAB) (C) counts during soaking.

[1]Colony forming units. [abc]Values followed by a different letter in each time interval indicate statistical difference between treatments ($P<0.05$). [xyz]Values followed by a different letter in the timeline for each acid indicate statistical difference ($P<0.05$).

LAB counts also presented significant differences for acid treatments (Figure 2), soaking time, and the interaction between them (P<0.05). All treatments experienced a similar pattern of growth in the first 24 hours and decline in the second day of soaking (48 h). Initially, acetic acid samples showed the highest increase in LAB counts (40%) in the first day of soaking, but also the highest decline in the second day (40%) to result in no differences with the initial population. Ascorbic acid samples experienced a similar pattern but with less increase (24 h) and decrease over time (21%) to result in no differences with the initial population. Although citric acid samples and controls showed lower increases in the first 24 hours of soaking (10% and 19%, respectively), significant reductions were observed in the second day resulted in overall decreases of LAB population (10% and 20%, respectively) which were significantly lower compared to initial counts and acetic acid and ascorbic acid samples at 48 hours of soaking.

3.2 Sensory evaluation (cupping).

Evaluations were carried out after drying, hulling and removal of all primary and secondary defects of coffee samples. No significant differences (P>0.05) were observed for acidity, body, balance, uniformity, cleanliness, sweetness. However, fermentation resulted in higher scores (P<0.05) for aroma, flavor, and aftertaste (Table 3). No differences were observed due to soaking or the application of acids during this procedure. However, the use of soaking increased the scores in certain attributes making them similar to the control (fermentation), but without being different from the rest of treatments. Only in the attribute of aftertaste, soaking for 48 hours with ascorbic acid proved to have a similar score as the control (fermentation), which was different from other treatments. For the overall score, all samples could be categorized as special coffee given their scores over 80 points (83.4-84.4). In general, no differences were observed with only soaked samples in ascorbic acid (24 h) and acetic acid (48 h) showing differences compared to the controls with no soaking.

3.3 Chemical analyses

Various components were quantified including caffeine, polyphenols, flavonoids, sugars and chlorogenic acids which were identified in addition to their quantification during these experiments.

3.4 Caffeine

Identification and quantification of caffeine was conducted through high performance liquid chromatography (HPLC) of aqueous samples. Caffeine was identified at approximately 272 nm and the output was close to min 40, where its maximum absorbance peak occurred (Figure 3). Caffeine content ranged from 11.5 to 12.4 mg/g and no differences were observed between demucilagination techniques, application of soaking, nor type of acid solutions used.

3.5 Chlorogenic Acids

Eight chlorogenic acid isomers were identified and quantified at 324 nm (Figure 4). Six caffeoylquinic acids (CQA) were identified (3-CQA, 5-CQA, 4-CQA, 3.4-diCQA, 3.5-diCQA acid and, 4.5-diCQA) and two minor feruloylquinic acids (FQA) were detected in samples (3-FQA and 5-FQA). Peak profiles were consistent among samples tested and followed a similar pattern as reported in previous studies for green coffee bean extracts (Craig et al., 2016), and in different types of coffee-related beverages (Jeon et al., 2019).

The most prevalent chlorogenic acid in samples was 5-CQA followed by 4-CQA, 3.4-diCQA, and 3-CQA (Table 4). Minor quantities of the rest of chlorogenic acids (4)

Table 3: Final cupping scores of samples after demucilagination and soaking.

Demuc[1]	Acid	Soaking time (h)	Aroma	Flavor	Aftertaste	Overall score[5]
Mechanical	Citric	24	7.80±0.05[b]	7.75±0.08[ab]	7.58±0.00[ab]	83.7±0.41[ab]
		48	7.80±0.05[b]	7.70±0.05[b]	7.58±0.09[ab]	83.6±0.25[ab]
	Ascorbic	24	7.86±0.05[ab]	7.64±0.05[b]	7.47±0.05[b]	83.5±0.39[b]
		48	7.78±0.05[b]	7.78±0.05[ab]	7.69±0.10[a]	84.0±0.51[ab]
	Acetic	24	7.86±0.05[ab]	7.75±0.08[ab]	7.61±0.05[ab]	83.9±0.42[ab]
		48	7.78±0.13[b]	7.67±0.09[b]	7.50±0.08[b]	83.4±0.59[b]
	Water (no acid)	24	7.92±0.17[ab]	7.69±0.10[b]	7.47±0.09[b]	83.6±0.46[ab]
		48	7.80±0.05[b]	7.75±0.08[ab]	7.61±0.13[ab]	83.9±0.33[ab]
	N/A[4]	-	7.81±0.10[b]	7.70±0.05[b]	7.53±0.09[b]	83.7±0.73[a]
Biological[2]	N/A[4]	-	7.97±0.13[a]	7.95±0.21[a]	7.67±0.09[a]	84.4±0.51[a]
CV[3] (%)			1.05	1.09	1.00	0.44

[1]Demucilagination, [2]Demucilagination through fermentation (24-36 h) and washing, [3]Coeficient of variation. [4]Control treatments with no soaking, 5Cupping scores were recorded according to SCA methodology, [ab]Values followed by a different letter in each column indicate statistical difference between treatments (P<0.05).

were detected. In general, mechanically demucilaginated samples soaked in acetic acid (24 and 48 h) and fermented samples (control) presented the highest concentrations of chlorogenic acids (48.7±1.7 mg/g, 48.4±1.0 mg/g, and 48.3±0.4 mg/g, respectively), which in average were 5% higher than the rest of treatments. The sample soaked for 48 hours in citric acid presented the next highest concentration of chlorogenic acids (46.9±1.0 mg/g) and it is more evident in 3.4-diCQA where it had the highest concentration of all samples which were comparable to the concentrations of samples soaked in acetic acid and fermentation (control). No statistical differences were observed for 3-CQA, and minor differences were observed in the rest of chlorogenic acids evaluated (3.5-diCQA, 5-FQA, 3-FQA, and 4.5-diCQA).

3.6 Flavonoids

There were significant differences for the type of soaking ($P<0.05$). Treatments with mechanical demucilagination and soaking presented the highest flavonoids concentration, independent of the type of acid applied during this process (Table 5). Samples soaked in water with no acid had similar characteristics compared to other soaked samples in the first 24 hours of soaking. However, flavonoid concentration was reduced (17%) during the second day of soaking whereas samples soaked with acids maintained flavonoid concentrations during the duration of this experiment (48 h). Samples with no soaking presented significantly less flavonoids regardless of their type of demucilagination (mechanical or biological).

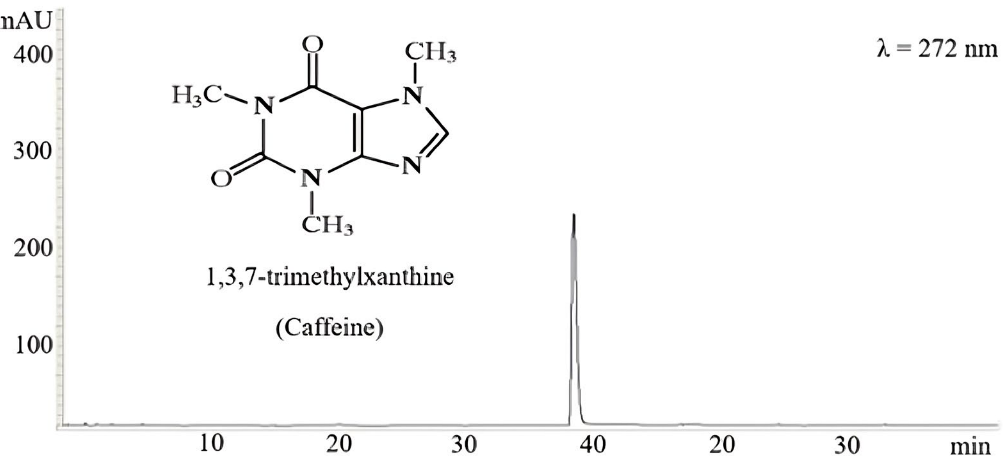

Figure 3: HPLC chromatogram of caffeine.

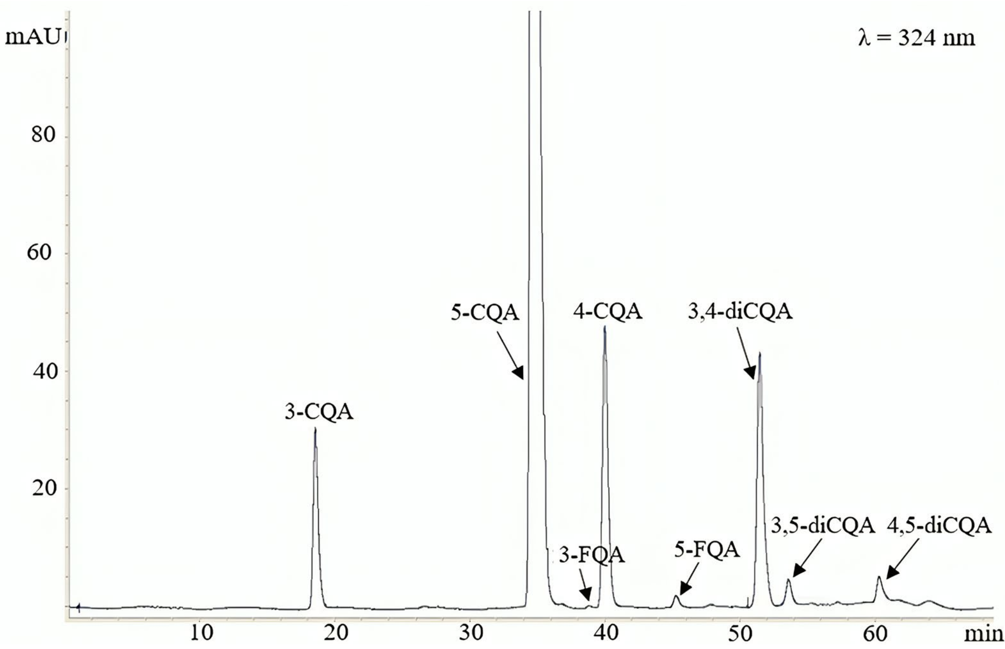

Figure 4: HPLC chromatogram of chlorogenic acids.

Table 4: Chlorogenic acids content extracted via HPLC from green coffee beans soaked in organic acids and their controls without soaking or acid.

Demuc[1]	Acid	Soaking time (h)	Chlorogenic acids (mg 5-CQA equivalents/g of coffee)							
			3-CQA[5]	4-CQA	5-CQA	3.4-diCQA	3.5-diCQA	4.5-diCQA	3-FQA[6]	5-FQA
Mechanical	Citric	24	2.16±0.07^{a}	3.57±0.07^{cd}	34.9±0.70^{d}	3.25±0.13^{c}	0.31±0.01^{b}	0.09±0.01^{bc}	0.02±0.00^{a}	0.21±0.00^{c}
		48	2.18±0.15^{a}	3.70±0.12^{bc}	36.9±0.47^{bc}	3.52±0.08^{a}	0.33±0.02^{ab}	0.09±0.01^{c}	0.02±0.00^{ab}	0.25±0.01^{ab}
	Ascorbic	24	2.22±0.09^{a}	3.70±0.06^{bc}	36.2±1.88^{c}	3.45±0.06^{ab}	0.34±0.02^{a}	0.10±0.01^{abc}	0.02±0.00^{ab}	0.24±0.02^{b}
		48	2.15±0.11^{a}	3.35±0.07^{d}	36.0±0.35^{cd}	3.39±0.08^{abc}	0.34±0.02^{a}	0.10±0.01^{abc}	0.02±0.00^{ab}	0.22±0.01^{c}
	Acetic	24	2.27±0.08^{a}	4.01±0.25^{a}	38.4±0.99^{a}	3.34±0.13^{bc}	0.33±0.02^{ab}	0.10±0.01^{abc}	0.02±0.00^{ab}	0.26±0.01^{a}
		48	2.25±0.08^{a}	4.01±0.19^{a}	38.1±0.41^{a}	3.36±0.03^{bc}	0.31±0.01^{b}	0.10±0.01^{ab}	0.02±0.00^{ab}	0.25±0.01^{ab}
	Water (no acid)	24	2.19±0.14^{a}	3.72±0.27^{bc}	36.5±1.31^{c}	3.37±0.09^{bc}	0.33±0.02^{ab}	0.11±0.01^{a}	0.02±0.00^{ab}	0.24±0.02^{ab}
		48	2.21±0.11^{a}	3.86±0.20^{ab}	36.7±0.40^{c}	3.36±0.11^{bc}	0.33±0.02^{ab}	0.10±0.01^{ab}	0.02±0.00^{ab}	0.26±0.02^{a}
	N/A[4]	-	2.19±0.15^{a}	3.70±0.10^{bc}	36.3±0.58^{c}	2.93±0.10^{d}	0.31±0.03^{b}	0.09±0.00^{bc}	0.02±0.00^{ab}	0.20±0.01^{d}
Biological[2]	N/A[4]	-	2.25±0.05^{a}	3.89±0.30^{ab}	38.1±0.38^{ab}	3.35±0.10^{bc}	0.32±0.02^{ab}	0.10±0.00^{ab}	0.02±0.00^{c}	0.23±0.00^{b}
CV[3] (%)			1.86	5.41	3.03	4.70	3.67	4.94	4.70	3.67

[1]Demucilagination, [2]Demucilagination through fermentation (24-36 h) and washing, [3]Coeficient of variation. [4]Control treatments with no soaking, [5]Caffeoylquinic acid, [6]Feruloylquinic acid, [a-d]Values followed by a different letter in each column indicate statistical difference between treatments ($P<0.05$).

Table 5: Flavonoid content, total polyphenol content, and antioxidant capacity from green coffee beans soaked in organic acids and their controls without soaking or acid.

Demuc[1]	Acid	Soaking time (h)	Flavonoids (mg CE[2]/g)	Total polyphenols (mg GAE[3]/g)	Antioxidant capacity (%)
Mechanical	Citric	24	33.0±3.04^{a}	46.4±0.98^{f}	86.2±0.83^{a}
		48	35.1±2.31^{a}	48.5±1.96^{ef}	82.9±2.01^{cd}
	Ascorbic	24	32.4±2.49^{ab}	53.3±3.12^{cd}	83.9±2.49^{bcd}
		48	33.0±3.04^{a}	46.7±3.07^{f}	83.0±1.86^{bcd}
	Acetic	24	31.8±3.08^{ab}	64.4±1.40^{a}	84.3±0.69^{abc}
		48	32.4±3.04^{ab}	63.4±2.42^{a}	82.3±1.13^{de}
	Water (no acid)	24	35.1±3.49^{a}	56.0±1.40^{bc}	80.9±0.54^{e}
		48	29.0±2.56^{bc}	58.4±2.93^{b}	82.8±1.94^{cde}
	N/A[6]	-	22.0±1.59^{d}	50.7±3.03^{de}	82.9±0.74^{cd}
Biological[4]	N/A[6]	-	27.7±1.78^{c}	62.2±3.69^{a}	84.3±0.69^{ab}
CV[5] (%)			1.57	1.09	0.65

[1]Demucilagination, [2]Catechin equivalents, [3]Gallic acid equivalents, [4]Demucilagination through fermentation (24-36 h) and washing, [5]Coeficient of variation. [6]Control treatments with no soaking, [a-f]Values followed by a different letter in each column indicate statistical difference between treatments ($P<0.05$).

3.7 Total Polyphenols

There were significant differences for the type of acid used during soaking ($P<0.05$). Consistent with chlorogenic acids, samples soaked in acetic acid and fermented samples (control) presented the highest polyphenol content being up to 25% higher than treatments soaked in citric acid which presented the lowest polyphenol content in this study (Table 5). Polyphenol content was maintained during soaking in all treatments except for ascorbic acid which presented a reduction (12%) during the second day of soaking.

3.8 Antioxidant activity

In general, samples had radical scavenging (antioxidant activities) greater than 80% and differences between treatments were harder to draw (Table 5). Fermentation resulted in one of the highest antioxidant activities observed. Samples soaked in citric and acetic acid also presented highest antioxidant capacities in the first 24 hours of soaking, but were significantly reduced in the second day of soaking (4% and 2%, respectively). Samples soaked in ascorbic acid and water (no acid) did not change over the duration of the experiment (48 h).

4 DISCUSSION

The reduction of fungi, yeasts and LAB populations after biological demucilagination (fermentation) demonstrated a microbial succession throughout the process due to solubilization of complex carbohydrates and usage of nutrients (Velmourougane, 2013; De Melo et al., 2017). High prevalence of LAB agreed with other studies reporting that low oxygen conditions favors the development of these bacteria (Shillinger et al., 2008; Silva, 2014) which then could favor the acidification and development of yeasts (Massawe et al., 2010). In parallel, physical removal of mucilage achieved reduction of microbiota present with similar final population for molds and smaller population for yeasts compared to fermentation (control). Although LAB counts showed no statistical differences after demucilagination, more than half a million colony forming units per gram of sample were lost during mechanical demucilagination. These results suggested adherence of microorganisms to the coffee parchment despite the force applied during mechanical removal of the mucilage after fermentation and washing in the case of the control. Authors have studied and demonstrated the adherence mechanisms of LAB probiotic bacteria and their competition with other microorganisms in different matrices (Lee et al., 2000; Borghesi et al., 2016). In parallel, results could also indicate that mucilage was not completely removed during mechanical demucilagination (Zhang et al., 2019a) thus removing microorganisms only partially.

Changes observed in microbial populations during soaking could also be attributed to natural competition for nutrients which were limited given the removal of the mucilage. As mold counts reduced over 48 hours of soaking, LAB population was predominant and increased during the first 24 hours of soaking in all treatments to finally return to, initial populations (acetic and ascorbic) or less (citric and only water), by the end of soaking (48 h). This can be explained by the property of LAB to grow quickly if fermentable carbohydrates are present (Zhang et al., 2019b) and to inhibit growth of competitors, combining a rapid utilization of sugars and the accumulation of lactic and acetic acid (Gaenzle, 2015). These results also support the idea that mucilage was still in contact with coffee beans as a source of nutrients during soaking (Zhang et al., 2019a). Another idea is that minor components could have been absorbed and deposited between coffee endosperm and parchment and slowly released during soaking (Zhang et al., 2019b).

Undoubtedly, coffee demucilagination through fermentation and the variants of this process using different methods induce different metabolic responses in coffee that can change chemical composition of the beans, and have consequences on sensory quality (Bytof, 2007). During this process, microorganisms such as fungi, yeasts, and bacteria play a leading role in the production of enzymes, acids, and alcohols that degrade the mucilage (Haile; Kang, 2019). By physically removing the mucilage from the coffee mass, the microbial load was reduced since this sugar-rich substrate was not present or was present in smaller amounts. Authors suggested that early removal of mucilage by other methods such as mechanical demucilagination fails to develop aromas and flavors of fermented coffees due to the absence of these microorganisms and their action (Velmourougane et al., 2008). While spontaneous fermentation in Arabica species lasts approximately 24 h, microbial succession is more dynamic, generating better cup quality (Velmourougane, 2013). However, through the results obtained in this study soaking in various acids demonstrated similar cupping scores compared to fermented samples (control). Fermentation yielded better cupping scores in three attributes. This evidenced the role of microorganisms, especially yeasts producing superior flavors, aromas and sensory characteristics as proposed by Elhalis et al. (2020) who tested the inhibition of yeast populations during fermentation, obtaining lower scores than treatments with active yeasts. In addition, they found that in the presence of yeasts, glycerol levels were found in a higher proportion, glycerol being produced by the sugar metabolism of yeasts and having a sweet taste and a soft sensation on the palate (Van Wyk et al., 2019). However, it is important to note that after mechanical demucilagination microorganisms continue to thrive which could have an effect of coffee sensory characteristics. Additionally, there were no differences in seven sensory descriptors, including overall score between all evaluated samples. Soaking coffee resulted in less use of water compared to wet processing of coffee and if adjusted to 24 hours or less, could also result in less time before drying. Additionally, results showed that coffee processed with soaking resulted in similar cupping scores compared to fermentation. Liu et al. (2019) evaluated soaking of Robusta coffee in a solution with acetic acid (2%) to modify the sensory properties reaching higher levels than Arabica coffee. These results allowed the increase of Robusta in commercial blends without consumers noticing any difference.

Although there were no numerical differences in samples with soaking, 75% of coffees soaked in citric acid presented citrus and mild citric flavors according to panelists (Q graders). This can be attributed to its flavor-enhancing properties in addition to its antimicrobial capacities (Nangare et al., 2021). The flavor characteristics of citric acid are well established in the non-alcoholic beverage industry whose results are known to add fruit flavors to final products (Berovic; Legisa, 2007; Senide; Chambers, 2020). In contrast, flavor descriptors that characterized samples soaked in organic acids were dry herbal and leached aftertaste, which according to quality standards is not characteristic of specialty coffees. Therefore, it is necessary to continue testing soaking with acid

solutions together with variations in roasting and beverage brewing, since all these factors alter the flavor descriptors (Hečimović et al., 2011).

Nearly 90% of the chlorogenic acids identified and quantified in this study corresponded to the group of caffeoylquinic acids (5-CQA, 4-CQA, and 3-CQA). The proportions of di-caffeoylquinic and feruloylquinic acids during this investigation was on average 8% and 1% respectively of the total chlorogenic acids. Farah and Donangelo (2006) reported 56-62% of caffeoylquinic acids, 15-29% di-caffeoylquinic acids, and 5-13% of feruloylquinic acids. It was clearly observed that the acidification of the medium where the coffee was soaked changed the distribution of chlorogenic acids, observing a migration or accumulation towards caffeoylquinic acids and a reduction in the other groups that make up total chlorogenic acids.

One of the hypotheses by which soaking in acetic acid obtained higher contents of chlorogenic acids may be the absorption of this compound in the seed and concentrated during drying; this idea may be supported by the ability of acetic acid to increase the selectivity factors and retention of analytes such as chlorogenic acids and provide better resolution when added up to 2% to the mobile phase with acetonitrile (Li et al., 2005). In accordance, Xie et al. (2011) tested the stability of the isomers of 3-CQA, 4-CQA, and 5-CQA, finding that at pH 5.0, these acids were stable, as the buffer solution increased in pH, its stability decreased, and a positional isomerization was observed. Other study evaluated the extraction and stability of chlorogenic acids comparing fermentation and the use of organic acids (Silveira et al., 2019).

Phenolic compounds are products of the secondary metabolism of plants, which are closely related to beneficial effects in the body of those who consume them and are also responsible for other properties in the product, such as color and flavor (Rasouli; Farzaei; Khodarahmi, 2017). Therefore, phenolic compounds play an important role within the bioactive profile of coffee. The family of polyphenols is the largest group of non-energetic substances present in foods of plant origin such as berries, grapes, coffee beans, cocoa, tea, among others. In recent years, the benefits of drinking coffee and tea-based beverages have been pointed out and evaluated thanks to the presence of polyphenols and flavonoids (Farah; De Paula-Lima, 2019). Phenolic compounds in coffee and tea are classified into flavonoids, lignans, stibenes, chlorogenic acids and phenolic acids, with flavonoids presenting in higher concentrations, especially flavonols and phenolic acids (Wang; Ho 2009; Ferruzzi, 2010).

Results indicated that mechanical demucilagination with soaking for 24 to 48 hours had a positive effect on the chemical characteristics of green coffee. This is consistent with (Velmourougane, 2011), who indicated that, during wet processing, soaking in water was one of the important steps followed in the production of quality coffee in India, since it allowed the leaching of some chemical compounds (diterpenes, polyphenols, tannins) responsible for the bitterness and darkening of coffee beans, increasing compounds such as flavonoids. This agrees with other authors who indicated that the application of organic acids generated a reaction that could increase the content of phenolic compounds and therefore total flavonoids (García-Mier et al., 2013).

Oxidants are compounds with a tendency to donate oxygen to other substances. Many reactive oxygen species are free radicals. A free radical is any chemical species that has one or more unpaired electrons Benítez-Estrada et al. (2020). Antioxidants are molecules capable of slowing or preventing the oxidation of other molecules, counteract free radicals and other reactive oxygen species, and are believed to help prevent or slow the progression of many noncommunicable diseases that affect humans (Ayoka et al., 2022). Antioxidant capacity is defined as the potential of a substance or compound to inhibit or hinder the oxidation of a substrate even in very small amounts (<1%, commonly 1-1000 mg/L). It is the property of a molecule of biological or synthetic origin to neutralize the action exerted by a free radical. Its measurement is useful to assess the quality of a food, the amount of antioxidants in a system, or the bioavailability of antioxidant compounds in the human body (López-Alarcón; Denicola, 2013).

No clear differentiation was observed between samples and all presented antioxidant activity over 80% which coincided with those reported by Masek et al. (2020), where they also found an antioxidant capacity of 81.6% in the aqueous extracts of green coffee capable of reducing DPPH radicals. Green coffee has a greater antioxidant capacity than roasted coffee. This can be attributed to the fact that the antioxidant capacity or activity is proportional to the chlorogenic acid content. This information was corroborated by Lazcano-Sánchez et al. (2015), who indicated that there is a decrease in the chlorogenic acid content during roasting which directly affects antioxidant activity.

5 CONCLUSIONS

Soaking coffee in organic acids modified the population dynamics of the organisms present by changing the pH conditions. This opens the possibility of trying other types of acids or additives that modify the populations and the microbial succession of the soaking water, as well as any other physical property that might affect their metabolism.

Soaking coffee in acid solutions maintained the sensory characteristics with respect to the fermented coffee, even though very specific aftertaste flavor descriptors such as leached, dry and herbal were obtained. Although the numerical score was similar, different descriptors were reported depending on the treatment applied to the coffee.

The soaking time had no impact on the improvement of characteristics associated with the quality of the coffee, that is, extending the soaking for another 24 h did not have a greater impact. This is why soaking times of less than 24 h should be evaluated.

The type of acid was able to modify the content and proportion of various families of chlorogenic acids in green coffee and total polyphenols. Since the proportion of volatile compounds in coffee changes according to genetic aspects, degree of roasting, and field conditions, it is necessary to study the transformation of these compounds that favor the generation of flavors under different conditions.

The use of water for mechanical demucilagination plus soaking in organic acids did not represent even 10% of the total water needed for the conventional fermentation and washing process, thus maintaining the ecological and sustainable purpose by reducing water consumption and contamination in coffee postharvest.

Soaking coffee in organic acids resulted in higher flavonoid contents than the mechanical demucilaginated treatments without soaking and fermented/washed (control).

Organic acid soaking did not affect antioxidant activity in demucilaginated green coffee beans. It should be noted that the radical scavenging activity will depend on various factors from the harvest to the benefit and processing that is provided to the coffee.

6 AUTHORS' CONTRIBUTIONS

Conceptual Idea: Cardona, J.; Methodology design: Cardona, J.; Data collection: Cardona, J. Maldonado, L.; Data analysis and interpretation: Cardona, J. Maldonado, L.; and Writing and editing: Cardona, J. Maldonado, L.

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