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MICROBIAL COMMUNITIES AND BIOCHEMICAL COMPOUNDS INVOLVED IN COFFEE FERMENTATION FROM DIFFERENT ALTITUDES OF THE CAPARAÓ REGION

LAVRAS-MG
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Thesis presented to the Federal University of Lavras, as part of the requirements of the Postgraduate Program in Agricultural Microbiology, research area in Biotechnology of Microorganisms Applied to Agriculture and the Environment, to obtain the title of Doctor.

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COMUNIDADES MICROBIAIS E COMPOSTOS BIOQUÍMICOS ENVOLVIDOS NA FERMENTAÇÃO DE CAFÉ DE DIFERENTES ALTITUDES DA REGIÃO DE CAPARAÓ

Thesis presented to the Federal University of Lavras, as part of the requirements of the Postgraduate Program in Agricultural Microbiology, research area in Biotechnology of Microorganisms Applied to Agriculture and the Environment, to obtain the title of Doctor.

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ABSTRACT

Minas Gerais is the most coffee producing state of Brazil, mainly of Catuaí variety. After this state comes Espirito Santo, containing the Caparaó region known for its specialty coffees harvested in mountains located at altitudes from 700 to 1,400 m. The differences perceived in coffee during tasting derived from pre-and post-harvest factors. Among those factors are the producing regions, coffee variety, temperature, altitude, processing methods, and type of fermentation. As a result, those factors change either the fruits or beans chemical characteristics together with their microbiota. In this sense, the first article aimed to characterize microbiologically (target NGS) and chemically fermented coffees from different altitudes processed via natural. Altitude was an important variable that caused shifts in the microbial community and biochemical compounds content. Also, coffees from a lower altitude contained a high bacterial richness and volatile alcohols contents. While high altitude coffees contained high esters, aldehydes, and phenolics contents. The second article aimed to study the dominant communities and evaluate the effect of altitude in those communities and on the biochemical profile from fermented coffees processed via pulped natural. Low altitude coffees favored the richness of bacteria and fungi. The pulped natural process presented dominance of citric acid, volatile alcohols, and caffeine.

Keywords: Altitude. NGS. SIAF. Coffee fermentation. Biochemical compounds.

RESUMO

Minas Gerais é o maior produtor de café do Brasil, principalmente da variedade Catuaí. Depois desse estado segue o Espírito Santo, onde fica a região do Caparaó conhecida por seus cafés especiais colhidos em montanhas localizadas em altitudes de 700 a 1.400 m. As diferenças percebidas no café durante a degustação derivam de fatores pré e pós-colheita. Entre esses fatores estão as regiões produtoras, variedades de café, temperatura, altitude, métodos de processamento e tipo de fermentação. Como resultado, esses fatores alteram as características químicas dos frutos ou grãos juntamente com sua microbiota. Nesse sentido, o primeiro artigo teve como objetivo caracterizar microbiologicamente (NGS) e quimicamente os cafés fermentados de diferentes altitudes processados via natural. A altitude foi uma variável importante que causou mudanças no conteúdo das comunidades microbianas e nos compostos bioquímicos. Além disso, os cafés de baixa altitude tiveram uma alta riqueza bacteriana e teores de álcoois voláteis. Enquanto os cafés de alta altitude tiveram altos teores de ésteres, aldeídos e fenólicos. O segundo artigo teve como objetivo estudar as comunidades dominantes e avaliar o efeito da altitude nessas comunidades e no perfil bioquímico de cafés fermentados processados via natural descascado. Os cafés de baixa altitude favoreceram a riqueza de bactérias e fungos. O processo natural despolpado apresentou dominância de ácido cítrico, álcoois voláteis e cafeína.

Palavras-chave: Altitude. NGS. SIAF. Fermentação do café. Compostos bioquímicos.

SUMMARY

FIRST PART

1 INTRODUCTION

In Brazil, coffee production increases with the consumer's demand. In 2020 arabica production in Brazil was 48.767 thousand bags, and for conilon was 14.310 thousand bags (CONAB, 2021). Coffee fruits must go through a series of steps involving collection, selection, and processing. After harvesting, coffee fruits are processed via 3 methods: dry, semidry, or pulped natural, and wet. The dry or natural method used whole and washed fruits, which are fermented while they are dried in cement patios or platforms. Fruits in the wet method are depulped and demucilated, then they are fermented in tanks with large volumes of water (SCHWAN; SILVA; BATISTA, 2012; SILVA, 2015; VILELA *et al*., 2010). The semidry method is a variation of the wet method, where fruits depulped, fermented under the sun in cement patios or platforms.

The processing method is part of the post-harvest factors that alter the final beverage attributes. Pre-harvesting factors such as coffee variety and harvesting region also affect the attributes. In Brazil, the most coffee producing states are Minas Gerais, Espírito Santo, and Sao Paulo (CONAB, 2021). Each state has different geographical characteristics and coffee flavors that vary from floral, fruity, citric, caramel, nutty to chocolate (BSCA, 2018). The arabica coffee varieties harvested in those states include Bourbon Amarelo, Bourbon Vermelho, Mundo Novo, and Catuaí Amarelo. In addition, conilon coffee is also harvested, mainly in the state of Espírito Santo.

Both pre- and post-harvesting factors affect the microbiota, fermentation, and biochemical compounds. Fermentation is the most important step during processing since it allows precursors production that is transformed into coffee volatiles and non-volatiles. During fermentation, microorganisms (Gram-positive and negative bacteria, filamentous fungi, and yeasts) convert the carbohydrates or organic matter into other compounds through biochemical reactions (EVANGELISTA *et al*., 2014a; LEE *et al*., 2015; RIBEIRO *et al*., 2017). As a result, compounds such as acids, aldehydes, ketones, alcohols, among others, are produced. Moreover, fermentation is essential for obtaining specialty and good quality coffees.

Therefore, it becomes important to study natural microbiota in coffee fermentation. Although its frequently studied, new advances in techniques had allowed a deep understanding and discoveries. For example, new generation sequencing to study microbial communities and those used during compound and sensorial analysis have become more sensitive, relevant, and faster.

2 LITERATURE REVIEW

2.1 Rising and importance of coffee in Brazil

The first seeds of arabica coffee in Brazil were first introduced in 1727, derived from the cultivar Typica. Then, in 1852 the second cultivar Bourbon Vermelho was introduced. Later, in 1871 natural mutations occurred from cultivar Typica, resulting in a new cultivar called Amarelo de Botucatu. By 1896, a third arabica coffee was introduced, cultivar Sumatra. After, natural hybrids were selected from those cultivars until reaching the most important cultivars grown in Brazil Mundo Novo, Catuaí Vermelho, and Catuaí Amarelo (SAKIYAMA; GAVA, 2015).

During that period, coffee exportation in Brazil emerged from the exhaustion of gold and diamond exploitation in the second half of the 18th century, making the Brazilian economy dependent on cotton, rice, sugar, and later coffee (SKIDMORE, 1999). After its appearance, coffee had been the leader crop of Brazil's exportation economy, representing in 1925 around 70% of Brazil's total exports (PAIVA, 2000). At that time, coffee had many secondary effects on the economy, such as free immigrant labor, foreign investment in infrastructure, capital accumulation of coffee growers, and the derived industry growth (PAIVA, 2000). Additionally, the demand for free labor led many coffee producers in Brazil to participate in the slavery abolition campaign (PAIVA, 2000).

According to the Conselho dos Exportadores de Café do Brasil (CeCafé), from January through December of 2017, exportation was equivalent to 30.88 thousand bags of 60 Kg that included industrialized and green coffee which represented in profit 5.23 billion of dollars (CECAFÉ, 2018). Since coffee production in Brazil increases daily with consumers' demand, arabica production was 48.767 thousand bags, and for conilon was 14.310 thousand bags (CONAB, 2021).

2.2 Pre-harvest and post-harvest factors affecting the quality of beverage

2.2.1 Producing regions of Brazil

The most producing coffee regions are located in the states of Minas Gerais, Espírito Santo, São Paulo, Bahia, Paraná, Rodônia, and Rio de Janeiro.

2.2.1.1 Minas Gerais

The state of Minas Gerais presented the highest production of arabica for 2020 with 34.337 thousand bags of green beans (CONAB, 2021). Among the states, Minas Gerais produces 55% of the coffee in Brazil. Within Minas Gerais, the cultivation regions are Matas de Minas, Mantiqueira de Minas, Sul de Minas, Cerrado Mineiro, and others (FIGURE 1).

Figure 1 - Photos showing coffee plantations in the Sul de Minas: **A**, **B.** at a farm located in Três Pontas and Cerrado Mineiro region: **C.** at a farm located in Patos de Minas.

Source: From the author (2021).

The Matas de Minas region emerged for the market of specialty coffees, mostly found Bourbon Vermelho variety. Its main producing cities are Manhuaçu, Ervália, Araponga, and Viçosa. Harvesting is done in varying altitudes from 400 to 700 m. Coffee beverages from this region have a medium body, medium acidity, high sweetness with chocolate aroma, and citrus flavor (BSCA, 2018).

Mantiqueira de Minas has been considered a tranditional region that produces high quality coffees and is the most awarded in Brazil. Until now the city of Carmo de Minas has won many awards, but there are others that stand out due to production such as Conceição das Pedras, Paraisópolis, Jesuânia, Lambari, Cristina, Dom Viçoso, and Pedralva (BSCA, 2018).

In the Sul de Minas region, many favorable factors like climate and relief produce craft beverages that result in awards. The variety cultivated is Bourbon Amarelo, and the producing cities are Guaxupé, Varginha, and Três Pontas (BSCA, 2018). Harvesting is done in varying altitudes from 700 to 1,080 m.

The Cerrado Mineiro has a dry climate that favors harvesting period. Producers cultivate mostly Bourbon Vermelho variety and the main producing cities are Patrocínio, Monte Carmelo, Araguari, Patos de Minas, Campos Altos, Unaí, Serra do Salitre, São Gotardo, Araxá, and Carmo do Paranaíba (BSCA, 2018). Harvesting is done in varying altitudes from 820 to 1,100 m.

2.2.1.2 Espírito Santo

The second state had the highest production of coffee for the year 2020, with 4.765 thousand bags for arabica coffee and 9.193 thousand bags for conilon coffee (CONAB, 2021).

2.2.1.2.1 Caparaó region

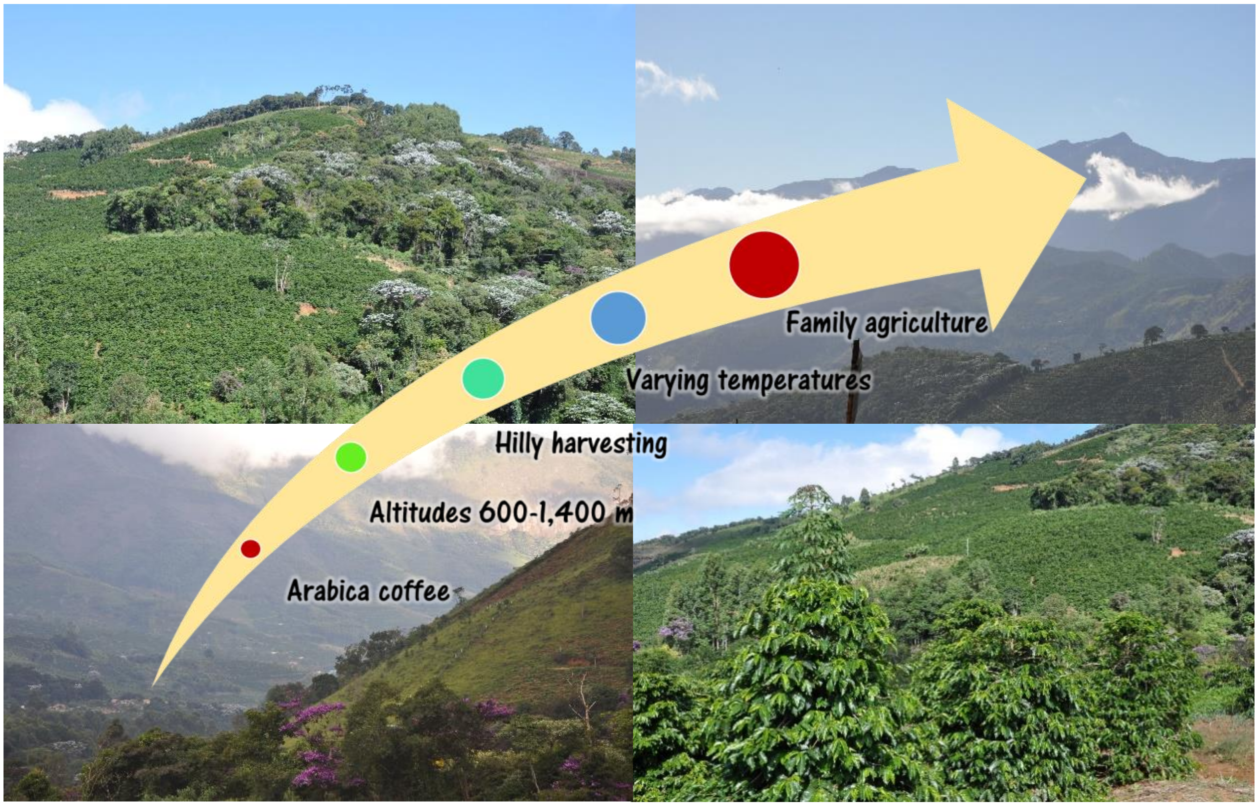
The Caparaó is a mountainous region that shares its territory with Espírito Santo, Minas Gerais, and Rio de Janeiro. Within, is found the Caparaó National Park (Parque Nacinal do Caparaó- PARNA Caparaó), that is a preservation area (ASSIS *et al*., 2017). Coffee harvesting in this region is mainly done by families, in hills, and from 600 to 1,400 m of altitude (FIGURE 2).

For years, coffee farmers from this region have won several quality awards, possibly due to its favorable conditions such as landscape characteristics and climate. Ninety percent of the coffee grown is Arabica coffee and represents 40% of Espírito Santo total production (SANTOS *et al*., 2017).

After harvest, most of the coffee from this region is processed via natural method (75%). That means around 42% of producers dry cherries without washing, 33% wash then dry, 16%

wash and remove the pulp before drying, and 9% wash and remove the skin without removing the pulp before drying (PASCHOA *et al*., 2017).

Figure 2 - Photos showing the landscapes of coffee plantations in the Caparaó region of Espírito Santo and their outlined characteristics.



Source: From the author (2021).

Based on an survey in the Caparaó region, 65% of coffee is dried on cement patios or another without being of soil, 19% use a mechanical dryer, 10% use soil patios to dry, 4% use suspended platforms, and 2% dry using hybrid structures (patios and suspended platforms) (PASCHOA *et al*., 2017).

2.2.1.3 São Paulo

This state occupies the third position of coffee production, with 6.180 thousand bags of arabica for 2020 (CONAB, 2021). Within this state, the Alta Mogiana region is known for producing traditional coffees, and its characteristics generate medium acidity, fruity aroma, and caramel-chocolate notes in the beverage (BSCA, 2018).

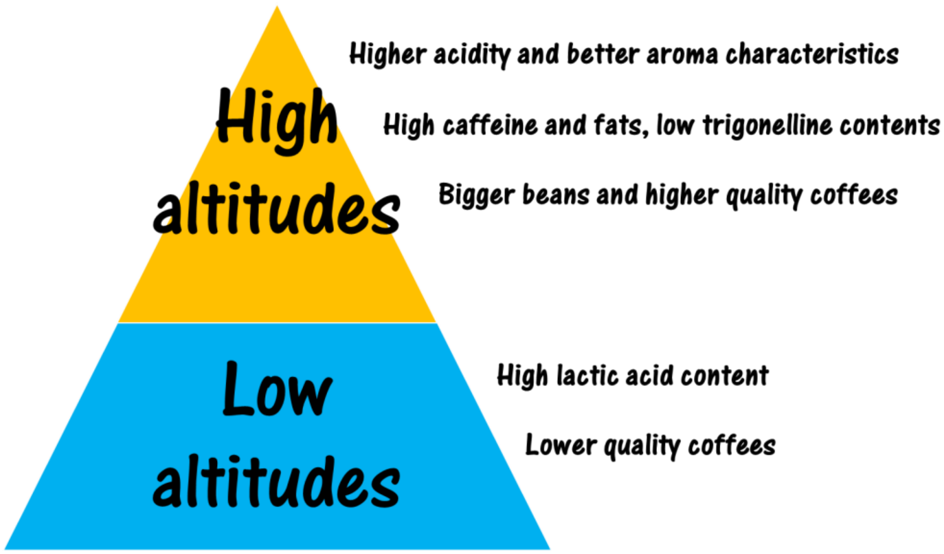
2.2.2 Temperature and altitude

In pre-harvesting, temperature affects the coffee plant development and consequently its fruits. Lower temperatures have been suggested to lengthen the maturation period of coffee fruits, which leads to a higher accumulation of aroma precursors (VAAST *et al*., 2006; WORKU *et al*., 2018). During fermentation, the temperature is essential because it is an indicator that microbiota is working. Also, it determines when fermentation must stop once the temperature had stabilized or decreased. Low temperatures can slow microbial growth and metabolism, continuing with their enzyme activity and organic acids and volatiles production.

According to Puerta-Quintero and Ríos-Arias (2011), at a temperature of 20.5 ºC, carbohydrates on the mucilage decrease together with an acidity increase, ethanol production, and a 40% lipid reduction. Meaning enzymes, microbiota, and lipases were active. On the contrary, at a temperature of 6.6 ºC, these changes are much slower, delaying lactic and alcoholic fermentation, and mucilage composition is preserved after some time.

The material of the recipients or bioreactors and volume used for fermentation also influences the coffee mass's temperature. In close batch fermentations conducted under 300 L stainless steel bioreactors, temperatures of the coffee mass varied from 23 to 27 ºC, which were sufficient to generate specialty coffee with notes above 82.60 (MARTINEZ *et al*., 2021a). However, with 20 L polypropylene bioreactors, temperatures varied from 18 to 24.5 ºC (MARTINS *et al*., 2020).

Figure 3 - Characteristics found in coffees from high and low altitudes.



Source: Alpizar and Bertrand (2004), Worku *et al*. (2018), Guimarães *et al*. (2019) and Da Mota *et al.* (2020).

Temperature is dependent of altitude. At high altitudes, the ambient temperature is lower and promotes heat-induced stress in plants while increasing the leaf and fruit ratio and photosynthetic rate, prolonging the maturation period and providing more carbohydrate and lipids (VAAST *et al*., 2006; WORKU *et al*., 2018).

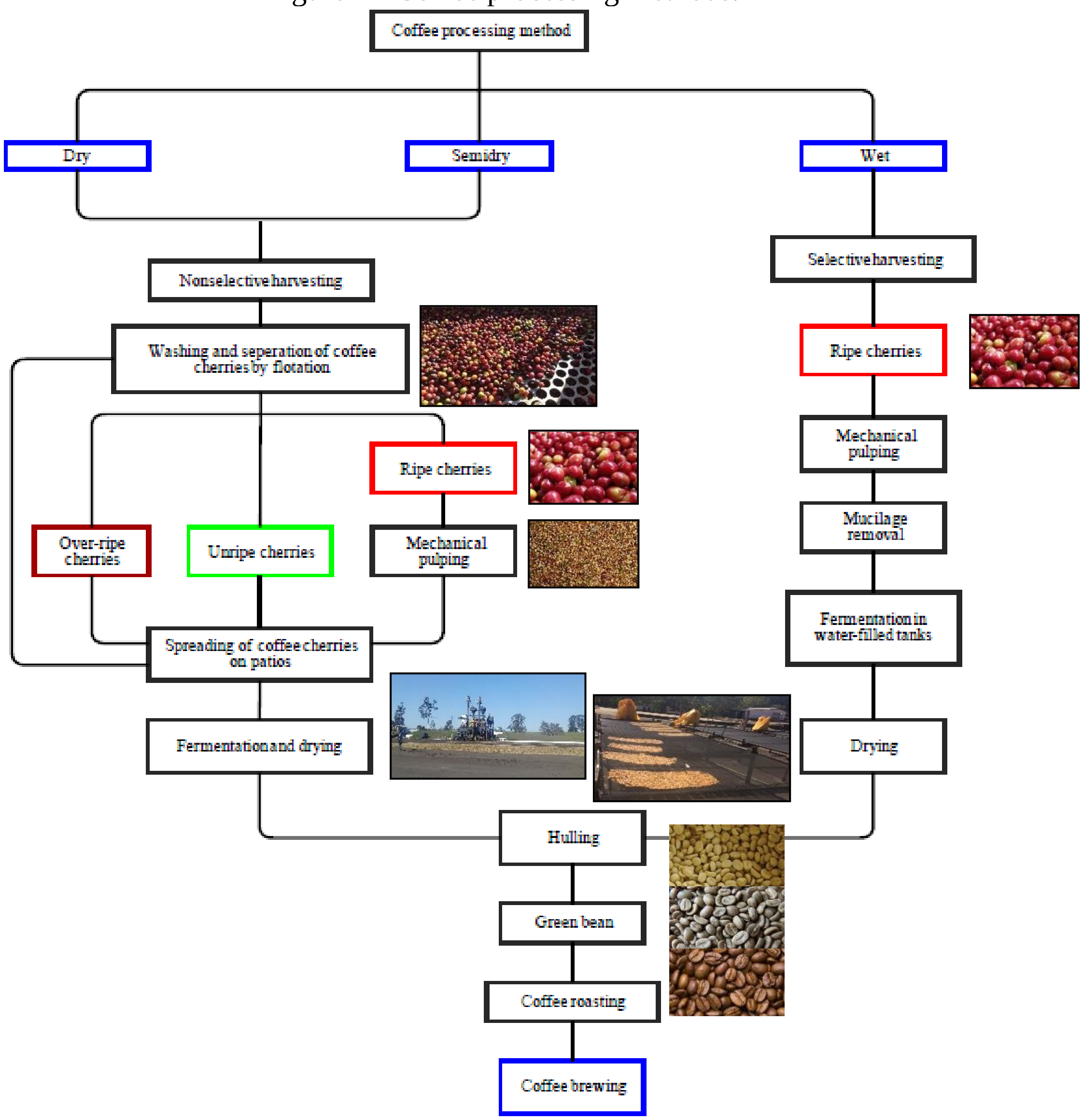
Some general characteristics of coffees from high and low altitudes are illustrated in Figure 3. Additionally, coffee grown at altitudes above 1,000 m have aromatic characteristics, low bitterness, and good acidity and body, while coffees grown below 850 m present high bitterness, grassy attributes, low aroma, and astringency (DECAZY *et al*., 2003). Apart from the sensorial perception, the microbial populations are susceptible to altitude changes, also their richness and abundances (MARTINEZ *et al.*, 2021b; MARTINS *et al.*, 2020).

2.2.3 Processing methods

After harvesting, cherries are either processed via dry, wet, and semi-dry (EVANGELISTA *et al*., 2014a, 2015; SILVA *et al*., 2000; VILELA *et al*., 2010) (FIGURE 4).

The dry method, also known as natural processing, is implemented in countries like Brazil and Ethiopia, which have extended periods of sunshine (LEE *et al*., 2015). For this process, coffee fruits are either handpicked or machine-harvested when most of them are mature. Consequently, the levels of maturity are not consistent among the harvested coffee fruits. Following harvesting, coffee fruits are transferred to patios and left to dry under the sun in layers of approximately 5-8 or 10 cm for 10–25 days, where they constantly heap and re-spread until reaching moisture of 11-12%. Along with drying, natural microbial fermentation occurs, and enzymatic hydrolysis leads to the breakdown of the pulp and mucilage within the coffee cherry, leaving it intact (LEE *et al*., 2015; SILVA *et al*., 2013).

Figure 4 - Coffee processing methods.



Source: Adapted from Dias (2015).

Coffee processed by the wet method is called washed or parchment coffee; it requires reliable pulping equipment and an adequate supply of clean water (EVANGELISTA *et al*., 2015). In this process, the objective is to remove both pulp and mucilage covering the seeds in an environmentally friendly way before drying. For initiation, only ripe cherries are used. Depending on the product harvested, separation may vary (mechanically or not); when mechanically, pulp from cherries is removed in a water flow. Then, mucilage is removed by fermentation on tanks followed by washing or machines (BRANDO; BRANDO, 2015).

On the other hand, the semi-dry or pulped natural method is a variation of wet and dry processes initiated in Brazil in the early 1990s. This process aims separation of ripe and unripe cherries by flotation using water. The pulp of ripe cherries is removed, and beans are dry with the remaining mucilage not removed surrounding the parchment. The fermentation process occurs directly under the sun. Coffee seeds resulting from this method are call pulped natural coffees (DUARTE; PEREIRA; FARAH, 2010; EVANGELISTA *et al*., 2014a).

2.2.4 Coffee fermentation

In all processing methods, the objective of fermentation is to remove the mucilage layer from the seeds to which it adheres and reduce processing time. While mucilage is degraded, cherries are simultaneously dried to 11-12% of moisture. It is a step that occurs naturally regardless of the processing method. During fermentation, physicochemical changes occur in beans, such as reducing water content, simple sugars, and formation of aroma and flavor precursors (SILVA, 2015; SILVA *et al*., 2013).

The optimum temperature for fermentation is 30–35 ºC. The coffee masses are stirred 2–3 times during the fermentation period. Mucilage degradation takes approximately 24–36 h for arabica and 72 h for robusta, depending on the inherent concentration of pectinolytic enzymes, environmental temperature, and elevation (MURTHY *et al*., 2012; SCHWAN; SILVA; BATISTA, 2012; SILVA, 2015).

2.2.4.1 Microbiota

Fermentation is carried out by the natural microbial diversity in coffee cherries, including yeasts, filamentous fungi, and bacteria (SILVA *et al*., 2000). Either type of microorganism can execute different roles during fermentation: pectinolytic enzyme production to degrade the mucilage and pulp, spoilage, mycotoxins production due to insufficient drying and storage (SILVA *et al*., 2000), and enrich flavor precursors. Apart from the environmental factors like temperature, pressure, altitude, and moisture, the fermentation methods: aerobic (spray inoculation), semi-anaerobic or open batch, and anaerobic fermentation or close batch (self-induced anaerobic fermentation (SIAF)) also affect the microbial diversity.

Bacteria are mainly responsible for producing organic acids like lactic and acetic acid (MARTINEZ *et al*., 2019), which are needed to lower the pH from the coffee mass and favor the yeasts growth. Yeast are mainly responsible for producing pectnolytics enzymes, volatiles precursors of high quality coffees, and inhibiting undesired microogasnisms.

Figure 5 - **A.** Aerobic fermentation: spontaneous fermentation or with starters (spray inoculation method: microbial solution sprayed on coffee fruits) in suspended platforms or terraces. **B.** Semi-anaerobic fermentation: spontaneous fermentation or with starters in open recipients or bioreactors with/without water. **C.** Anaerobic fermentation: spontaneous fermentation or with starters in close recipients and bioreactors (SIAF method: anaerobic fermentation induced by microorganisms which generate CO_2).

Source: From the author (2021).

2.2.4.1.1 Aerobic fermentation: spray inoculation

Coffee can be aerobically fermented spontaneously or with microbial starters in suspended platforms or terraces. The second is called the spray inoculation method. This method consists of spraying a microbial solution on the coffee fruits or beans surface placed on suspended platforms (FIGURE 5A). The method was implemented firstly by Evangelista *et al*. (2014b). Depending on the microbial starter can either favor bacteria or yeast populations, as observed in Martinez *et al*. (2017) and Evangelista *et al*. (2014b). Moreover inoculation with yeast increases the sensory notes and generate differentiated flavors, for example in Evangelista *et al*. (2014b) spontaneous fermentations exihibited bitterness, acidity, and chocolates attributes while with yeast starters beverages exihibited caramel and a more intense chocolate attribute during tasting.

Bacteria

A list of bacteria have been identified in aerobic fermentations: *Acetobacter pasteurianus*, *Acinetobacter* sp., *Acinetobacter* spp., *Aeromonas*, *Arthrobacter*, *Arthrobacter* sp., *Bacillus anthracis*, *Bacillus cereus*, *Bacillus laterosporus*, *Bacillus macerans*, *Bacillus megaterium*, *Bacillus polymyxa*, *Bacillus* sp., *Bacillus stearothermophilus*, *Bacillus subtilis*, *Brochothrix*, *Chromobacter violaceum*, *Chryseobacterium*, *Citrobacter freundi*, *Citrobacter* sp., *Dermabacter*, *Enterobacter*, *Enterobacter aerogenes*, *Enterobacter agglomerans*, *Enterobacter cloacae*, *Enterobacter gergoviae*, *Enterobacter sakazakii*, *Enterobacteriaceae*, *Erwinia herbicola*, *Escherichia coli*, *Flavobacterium odoratum*, *Gluconobacter cerevisiae*, *Gluconobacter cerinus*, *Gluconobacter frateurii*, *Gluconobacter oxydans*, *Hafnia alvei*, *Klebsiella oxytoca*, *Klebsiella ozaenae*, *Klebsiella pneumoniae*, *Kozakia baliensis*, *Lactobacillus*, *Lactobacillus brevis*, *Lactobacillus hordei*, *Lactobacillus mali*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Microbacterium*, *Pantoea* sp., *Pasteurella haemolytica*, *Pseudomonas*, *Pseudomonas aeruginosa*, *Pseudomonas cepacian*, *Pseudomonas fluorescens*, *Pseudomonas paucimobilis*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas vesicularis*, *Ralstonia* sp., *Salmonella choleraesuis*, *Salmonella enterica*, *Salmonella paratyphi*, *Serratia liquefaciens*, *Serratia marcescens*, *Serratia plymuthica*, *Serratia* sp., *Shigella dysenteriae*, *Tatumella ptyseos*, *Weissella*, and *Yersinia* sp., *Erwinia billingiae*, *Pantoea agglomerans*, *Pantoea brenneri*, *Pantoea dispersa*, Pantoea eucrina (DE BRUYN *et al*., 2017; EVANGELISTA *et al*., 2014b; HAMDOUCHE *et al*., 2016; LEONG *et al*., 2014; SILVA *et al*., 2000, 2008; VILELA *et al*., 2010).

Fungi

Some fungi include: *Arthrobotrys* spp., *Arxula adeninivorans*, *Arxula* sp., *Aspergillus*, *Aspergillus chevalieri*, *Aspergillus flavus*, *Aspergillus foetidius*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus ochraceus*, *Aspergillus* sp., *Aspergillus sydowii*, *Aspergillus tamarii*, *Aspergillus tubingensis*, *Aspergillus versicolor*, *Blastobotrys proliferans*, *Candida auringiensis*, *Candida carpophila*, *Candida ernobii*, *Candida fermentati*, *Candida fukuyamaensis*, *Candida glucosophila*, *Candida incommunis*, *Candida membranifaciens*, *Candida paludigena*, *Candida quercitrusa*, *Candida saitoana*, *Candida schatarii*, *Candida vartiovaarae*, *Citeromyces matritensis*, *Cladosporium*, *Cladosporium cladosporioides*, *Cladosporium macrocarpum*, *Cladosporium* sp., *Cylindrocarpon* sp., *Debaryomyces hansenii*, *Debaryomyces polymorphus*, *Eurotium chevalieri*, *Fusariella* sp., *Fusarium*, *Fusarium chlamydosporum*, *Fusarium lateritium*, *Fusarium nivale*, *Fusarium semitectum*, *Fusarium solani*, *Fusarium* sp., *Fusarium sporotrichioides*, *Fusarium stilboides*, *Geotrichum fermentans*, *Geotrichum* sp., *Hanseniaspora uvarum*, *Kloeckera* sp., *Kluyveromyces* sp., *Meyerozyma caribbica*, *Monilia* spp., *Mucor hiemalis*, *Penicillium*, *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Penicillium citrinum*, *Penicillium commune*, *Penicillium corylophilum*, *Penicillium crustosum*, *Penicillium decumbens*, *Penicillium fellutanum*, *Penicillium implicatum*, *Penicillium restrictum*, *Penicillium roqueforti*, *Penicillium solitum*, *Penicillium* sp., *Phoma* sp., *Pichia acacia*, *Pichia anomala*, *Pichia burtonii*, *Pichia caribbica*, *Pichia ciferii*, *Pichia fermentans*, *Pichia guilliermondii*, *Pichia jadinii*, *Pichia kluyveri*, *Pichia lynferdii*, *Pichia ofunaensis*, *Pichia subpelliculosa*, *Pichia sydowiorium*, *Rhizoctoni*a spp., *Rhodotorula mucilaginosa*, *Saccharomyces bayanus*, *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Saccharomycopsis crataegensis*, *Saccharomycopsis fermentans*, *Saccharomycopsis fibuligera*, *Schizosaccharomyces pombe*, *Sordariomycetes* sp., *Sporopachydermia cereana*, *Starmerella bacillaris*, *Stephanoascus smithiae*, *Sugiyamaella smithiae*, *Torulaspora delbrueckii*, *Trichosporonoides oedocephales*, and *Williopsis saturnus sargentensis* (DE BRUYN *et al*., 2017; EVANGELISTA *et al*., 2014b; HAMDOUCHE *et al*., 2016; SILVA *et al*., 2000, 2008; VILELA *et al*., 2010).

2.2.4.1.2 Semi-anaerobic fermentation or open batch fermentation

This method consists of fermenting coffee in open recipients-bioreactors with or without using microbial suspensions (FIGURE 5B). It was first done by Martinez *et al*. (2017) in coffee processed via semidry and Bressani *et al*. (2018) in coffee processed via dry. Favors semi-anaerobic conditions and allows a constant microbial dynamic. Inoculation of the starter solution is added in fruits or beans while coffee is being placed inside the reipients or bioreactors and left for fermentation. Also fermentations can be carried with (wet method) or without water.

Bacteria

The following bacteria have been identified in semi-anaerobic fermentations: *Acetobacter*, *Acetobacter cerevisiae*, *Acetobacter cibinogensis*, *Acetobacter fabarum*, *Acetobacter ghanensis*, *Acetobacter indonesiensis*, *Acetobacter lambici*, *Acetobacter malorum*, *Acetobacter okinawensis*, *Acetobacter orientalis*, *Acetobacter papaya*, *Acetobacter pasteurianus*, *Acetobacter persici*, *Acetobacter senegalensis*, *Acetobacter thailandicus*, *Acetobacteraceae*, *Acinetobacter*, *Acinetobacter lwoffii*, *Acinetobacter schindleri*, *Actinobacterium* sp., *Actinomycetaceae*, *Aeromonas*, *Aeromonas schubertii*, *Alcaligenaceae*, *Alteromonadaceae*, *Amycolatopsis orientalis*, *Anabaena*, *Arthrobacter gandavensis*, *Arthrobacter koreensis*, *Arthrobacter luteolus*, *Asaia* sp., *Bacillaceae*, *Bacillus*, *Bacillus amyloliquefaciens*, *Bacillus asahii*, *Bacillus cereus*, *Bacillus clausii*, *Bacillus humi*, *Bacillus licheniformis*, *Bacillus safensis*, *Bacillus simplex*, *Bacillus* sp., *Bacillus subtilis*, *Bacteroidaceae*, *Bdellovibrionaceae*, *Beijerinckiaceae*, *Beutenbergiaceae*, *Bifidobacteriaceae*, *Blattabacteriaceae*, *Bradyrhizobiaceae*, *Brevibacillus parabrevis*, *Brevibacteriaceae*, *Brucellaceae*, *Burkhoolderiaceae*, *Campylobacteraceae*, *Cardiobacteriaceae*, *Caulobacteraceae*, *Cellulomonadaceae*, *Cellulosimicrobium cellulans*, *Cellulosimicrobium funkei*, *Chitinophagaceae*, *Chryseobacterium taichungense*, *Chrysomonas luteola*, *Chthoniobacteraceae*, *Citrobacter*, *Citrobacter freundii*, *Citrobacter koseri*, *Clostridiaceae*, *Comamonadaceae*, *Corynebacteriaceae*, *Corynebacterium bovis*, *Cronobacter muytjensii*, *Curtobacterium* sp., *Cytophagaceae*, *Deinococcaceae*, *Dermabacteraceae*, *Dyella kyungheensis*, *Enterobacter*, *Enterobacter agglomerans*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter dissolvens*, *Enterobacter kobei*, *Enterobacter lignolyticus*, *Enterobacter ludwigii*, *Enterobacter massiliensis*, *Enterobacter mori*, *Enterobacter* sp., *Enterobacteriaceae*,

Enterobacteriaceae bacterium, *Enterococcaceae*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus hirae*, *Enterococcus raffinosus*, *Enterococcus* sp., *Erwinia*, *Erwinia herbícola*, *Erwinia persicina*, *Erwinia soli*, *Escherichia coli*, *Escherichia hermannii*, *Fimbriimonadaceae*, *Flavobacteriaceae*, *Flavobacterium*, *Flavobacterium odoratum*, *Flavobacterium* sp., *Frateuria aurantia*, *Fusobacteriaceae*, *Gemmataceae*, *Gemmatimonadaceae*, *Geodermatophilaceae*, *Gluconobacter albidus*, *Gluconobacter cerevisiae*, *Gluconobacter cerinus*, *Gluconobacter frateurii*, *Gluconobacter japonicus*, *Gluconobacter kondonii*, *Gluconobacter nephelii*, *Gluconobacter oxydans*, *Hafnia*, *Hyphomicrobiaceae*, *Intrasporangiaceae*, *Kineosporiacea*, *Klebsiella*, *Klebsiella oxytoca*, *Klebsiella ozaenae*, *Klebsiella pneumoniae*, *Kocuria* sp., *Kozakia*, *Lachnospiraceae*, *Lactobacillaceae*, *Lactobacillus*, *Lactobacillus lactis*, *Lactobacillus plantarum*, *Lactobacillus bifermentans*, *Lactobacillus brevis*, *Lactobacillus coryniformis*, *Lactobacillus fabifermentans*, *Lactobacillus fermentum*, *Lactobacillus futsaii*, *Lactobacillus helveticus*, *Lactobacillus hordei*, *Lactobacillus lactis subsp hordniae*, *Lactobacillus lactis subsp. Lactis*, *Lactobacillus manihotivorans*, *Lactobacillus parafarraginis*, *Lactobacillus paraplantarum*, *Lactobacillus plantarum*, *Lactobacillus satsumensis*, *Lactobacillus siliginis*, *Lactobacillus similis*, *Lactobacillus* sp., *Lactobacillus suebicus*, *Lactobacillus vaccinostercus*, *Lactobacillus xiangfangensis*, *Lactococcus*, *Lactococcus hircilactis*, *Lactococcus lactis*, *Lactococcus lactis subsp. Lactis*, *Legionellaceae*, *Leminorella grimontii*, *Leuconostoc citreum*, *Leuconostoc fallaxm*, *Leuconostoc gelidum subsp gasicomitatum*, *Leuconostoc holzapfelii*, *Leuconostoc inhar*, *Leuconostoc kimchii*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Leuconostoc mesenteroides dextranicum*, *Leuconostoc mesenteroides sobsp. Cremoris*, *Leuconostoc pseudomesenteroides*, *Leuconostoc* sp., *Leuconostocaceae*, *Lysinibacillus fusiformis*, *Lysinibacillus macroides*, *Methylobacteria radiotolerans*, *Methylobacteriaceae*, *Methylocystaceae*, *Microbacteriaceae*, *Microbacterium laevaniformans*, *Microbacterium paraoxydans*, *Microbacterium* sp., *Microbacterium testaceum*, *Micrococcaceae*, *Micrococcus*, *Micromonsporaceae*, *Moraxellaceae*, *Mycobacteriaceae*, *Nannocystaceae*, *Neisseriaceae*, *Nitrospiraceae*, *Nocardiaceae*, *Nocardioidaceae*, *Ochrobactrum pseudogrignonense*, *Ochrobactrum rhizosphaerae*, *Oenococcus oeni*, *Oeonococcus alcoholitolerans*, *Oeonococcus kitahare*, *Oxalobacteraceae*, *Paenibacillaceae*, *Paenibacillus amylolyticus*, *Paenibacillus cookii*, *Paenibacillus konsidensis*, *Paenibacillus lactis*, *Pantoea agglomerans*, *Pantoea dispersa*, *Pantoea rodasii*, *Pantoea rwandensis*, *Pasteurellaceae*, *Patulibacteraceae*, *Pectobacterium carotovorum actinidae*, *Pectobacterium carotovorum carotovorum*, *Pectobacterium carotovorum odoriferum*, *Pectobacterium carotovorum brasiliense*, *Pediococcus pentosaceus*, *Phyllobacteriaceae*, *Planctomycetaceae*, *Porphyromonadaceae*, *Prevotellaceae*, *Proteus*, *Proteus penner*, *Pseud monteilii*, *Pseudomonas*, *Pseudomonas cepaciae*, *Pseudomonas delafieldii*, *Pseudomonas entomophila*, *Pseudomonas fluorescens*, *Pseudomonas* sp., *Pseudonocardiaceae*, *Ralstonia* sp., *Raoultella planticola*, *Rhizobiaceae*, *Rhizobium pusense*, *Rhodobacteraceae*, *Rhodococcus pyridinivorans*, *Rhodococcus rhodochrous*, *Rhodospirillaceae*, *Rickettsiaceae*, *Roseateles aquatilis*, *Ruminococcaceae*, *Salmonella entérica*, *Samonella* sp., *Serratia*, *Serratia marcescens*, *Sinobacteraceae*, *Sphingobacteriaceae*, *Sphingomonadaceae*, *Staphylococcus*, *Staphylococcus warneri*, *Staplylococcus aureus*, *Stenotrophomonas maltophilia*, *Streptococcaceae*, *Streptococcus*, *Streptococcus faecalis*, *Streptococcus macedonicus*, *Streptomycetaceae*, *Tatumella morbirosei*, *Tatumella ptyseos*, *Trueperaceae*, *Turicibacteraceae*, *Weeksellaceae*, *Weissella*, *Weissella cibaria*, *Weissella confusa*, *Weissella hellenica*, *Weissella paramensenteoides*, *Weissella soli*, *Williamsiaceae*, *Xanthobacteraceae*, *Xanthomonadaceae*, and *Yersinia mollaretii* (AVALLONE *et al*., 2001; DE BRUYN *et al*., 2017; ELHALIS; COX; ZHAO, 2020; EVANGELISTA *et al*., 2015; HAMDOUCHE *et al*., 2016; HATININGSIH *et al*., 2018; JUNQUEIRA *et al*., 2019; NASANIT; SATAYAWUT, 2015; POTHAKOS *et al*., 2020; PUERTA-QUINTERO; MEJIA; BETANCUR, 2012; RIBEIRO *et al*., 2018; VELMOUROUGANE, 2013; ZHANG *et al*., 2019a, 2019b).

Fungi

The following fungi have been identified in previous semi-anaerobic fermentations: *Acremonium*, *Aspergillus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus* sp., *Aspergillus tamarii*, *Aspergillus terréus*, *Bensingtoni*, *Candida*, *Candida albicans*, *Candida ethanolica*, *Candida glabrata*, *Candida guilliermondii*, *Candida humilis*, *Candida krusei*, *Candida orthopsilosis*, *Candida pseudointermedia*, *Candida qinlingensis*, *Candida quercitrusa*, *Candida railenensis*, *Candida solani*, *Candida* sp., *Candida tropicalis*, *Candida vanderwaltii*, *Candida xylopsoci*, *Cladosporium*, *Cladosporium* sp., *Cladosporium sphaerospermum*, *Classiculaceae*, *Cordyceps brongniartii*, *Cryptocccus albidus*, *Cryptococcus laurentii*, *Cryptococcus terreus*, *Debaromyces hansenii*, *Dipodascus tetrasporeus*, *Fusarium*, *Fusarium* sp., *Hanseniaspora* *opuntiae*, *Hanseniaspora uvarum*, *Hanseniaspora vineae*, *Issatchenkia orientalis*, *Kazachstania exigua*, *Kloeckera apis apiculate*, *Kluyveromyces marxianus*, *Kluyveromyces* sp., *Lachancea lanzarotensis*, *Leucospridiella*, *Lobulomycetales*, *Malassezia* sp., *Martiniozyma asiática*, *Meyerozyma caribbica*, *Mitchella repens*, *Mucor* sp., *Neodevriesia*, *Papiliotrema flavescens*, *Papiliotrema terrestres*, *Penicillium*, *Penicillium* sp., *Physciceae*, *Pichia*, *Pichia anomala*, *Pichia caribbica*, *Pichia fermentans*, *Pichia guilliermondii*, *Pichia kluyveri*, *Pichia kudriavzevii*, *Pichia nakasei*, *Pichia ohmeri*, *Pichia* sp., *Pleosporales*, *Rhizopus* sp., *Rhodotorula*, *Rhodotorula mucilaginosa*, *Rhodotorula* spp., *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Saccharomycetes*, *Saccharomycopsis crataegensis*, *Saccharomycopsis fibuligera*, *Saccharoycopsidaceae*, *Schwanniomyces* sp., *Shizosaccharomyces* sp., *Sordariomycetes* sp., *Sporidiobolus*, *Starmerella*, *Starmerella bacillaris*, *Torulaspora delbrueckii*, *Torulopsis pintolopessi*, *Tremellaceae*, *Trichoderma*, *Vishniacozyma*, *Wickerhamomyces anomalus*, and *Wickerhamomyces ciferrii* (AVALLONE *et al*., 2001; DE BRUYN *et al*., 2017; DE MELO PEREIRA *et al*., 2014; ELHALIS; COX; ZHAO, 2020; EVANGELISTA *et al*., 2015; HAILE; KANG, 2019; HAMDOUCHE *et al*., 2016; HATININGSIH *et al*., 2018; JUNQUEIRA *et al*., 2019; MASOUD *et al*., 2004; NASANIT; SATAYAWUT, 2015; POTHAKOS *et al*., 2020; PUERTA-QUINTERO; MEJIA; BETANCUR, 2012; VELMOUROUGANE, 2013; ZHANG *et al*., 2019a, 2019b).

2.2.4.1.3 Anaerobic fermentation or close batch: SIAF

This method is also considered a close batch method consisting of fermenting coffee in close recipients-bioreactors with or without microbial suspensions. It favors anaerobic conditions, yeast activity, and CO_2 release. It was first introduced by Martins *et al*. (2020) and Da Mota *et al*. (2020) in coffees processed via dry and semidry.

Bacteria

Some bacteria identified along this process include *Acetobacter*, *Acinetobacter pittii*, *Acinetobacter radioresistens*, *Actinomycetospora*, *Actinoplanes*, *Agrobacterium tumefaciens*, *Arthrobacter*, *Arthrobacter sulfonivorans*, *Aureimonas*, *Beijerinckia*, *Brevundimonas*, *Cellulosimicrobium cellulans*, *Clavibacter*, *Corynebacterium*, *Curtobacterium*, *Curtobacterium* *flaccumfaciens*, *Devosia*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Erwinia persicina*, *Fimbriimonas*, *Fructobacillus*, *Geodermatophilus*, *Gluconobacter*, *Gluconobacter cerinus*, *Hartmannibacter*, *Lactococcus lactis*, *Leclercia*, *Leuconostoc*, *Leuconostoc mesenteroides*, *Lysinimonas soli*, *Methylobacterium*, *Microbacterium foliorum*, *Microbacterium testaceum*, *Micrococcus lactis*, *Micrococcus luteus*, *Moraxella osloensis*, *Nakamurella*, *Neorhizobium*, *Novosphingobium*, *Pantoea agglomerans*, *Phenylobacterium*, *Pirellula*, *Pluralibacter*, *Pseudomonas extremaustralis*, *Pseudomonas oryzihabitans*, *Pseudonocardia*, *Rhizobium*, *Rhizorhabdus*, *Roseomonas*, *Serratia marcescens*, *Sphingomonas*, *Sphingomonas desiccabilis*, *Staphylococcus epidermidis*, *Tatumella terrea*, *Weissella*, *Weissella paramesenteroides*, and *Xanthomonas oryzae* (MARTINEZ *et al*., 2021b; MARTINS *et al*., 2020).

Fungi

The following fungi have been previously identified in anaerobic fermentations: *Acremonium furcatum*, *Acremonium hennebertii*, *Acrocalymma fici*, *Acrocalymma walkeri*, *Alfaria terrestres*, *Alternaria argyroxiphii*, *Antennariella placitae*, *Apiotrichum laibachii*, *Aplosporella yalgorensis*, *Articulospora proliferata*, *Aspergillus westerdijkiae*, *Aureobasidium pullulans*, *Bannoa ogasawarensis*, *Barnettozyma californica*, *Biatriospora mackinnonii*, *Blastobotrys buckinghamii*, *Boeremia exigua*, *Botrytis caroliniana*, *Brachyphoris oviparasitica*, *Bulleromyces albus*, *Candida blattae*, *Candida orthopsilosis*, *Candida parapsilosis*, *Candida quercitrusa*, *Candida railenensis*, *Candida sake*, *Candida saopaulonensis*, *Candida tropicalis*, *Capitofimbria compacta*, *Capnodium coffeae*, *Catenulostroma hermanusense*, *Citeromyces matritensis*, *Cladosporium aphidis*, *Cladosporium delicatulum*, *Cladosporium dominicanum*, *Cladosporium flabelliforme*, *Cladosporium halotolerans*, *Cladosporium sphaerospermum*, *Claviceps maximensis*, *Clavispora lusitaniae*, *Clonostachys compactiuscula*, *Clonostachys miodochialis*, *Clonostachys rosea*, *Clonostachys wenpingii*, *Colletotrichum annellatum*, *Colletotrichum lupini*, *Colletotrichum theobromicola*, *Coniothyrium sidae*, *Cryptococcus dimennae*, *Cryptococcus flavescens*, *Cryptococcus randhawai*, *Cryptococcus saitoi*, *Curvibasidium cygneicollum*, *Curvularia americana*, *Cutaneotrichosporon jirovecii*, *Cutaneotrichosporon moniliiforme*, *Cutaneotrichosporon terricola*, *Cyberlindnera fabianii*, *Cyphellophora eucalypti*, *Cyphellophora europaea*, *Cyphellophora fusarioides*, *Cyphellophora laciniata*, *Cyphellophora vermisporam*, *Cystobasidium oligophagum*, *Cystofilobasidium* *alribaticum*, *Cystofilobasidium capitatum*, *Cystofilobasidium ferigula*, *Cystofilobasidium infirmominiatum*, *Cystofilobasidium intermedium*, *Debaryomyces hansenii*, *Debaryomyces nepalensis*, *Deltopyxis triangulispora*, *Derxomyces anomalus*, *Didymella calidophila*, *Didymella coffeae-arabicae*, *Didymella nigricans*, *Dimennazyma cistialbidi*, *Dioszegia var. yunnanensis*, *Diutina catenulata*, *Epicoccum draconis*, *Epicoccum nigrum*, *Erythrobasidium hasegawianum*, *Eupenidiella venezuelensis*, *Euteratosphaeria verrucosiafricana*, *Exophiala castellanii*, *Exophiala phaeomuriformis*, *Exophiala salmonis*, *Fellomyces borneensis*, *Fellomyces mexicanus*, *Filobasidium chernovii*, *Filobasidium floriforme*, *Fusarium acutatum*, *Fusarium asiaticum*, *Fusarium delphinoides*, *Fusarium penzigii*, *Fusarium proliferatum*, *Fusarium solani*, *Gibberella intricans*, *Hannaella kunmingensis*, *Hannaella luteola*, *Hannaella oryzae*, *Hannaella siamensis*, *Hannaella sinensis*, *Hannaella zeae*, *Hanseniaspora uvarum*, *Hansfordia pulvinate*, *Holtermanniella wattica*, *Kazachstania exigua*, *Kazachstania gamospora*, *Knufia tsunedae*, *Kodamaea ohmeri*, *Lactarius saponaceus*, *Lecanicillium antillanum*, *Lectera colletotrichoides*, *Leptoxyphium madagascariense*, *Lodderomyces elongisporus*, *Lodderomyces elongisporus*, *Lophiotrema rubi*, *Macroventuria anomochaeta*, *Meyerozyma caribbica*, *Meyerozyma guilliermondii*, *Mortierella ambigua*, *Musicillium theobromae*, *Mycosphaerella ellipsoidea*, *Myrmaecium fulvopruinatum*, *Myxospora aptrootii*, *Naganishia albida*, *Naganishia diffluens*, *Naganishia randhawae*, *Nakazawaea holstii*, *Nectria balansae*, *Neoascochyta paspali*, *Neodevriesia modesta*, *Neonectria major*, *Nigrospora oryzae*, *Occultifur externus*, *Papiliotrema flavescens*, *Papiliotrema laurentii*, *Papiliotrema perniciosus*, *Paraconiothyrium archidendri*, *Paraconiothyrium fungicola*, *Paraconiothyrium variabile*, *Penicillium kongii*, *Penicillium solitum*, *Peniophora albobadia*, *Peniophora laxitexta*, *Periconia byssoides*, *Periconia cookie*, *Periconia macrospinosa*, *Phacidiella eucalypti*, *Phaeosphaeria caricis*, *Phaeosphaeria podocarpi*, *Phialemoniopsis ocularis*, *Phoma omnivirens*, *Pichia kluyveri*, *Pilidium concavum*, *Plectosphaerella cucumerina*, *Pleurotus pulmonarius*, *Polyporus tricholoma*, *Psathyrella luteopallida*, *Pseudocercospora bixae*, *Pseudomerulius curtisii*, *Pseudophaeomoniella oleae*, *Pseudoplectania affinis*, *Pseudorobillarda phragmitis*, *Pseudoteratosphaeria ohnowa*, *Pyrenochaetopsis leptospora*, *Rachicladosporium cboliae*, *Rachicladosporium paucitum*, *Resinicium friabile*, *Rhodosporidiobolus fluvialis*, *Rhodosporidiobolus lusitaniae*, *Rhodosporidiobolus odoratus*, *Rhodosporidiobolus ruineniae*, *Rhodotorula araucariae*, *Rhodotorula babjevae*, *Rhodotorula dairenensis*, *Rhodotorula diobovata*, *Rhodotorula* *mucilaginosa*, *Rhodotorula taiwanensis*, *Rhynchogastrema complexa*, *Rhynchogastrema nanyangensis*, *Roussoella solani*, *Saccharomyces cerevisiae*, *Saitozyma flava*, *Saitozyma paraflava*, *Saitozyma podzolica*, *Sampaiozyma vanillica*, *Schizophyllum commune*, *Scolecobasidium terreum*, *Selenophoma mahoniae*, *Septoria create*, *Setophoma chromolaenae*, *Setophoma terrestris*, *Sirobasidium brefeldianum*, *Solicoccozyma terrea*, *Sphaeropsis citrigena*, *Sporobolomyces johnsonii*, *Sporobolomyces koalae*, *Strelitziana africana*, *Strelitziana eucalypti*, *Symmetrospora coprosmae*, *Symmetrospora vermiculata*, *Taphrina inositophila*, *Torulaspora delbrueckii*, *Toxicocladosporium irritans*, *Toxicocladosporium strelitziae*, *Trametes hirsuta*, *Trichomerium foliicola*, *Trichosporon asahii*, *Trichosporon coremiiforme*, *Udeniomyces pyricola*, *Vishniacozyma dimennae*, *Vishniacozyma foliicola*, *Vishniacozyma heimaeyensis*, *Vishniacozyma taibaiensis*, *Vishniacozyma victoriae*, *Volutella consors*, *Wallemia hederae*, *Wickerhamomyces anomalus*, *Wickerhamomyces ciferriim*, *Wickerhamomyces lynferdii*, *Wickerhamomyces pijperi*, *Wickerhamomyces sydowiorum*, *Wickerhamomyces xylosica*, *Xeromyces bisporus*, and *Zymoseptoria verkleyi* (MARTINEZ *et al*., 2021b; MARTINS *et al*., 2020).

2.3 Biochemical compounds

Compounds in coffee are produced along with the fermentation and roasting phase, most through Maillard reactions, Strecker degradation, breakdown of amino acids, degradation of trigonelline, quinic acid, pigments, lipids, and interaction between intermediate products (SUNARHARUM; WILLIAMS; SMYTH, 2014). Other compounds take part naturally in fruits; these are proteins, sugars, tannins, nitrogen compounds, pectin's, caffeine, and chlorogenic acids. Differences between compounds during fermentation and after roasting will determine the perceived attributes during tasting. Volatile or non-volatile compounds are identified by gas chromatography-mass spectrometry analysis (GC–MS) and high-performance liquid chromatography (HPLC).

More detailed chemical composition of fruits, pulp, and mucilage are shown in Table 1.

Table 1 - Chemical composition of pulp and mucilage from coffee fruits before the fermentation process.

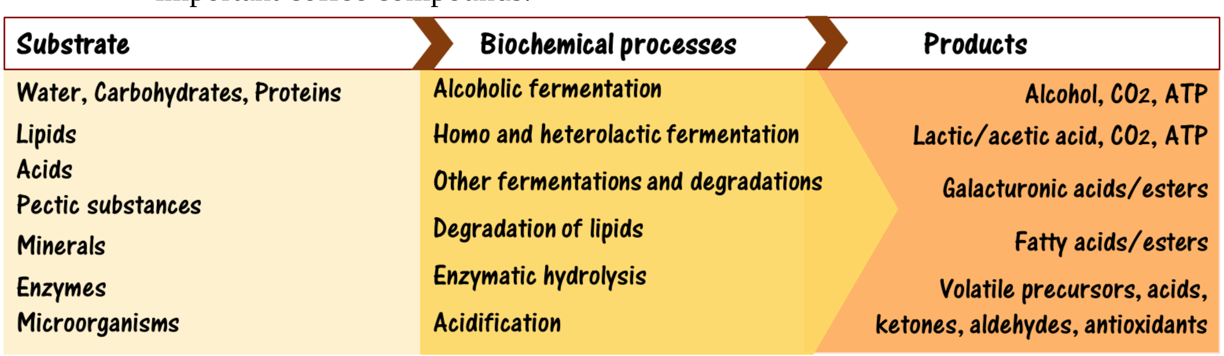
	Components (% Dry weight)							
	Protein	**Nitrogen free extract**	**Tannins**	**Total pectin substance**	**Reducing sugar**	**Non-reducing sugar**	**Caffeine**	**Chlorogenic acid**
Pulp	10.0	44.0	1.8-8.6	6.5	12.4	2.0	1.3	2.6
Mucilage	8.9	35.8	0	35.8	30.0	20.0	0	0
Fruit	7.5	1.2-8	7.7	0.88	0.05-0.20	8.0	0.73	6-7

Source: Adapted from Silva (2015).

2.3.1 During fermentation

Microorganisms in the fermentation stage can either contribute positively or negatively to coffee flavor and aroma. The positive contribution confers additional flavor notes due to compounds produced by fermentation. For example, free sugars and amino acid concentration are determined by the microbial activity and extent of fermentation. While coffee is fermented, mucilage and water content are reduced, facilitating drying. Drying increases glucose and fructose contents in fruits or beans. Subsequently, coffee beans obtain by fermentation have higher volatiles concentration such as alcohols, acids, esters, aldehydes, and ketones that resulted in more pleasant coffee aromas (SILVA, 2015). A summarized comprehension of the biochemical processes happening during fermentation and the resulted products is illustrated in Figure 6.

Figure 6 - Available substrates in a coffee induced several biochemical reactions resulting in important coffee compounds.



Source: Adapted from Puerta-Quintero and Molina (2015).

Although specific volatile compounds change along the fermentative process, some chemical groups dominate this phase, and others dominate after roasting. Table 2 illustrates the volatile chemical groups present in coffee and those that are the highest after natural (control) and inoculated fermentations. Independent of inoculation, the dominant groups are acids, alcohols, aldehydes, and hydrocarbons (only for Ouro Amarelo Variety).

Table 2 - Variations within the different chemical volatile groups found in several studies during fermentation.

Variety	Treatment	Ac	Alc	Ald	Hyd	Ket	Est	Phe	Fur	Pyre	Pyrs	Pyro	Terp	Lact	Reference
Acaiá	Control	+	+				+								EVANGELISTA et al. (2015) Wet process
Catuaí Amarelo	Control	+	+	+											MARTINEZ et al. (2017) Pulped natural process
	CCMA 0543	+	+	+											
	CCMA 0544	+	+	+											
	CCMA 0684	+	+	+											
Ouro Amarelo	Control	+	+	+	+										RIBEIRO et al. (2017) Pulped natural process
	CCMA 0543	+	+	+	+										
	CCMA 0200	+	+	+	+										
Mundo Novo	Control	+	+	+											
	CCMA 0543	+	+	+											
	CCMA 0200	+	+	+											
Ouro Amarelo	Control		+	+	+										RIBEIRO et al. (2018) Wet process
Mundo Novo	Control		+	+	+										
Catuaí Vermelho	Control		+	+	+										
Bourbon Amarelo	Control	+	+	+											BRESSANI et al. (2020) Natural and pulped natural process
	CCMA 0543	+	+	+											
	CCMA 0198	+	+	+											
	CCMA 0544	+	+	+											
	CCMA 0684	+		+											
Canário Amarelo	Control	+	+	+											
	CCMA 0543	+	+	+											
	CCMA 0198	+	+	+											
	CCMA 0544	+	+	+											
	CCMA 0684	+	+	+											

The filled spaces with color indicate the chemical volatiles groups detected in each study. + Indicates the most representative volatile chemical groups out of the total chemical groups detected. Ac: Acids, Alc: Alcohols, Ald: Aldehydes, Hyd: Hydrocarbons, Ket: Ketones, Est: Esters, Phe: Phenols, Fur: Furans, Pyre: Pyrazines, Pyrs: Pyrans, Pyro: Pyrones, Terp: Terpenes, and Lact: Lactones. Yeasts: CCMA 0200 and CCMA 0543: *Saccharomyces cerevisiae*, CCMA 0544: *Candida parapsilosis*, CCMA 0684: *Torulaspora delbrueckii*, and CCMA 0198: *Meyerozyma caribbica*.

Source: From the author (2021).

2.3.2 Roasting

Roasting is a process that involves beans exposure to high temperatures above 300 ºC (TOLEDO *et al*., 2016). During this process, the main volatile products of the Maillard reactions are pyridines, pyrazines, furans, and pyrroles, from the Strecker reactions include aldehydes, ammonia, and carbon dioxide, and from pyrolysis are phenolic derivates (TOLEDO *et al*., 2016).

According to Sunarharum, Williams and Smyth (2014), among all the groups found in roasted coffee, furans are the most abundant group. They are produced through thermal degradation and exhibit caramel-like, malty, and sweet roasted aromas. Pyrazines is another group that increases with roasting, and they are essential for flavor since they exhibit nutty, earthy, roasty, and green aromas. Another group released during roasting is phenols, particularly guaiacol, 4-ethyl guaiacol, 4-vinylguaiacol, and vanillin. Moreover, the thermal degradation of trigonelline produces pyridines.

Important compounds detected in roasted coffee are described in Table 3.

Table 3 - List of potent compounds detected in roasted coffee (Continued).

Compound	Sensory descriptor
Acids	
2-Methyl-1-butanoic acid	Sweaty, acidic
3-Methyl-1-butanoic acid	Sweaty, acidic
Acetic acid	Pungent, sour
Furans	
2,5-Dimethylfuran	Coffee
2-Vinyl-5-methylfuran	Coffee
5-Methylfurfural	Caramel
Furfuryl acetate	Nutty
2-Furfuryl methyl sulfide	Coffee
Furfural	Almond bitter
Furanones	
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	Caramelic
3-Hydroxy-4,5-dimethyl-2(5H)-furanone	Seasoning-like, spicy
5-Ethyl-3-hydroxy-4-methyl-2(5H)-furanone	Seasoning-like
Ketones	
2,3-Butanedione	Buttery, caramel-like
2,3-Pentanedione	Buttery, caramel-like
2-Hydroxy-3,4-dimethyl-2-cyclo-penten-1-one	Caramel-like
2-Ethyl-4-methyl-2,5-furanedione	Coffee
Phenols	
Phenol	astringent
Guaiacol	Phenolic, burnt
4-Ethylguaiacol	Spicy, flower
p-Vinylguaiacol	Spicy
Vanillin	Vanilla-like
Pyrazines	
3-Isopropyl-2-methoxypyrazine	Earthy, roasty
2-Ethyl-3,5-dimethyl pyrazine	Earthy, roasty
2,3-Diethyl-5-methylpyrazine	Earthy, roasty
3-Isobutyl-2-methoxypyrazine	Earthy

Table 3 - List of potent compounds detected in roasted coffee (Conclusion).

Compound	Sensory descriptor
Pyrazines	
Pyrazine	Coffee
2-Methylpyrazine	Toasted
2,5 Dimethylpyrazine	Toasted, roasty, nuts
2,6-Dimethylpyrazine	Nutty, sulfur like
2,3,5-Trimethyl pyrazine	Herbs, earthy, musty
Ethyl pyrazine	Toasted, caraway
Sulfur compounds	
Methional	Boiled potato-like
Bis(2-methyl-3-furyl)disulphide	Meat-like
Aldehydes	
2-Methylbutanal	Sweet
3-Methylbutanal	Sweet
3-Methylbutanal	Malty
Phenylacetaldehyde	Sweet, fruity
Esters	
Methyl acetate	Pleasant
Ethyl acetate	Fruity
Ethyl-2-butenoate	Floral
Ethyl-3-methylbutyrate	Fruity
Ethyl-2-methylbutyrate	Fruity
Miscellaneous	
Butyrolactone	Sweet
3-Methylthiophene	Roasty
(E)-β-damascenone	Floral, honey

Source: Adapted from Lee *et al*. (2015) and Hameed *et al*. (2018).

Several studies have demonstrated that the chemical groups present in roasted beans is more diverse as shown in Table 4. Independent of the coffee variety, process, and stater cultures implementation, the groups dominating include pyrazines, acids, ketones, pyrroles, furans, and pyridines.

Table 4 - Variations within the different chemical volatile groups in roasted beans found in several studies.

Variety	Treatment	Ac	Alc	Ald	Ar	Am	Amd	Fure	Hyd	Ket	Est	Phe	Fur	Fura	Pyre	Pyri	Pyrs	Pyro	Pyrr	Sulf	Terp	Thiz	Thip	Lact	Reference
Acaiá	Control	+	+										+												EVANGELISTA et al. (2015) Wet process
Catuaí Amarelo	Control									+					+	+									MARTINEZ et al. (2017) Pulped natural
	CCMA 0543									+					+	+									
	CCMA 0544									+					+	+									
	CCMA 0684									+					+	+									
Ouro Amarelo	Control									+					+				+						RIBEIRO et al. (2017) Pulped natural process
	CCMA 0543									+					+				+						
	CCMA 0200									+					+				+						
Mundo Novo	Control									+					+				+						
	CCMA 0543									+					+				+						
	CCMA 0200									+					+				+						
Ouro Amarelo	Control									+					+				+						RIBEIRO et al. (2018) Wet process
Mundo Novo	Control									+					+				+						
Catuaí Vermelho	Control									+					+				+						
Catuaí Vermelho	Control																								MARTINS et al. (2019) Wet process
	CCMA 0684																								
	CCMA 0200																								
Mundo Novo	Control																								
	CCMA 0684																								
	CCMA 0200																								
Bourbon Amarelo	Control	+													+										BRESSANI et al. (2020) Natural and pulped natural process
	CCMA 0543	+													+										
	CCMA 0198	+													+										
	CCMA 0544	+													+										
	CCMA 0684	+													+										
Canário Amarelo	Control	+													+										
	CCMA 0543	+													+										
	CCMA 0198	+													+										
	CCMA 0544	+													+										
	CCMA 0684	+													+										
Bourbon Amarelo	Control																								DA MOTA et al. (2020) Natural and pulped natural process
	CCMA 0543																								
	CCMA 0684																								
Catuaí Amarelo	Control																								
	CCMA 0543																								
	CCMA 0684																								
Rubi	Control																								
	CCMA 0543																								
	CCMA 0684																								
Catuaí Vermelho	Control	+											+		+										MARTINEZ et al. (2021) Natural and pulped natural process
	CCMA 0543	+											+		+										
	CCMA 0535	+											+		+										

The filled spaces with color indicate the chemical volatiles groups detected in each study. + Indicates the most representative volatile chemical groups out of the total chemical groups detected. Ac: acids, Alc: alcohols, Ald: aldehydes, Ar: aromatics, Am: amines, Amd: amides, Fure: furanones, Hyd: hydrocarbons, Ket: ketones, Est: esters, Phe: phenols, Fur: furans, Fura: furaldehydes, Pyre: pyrazines, Pyri: pyridines, Pyrs: pyrans, Pyro: pyrones, Pyrr: pyrroles, Sulf: sulfur compounds, Terp: terpenes, Thiz: thiazoles, Thip: thiopenes, and Lact: lactones. Yeasts: CCMA 0200 and CCMA 0543: *Saccharomyces cerevisiae*, CCMA 0544: *Candida parapsilosis*, CCMA 0684: *Torulaspora delbrueckii*, CCMA 0198: *Meyerozyma caribbica*, and CCMA 0535: *Saccharomyces cerevisiae*.

Source: From the author (2021).

2.4 New identification technique for microbial communities

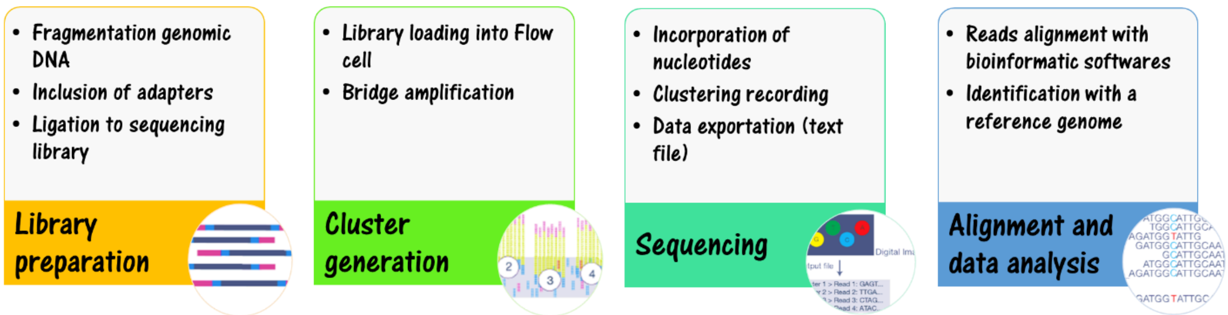
The use of culture-dependent and independent methods can give a complete scene of microbial diversity. Culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE), were developed to differentiate rRNA genes directly purified from complex microbial communities. DGGE had shown to be a good tool for monitoring microbial dynamics without cultivation, and PCR-amplified 26S rRNA genes from DGGE bands had provided a qualitative assessment of the yeast diversity in wine fermentation (COCOLIN; BISSON; MILLS, 2000). However, DGGE is not a technique used for quantifying. Therefore, for this purpose, quantitative real-time PCR (QPCR) is faster and advantageous because of its sensitivity (BATISTA *et al*., 2015). Nevertheless, there is a problem, this technique allows monitoring of one species at a time. Therefore, other techniques like next-generation sequencing allow whole-genome sequencing, amplicon sequencing, and identification of microbial diversity in a shorter time.

Sequencing took a revolution by the year 2005 due to the pyrosequencing technology release. The next-generation sequencing allows the generation of thousands to millions of short sequencing reads in a single machine run. Throughout time, other technologies have emerged, such as Solexa/Illumina sequencers (VINCENT *et al*., 2017).

For Illumina sequencing, first, a library must be prepared; this is by fragmentation of DNA sample and ligation of specialized adapters to both fragments ends. Then, the library is loaded into a flow cell, and the hybridization of fragments to the flow cell surface occurs. Each bound fragment is amplified through bridge amplification. Sequencing reagent and nucleotides label fluorescently are incorporated, and imaging of flow cell and clustering is recorded. After, reads are aligned to a reference sequence with bioinformatics software. The differences between the reference genome and the newly sequenced reads are identified (FIGURE 7).

This technology has become an aid to identify the diversity of bacteria in coffee fermentation, just as in Neto *et al*. (2018), which had a presence of over eighty bacterial genera, many of which have been detected for the first time during coffee bean fermentation, including *Fructobacillus*, *Pseudonocardia*, *Pedobacter*, *Sphingomonas*, and *Hymenobacter*.

Figure 7 - Next-Generation Sequencing Overview – four steps of Illumina sequencing: library preparation, cluster generation, sequencing, and alignment, and data analysis. Source: Illumina.



Source: From the author (2021).

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SECOND PART – ARTICLES

ARTICLE 1 - THE ALTITUDE OF COFFEE CULTIVATION CAUSES SHIFTS IN THE MICROBIAL COMMUNITY ASSEMBLY AND BIOCHEMICAL COMPOUNDS IN NATURAL INDUCED ANAEROBIC FERMENTATIONS

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Abstract

Coffee harvested in the Caparaó region (Minas Gerais, Brazil) is associated withhigh-quality coffee beans resulting in high-quality beverages. We characterize, microbiologically and chemically, fermented coffees from different altitudes through target NGS, chromatography, and conventional chemical assays. The genera *Gluconobacter* and *Weissella* were dominant in coffee’s fruits from altitudes 800 and 1,000 m. Among the Eukaryotic community, yeasts were the most dominant in all altitudes. The most dominant fungal genus was *Cystofilobasidium*, which inhabits cold environments and resists low temperatures. The content of acetic acid was higher at altitudes 1,200 and 1,400 m. Lactic acid and the genus *Leuconostoc* (Pearson: 0.93) were positively correlated. The relative concentration of volatile alcohols, especially of 2-heptanol, was high at all altitudes. Bacteria population was higher in coffees from 800 m, while at 1,000 m, fungi richness was favored. The altitude is an important variable that caused shifts in the microbial community and biochemical compounds content, even in coffees belonging to the same variety and cultivated in the same region under SIAF (self-induced anaerobic fermentation) conditions. Coffee from lower altitudes has higher volatile alcohols content, while high altitudes have esters, aldehydes, and total phenolics contents.

Keywords: Target sequencing, Caparáo region, microbial community, altitude, coffee fermentation

1. Introduction

The Caparaó is a region located in a mountainous territory shared by two Brazilian states, Minas Gerais and Espírito Santo (Assis et al., 2017; Campanha et al., 2017), and known for producing high-quality coffees. The coffee plants are owned by family farms grown at different altitudes and microclimates (Campanha et al., 2017). Ninety percent of the Caparaó region's production is of *Coffea arabica*, representing 40% of total production in the Espírito Santo state (Santos et al., 2017), and 75% of them are processed by the natural method (Paschoa et al., 2017).

Crop growing environment, plants genetic traits, and post-harvesting processes are among the essential drivers of coffee quality (De Bruyn et al., 2017; Borém et al., 2019), meaning that coffee is a *terroir* product, and care is needed to obtain specialty coffee beverages. Three methods are commonly used to process coffee: natural (dry), wet, and pulped natural (semi-dry). The natural method is the oldest process that uses whole intact fruits, directly placed on cement patios or suspended platforms for fermentation and drying until reaching 11–12% moisture (Schwan et al., 2012). In the wet method, fruits are depulped, then fermented in tanks with water, and placed directly for drying. While the pulped natural is a mixture of both methods where fruits are depulped and placed directly for fermentation and drying. Each method has shown differences in sensory perception and microbiota dynamics (Silva et al., 2000, 2008a; Avallone et al., 2001; Evangelista et al., 2014a,b; Bressani et al.,2018). A more recent method known as self-induced anaerobic fermentation (SIAF) showed promising results in Da Mota et al.(2020) and Martins et al. (2020).

During fermentation, microorganisms consume carbohydrates or other organic compounds and proliferate (Silva et al., 2000; Silva, 2015). Most microorganisms that participate in the process come from the environment like soil, air, plants, and other sources (Silva et al., 2008a; Silva, 2015). Yeasts from the genera *Saccharomyces*, *Pichia*, *Candida*, *Kluyveromyces*, *Hanseniaspora*, and bacteria belonging to *Leuconostoc*, *Lactobacillus*, *Bacillus*, *Flavobacterium*, *Serratia*, *Pseudomonas*, and *Weissella* are often found while fermentation in the different post-harvest processes (Silva et al., 2000; Avalloneet al., 2001; Masoud et al., 2004; Vilela et al., 2010; Silva, 2015).

Microbial communities usually change in response to the environmental conditions where fermentations are carried out and affect coffee quality. Those conditions include temperature, moisture, and altitude (Borém et al., 2019; Martins et al., 2020). Bertrand et al. (2006) observed that green coffee beans from the variety Caturra grew at high altitudes and processed via the wet method in Costa Rica have high caffeine and fats and low trigonelline contents. A study with Ethiopian arabica green coffee beans showed that an increase in altitude decreases caffeine and chlorogenic acids contents, while sucrose, acidity, and flavor increase (Worku et al., 2018). In Brazil, research conducted in the Matas de Minas region showed that yellow and red Catuaí coffee varieties from higher altitudes produce higher quality coffee beans (Silveira et al., 2016).

Further research regarding altitude vs. compounds content variation is needed because they directly influence the beverage flavor. For example, organic acids mainly affect the sweet flavor (Galli and Barbas, 2004) and acidity (Ribeiro et al., 2017). Bioactive compounds trigonelline and chlorogenic acids are precursors of volatile compounds that contribute to roasted coffees taste and aroma (Ribeiro et al., 2016), and volatile alcohol precursors produce rose-like and fruity-like flavors (Lee et al., 2015).

Recent advances in Next-Generation Sequencing (NGS) are now allowing a deep microbiota characterization during the fermentative process under different conditions in several countries (Cao et al., 2017; de Oliveira Junqueira et al., 2019; Zhang et al., 2019; Elhalis et al., 2020; Pothakos et al., 2020), but few studies have been carried out with Brazilian coffees.

The present study aimed to characterize the dominant microbial communities of bacteria and fungi present inself-induced anaerobic fermentations containing different altitudes coffees performed in the Caparaó region througha metataxonomic approach. Moreover, this study aimed to evaluate the effect of altitude and microbiota profile on the biochemical compounds profile (organic acids, bioactives, and volatiles) during the fermentative process.

2. Material and Methods

2.1 Pilot study on-farm: coffee process and fermentation

Ripe fruits of *Coffea arabica* cv Catuaí Vermelho IAC 44 were manually collected from different altitudes: 800, 1,000, 1,200, and 1,400 m, at the Caparaó region, located in Minas Gerais and Espírito Santo, Brazil. The coffee fruits were processed using the natural method. Then the fruits were transferred into 20 L bioreactors (polypropylene food buckets with lids), following the bioreactors were closed for SIAF. Fermentations were performed in triplicate.

The fermentative processes for all coffees from different altitudes were carried out simultaneously in close batches at a farm located at 1,200 m to avoid any environmental interference and favor controlled conditions. The bioreactors were placed under an open storage house built with fences for fermentation and suspended terraces. Before filling the bioreactors with coffee, portable data loggers (INKBIRD) were placed inside the bioreactors to register the mass temperature during fermentation. Fermentation lasted 72 h, and sub-samples of approximately 100 g were taken after 48 h of fermentation for dominant microbiota profiling and metabolites evaluation. Fruits' initial sugar content (Brix degree-°Bx) was measured with a refractometer (Sigma-Aldrich, Saint Louis, MO, USA).

2.2 Composition and abundance of bacteria and fungi communities

2.2.1 DNA extraction

Total DNA was extracted from 48 h fermented coffee fruits collected in fields at 800, 1,000, 1,200, 1,400 m of altitude. One hundred grams of coffee fruits were vortexed in 50 ml sterilized Milli-Q water for 10 min to detach the fruits' microbial cells. Then the resulted suspension was transferred to another tube and centrifuged (12,745 RCF for 10 min at 4 ºC) to separate the supernatant and obtain a pellet. After the supernatant was discarded, 30 mg of the remaining pellet was used for DNA extraction with the QIAamp DNA Mini Kit, following the "DNA Purification from Tissues" protocol (Qiagen, Hilden, Germany). The purity of the extracted DNA was checked with a Nanodrop Lite spectrophotometer (Nanodrop Technologies, Wilmington, DE, United States) (260/280 nm ratio), and it was quantified by Qubit® 4.0 fluorometer using the dsDNA HS Assay kit (Invitrogen™) according to the manual. The DNA integrity was also confirmed by electrophoresis in a 0.8 % agarose gel with 1 X TAE buffer.

2.2.2 Illumina high-throughput sequencing of bacterial/archaeal 16S rRNA genes and fungal internal transcribed spacer (ITS)

The NGS Soluções Genômicas performed sample preparation for sequence and sequencing in Piracicaba-Sao Paulo, Brazil. The V3-V4 regions of the 16S rRNA gene of bacteria and the ITS1 and ITS2 regions of fungi were amplified from the total DNA extracted. We used the primers 341F (5'- CCTACGGGNGGCWGCAG -3') and 806R (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013) for bacteria/archaea, and the ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA -3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (Gardes and Bruns, 1993; Smith and Peay, 2014) for fungi.

Samples were paired-ended sequenced (2x 250 bp) on an Illumina MiSeq platform using the V2 kit (Illumina Inc).

2.2.3 Data analysis

The raw.fastq files were used to build a table of amplicon sequence variants (ASVs) with dada2 version 1.12 (Callahan et al., 2016). Briefly, using default parameters, the raw data quality was evaluated, filtered, and trimmed. The filtering parameters (maxN= 0, truncQ= 2, rm.phix= TRUE, maxEE=(2,2), and truncLen (235, 230)) were applied before inputting the filtered reads into dada2's parametric error model. The truncLen parameter was not applied for ITS1, and ITS2 reads since the expected sequence length is variable for fungi. Later, the forward and reverse reads were merged to obtain a full denoised sequence, and a higher-resolution table of amplicon sequence variants (ASVs) was constructed. Only ASVs with total abundances higher than 0.1% are reported. Chimeric sequences were detected and removed. Taxonomy was assigned to each ASV using the RDP ribosomal RNA gene database (version 11.5) for the 16S rRNA gene and with UNITE database (version 8.2) for fungal ITS. Sequences were matched the reference sequence with 100% identity.

2.3 Biochemical analysis

2.3.1 Organic acids evaluation

Organic acids of coffee fruits were evaluated after 48 h of fermentation. Three grams of coffee fruits were vortexed in Falcon tubes containing 20 mL of 16 mM perchloric acid and Milli-Q water at room temperature (25 ºC) for 10 min. The resulted suspension (without the fruits) was transferred to another tube, centrifuged at 12,745 RCF for 10 min at 4 ºC to obtain the supernatant. The supernatant was transferred to a new tube, and then its pH was adjusted to 2.11 using perchloric acid and recentrifuged under the same conditions. The supernatant from the second centrifugation was filtered through a 0.22 μm cellulose acetate membrane (Merck Millipore, Germany) and directly injected (20 μL) chromatographic column.

The samples were analyzed using a high-performance liquid chromatography (HPLC) system (Shimadzu Corp., Japan) equipped with a detection system consisting of a UV–Vis detector (SPD 10Ai) and a Shimpack SCR-101H (7.9 mm 30 cm) column operating at 50 ºC, which was used to achieve chromatographic separation of water-soluble acids at a flow rate of

0.6 mL min^{-1}. The acids were identified by comparison with the retention times of authentic standards. The quantification was performed using calibration curves constructed with standard compounds [malic and citric acid were purchased from Merck (Darmstadt, Germany), lactic and tartaric acid were purchased from Sigma-Aldrich (Saint Louis, MO, USA), acetic and succinic acids were purchased from Sigma-Aldrich, isobutyric and butyric acid were purchased from Riedel-deHaen (Seelze, Germany)]. All analyses were performed in duplicate.

2.3.2 Caffeine, Trigonelline, and Chlorogenic acids by HPLC

The identification of caffeine, chlorogenic acid [5-CGA], and trigonelline was made using a Shimadzu liquid chromatography system (Shimadzu Corp., Japan) equipped with a C18 column, following the protocol proposed by Malta and Chagas (2009). 0.5 g of grounded coffee fruits were place in tubes containing 50 mL Milli-Q water and boiled for 3 min to extract total compounds. Then the suspension was filtered through a 0.22 μm cellulose acetate membrane (Merck Millipor). Identification and quantitative analysis were performed using caffeine calibration curves, trigonelline, and 5-CGA (Sigma-Aldrich). All analyses were performed in duplicate.

2.3.3 Total polyphenols and antioxidant activity

Coffee samples were defatted following the methodology described by Batista et al. (2016). One hundred fruits were grounded with liquid nitrogen per sample, then 4 g were weighted. Following, 20 mL of n-hexane (Merck) was added into the 4 g, vortexed for 5 min, and centrifuged at 4,200 x g for 10 min/4 ºC to separate the lipids from the grounded sample left in the supernatant. After discarding the supernatant, the same procedure was repeated three times. The resulted lipid-free samples were air-dried for 24 h to evaporate the residual organic solvent.

The polyphenols and antioxidants were extracted, according to Kim et al. (2018), with minor modifications. Fifty milliliter of distilled water at 90 °C were added in a tube containing 2.75 g lipid-free ground coffee. Then, the mixture was left standing at room temperature (25 ºC) for 20 min; after that period, the mixture was filtered through a Whatman No. 2 filter paper.

2.3.3.1 Determination of total polyphenol content (TPC)

The total polyphenol content (TPC) was determined by a spectrophotometric assay (UV-VIS Spectrum SP-2000 UV, Biosystems) following the Follin – Ciocalteau methodology (Singleton and Rossi, 1965). In brief, 500 μL of coffee extract, 2.5 mL of Folin–Ciocalteau reagent (10 %), and 2.0 mL of Na_2CO_3 (4 % w/v) were homogenized and incubated at room temperature (25 ºC), in the dark for 120 min. The absorbance of the samples was measured at 750 nm. The TPC concentrations were calculated based on the standard curve of gallic acid (ranging from 10 to 100 μg mL^{-1}) and expressed as milligrams of gallic acid equivalents per gram of ground coffee (mg GAE g^{-1}). All analyses were performed in triplicate.

2.3.3.2 Antioxidant Activity Assays

Two different methodologies were applied to measure the antioxidant activity of coffee extracts. In the first one, the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging assay was performed as follows: 0.1 mL of coffee extract was added to 3.9 mL of the DPPH radical solution (0.06 mM) and incubated at room temperature (25 ºC), in the dark for 120 min, then the absorbance was measured at 515 nm (Spectrophotometer UV-Vis Spectrum® SP-2000UV, Shanghai, China). Trolox was used as a standard. A calibration curve (y = -0.0004x + 0.6636) was assembled using a range of 10, 20, 30, 40, 50 and 60 μM Trolox with linearity R^2 = 0.9999 (Batista et al., 2016). The results were expressed as μM Trolox Equivalents (TE) per gram of ground coffee (μM TE g^{-1}).

The second assay was performed with a 2,2′-azinobis (3-ethylbenzothiazoline-6-suslfonic acid) (ABTS) stock solution reaction (7 mM) with potassium persulphate (140 mM). After, the mixture was left in the dark at room temperature (25 ºC) for 16 h before use. The ABTS solution was diluted in ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. Thirty microliters of the coffee extracts were added to 3.0 mL of the ABTS radical solution, and after 6 min, the absorbance was measured. Trolox was used as a standard. A calibration curve (y = -0.0003x + 0.6802) was assembled using a range of 100, 500, 1,000, 1,500 and 2,000 μM Trolox with linearity of R^2 =0.9983. The results were expressed as μM Trolox Equivalents (TE) per gram of ground coffee (μM TE g^{-1}).

2.3.4 Volatile compounds

Volatile compounds were extracted from 48 h fermented coffee fruits using a headspace solid-phase microextraction (HS-SPME). Coffee fruits (2 g) were macerated with liquid nitrogen and placed in a 15 ml hermetically sealed vial. After equilibration at 60 °C for 15 min., the volatile compounds were extracted at 60 °C for 30 min. The desorption time on the column was 2 min.

The compounds were analyzed using a Shimadzu QP2010 GC model equipped with mass spectrometry (MS) and a silica capillary Carbo-Wax 20M (30 m × 0.25 mm × 0.25 mm) column. The operation conditions of analysis consisted of maintaining the oven temperature at 50 °C for 5 min., then raised to 200 °C at 8 °C $min.^{-1}$ and maintained for 15 min. The injector and detector were kept at 230 and 200 °C, respectively, and He carrier gas was maintained at a flow rate of 1.9 ml $min.^{-1}$. The volatile compounds were identified by comparing their mass spectra against those available in the NIST11 library. The retention Index (RI) for each compound was calculated using an alkane series (C10–C40) compared with those found in the literature.

2.4 Statistical analysis

Alpha and beta diversity analyzes were performed for the evaluated microbial communities. Each altitude richness and abundance were used to calculate the bacterial and fungal Shannon and Simpson diversity indices. Moreover, the relative abundance was calculated, and ASVs profiles were clustered for each altitude using the XLSTAT software (Addinsoft, version 2020.1.3). Bray-Curtis-based non-metric multidimensional scaling (NMDS) was used to evaluate the dissimilarities between the fungal community and organic acids and volatile compounds with the XLSTAT software (Addinsoft, version 2020.1.3).

The raw data normal distribution for statistical analysis was evaluated with the Shapiro-Wilk and Anderson-Darling tests. All values in the figures are expressed as averages. Standard deviations were calculated using the XLSTAT software (Addinsoft, version 2020.1.3). The Tukey test was run with $p \leq 0.05$ in the SISVAR software (Ferreira, 2014) to evaluate the difference in acid concentration, volatiles relative concentration, and antioxidants' concentration and activity. The Pearson correlation coefficient was used to calculate the correlations between the bacterial genera, acids, and volatile compounds, using Origin software (version 2020). The principal component analysis was run on all altitudes, acids, antioxidants, and volatile compounds using XLSTAT software (Addinsoft, version 2020.1.3).

3. Results

3.1 Fruits Initial Sugar Content and Fermentation Temperature

The initial ºBrix value from coffee fruits was between 18 to 19.3 (Table 1). The coffee mass temperature varied from 18 to 25 ºC at 48 h (Table 1). The environmental temperature varied from 8 ºC to 23.1 ºC and relative moisture varied from 56.1 to 85% during fermentation.

Table 1. Fruit characteristics, coffee mass temperature, and microbial diversity indices. Data is expressed as Mean ± SD.

Coffee Altitude (m)	Initial Brix (Bx)	Coffee mass temperature (ºC)			Bacterial Diversity Indices		Eukaryotic Diversity Indices	
		0 h	24 h	48 h	Shannon	Simpson	Shannon	Simpson
800	18.6 ± 0.6	18 ± 0	22 ± 0.06	23 ± 0.15	2.281 ± 0.2	7.973 ± 1.7	3.334 ± 0.1	14.766 ± 2.0
1,000	18.6 ± 1.5	18 ± 0	24 ± 0.12	25 ± 0.06	2.018 ± 0.5	5.574 ± 2.2	2.721 ± 0.1	5.666 ± 0.3
1,200	19.3 ± 0.6	18 ± 0	20 ± 0.10	21.6 ± 0.06	2.005 ± 0.4	5.004 ± 2.0	0.924 ± 0.3	1.417 ± 0.2
1,400	18 ± 3.0	18 ± 0	22 ± 0.12	23 ± 0.15	1.661 ± 0.5	4.330 ± 1.3	2.983 ± 0.2	9.372 ± 1.0

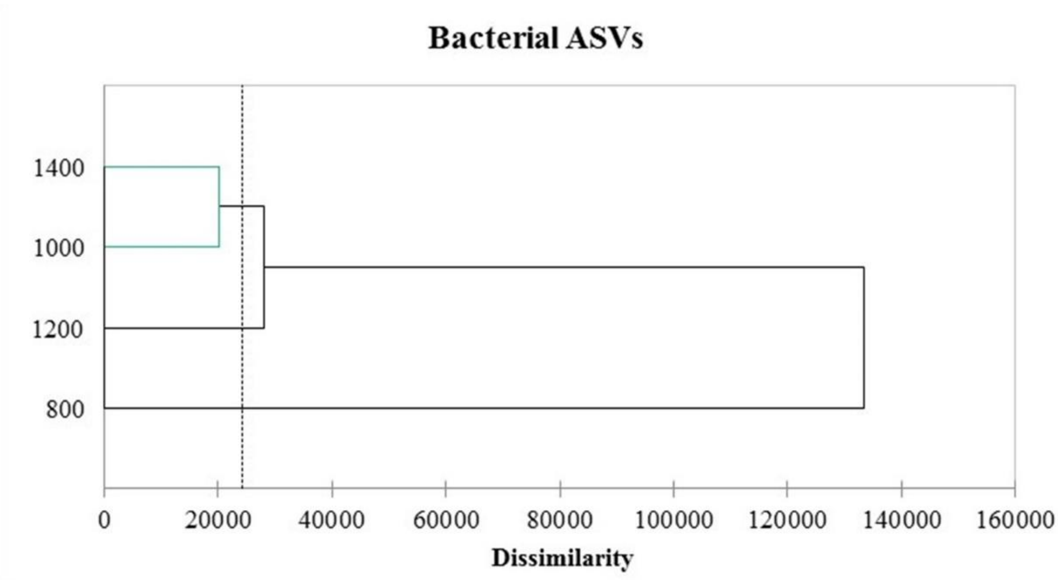
SD: Standard deviation

3.2 Microbial community profile

A total of 63.966, 16.346, 42.238, and 19.727 filtered 16S rRNA partial gene sequences and 104.719, 194.033, 263.884, and 119.571 filtered ITS sequences were obtained for the altitudes 800, 1,000, 1,200, and 1,400 m, respectively.

Among the altitudes, 800 m had the highest bacterial richness with 18 genera assigned, and 1,000 m had the highest fungal richness with 166 species assigned. Table 1 shows the bacterial and fungal diversity indices for all evaluated altitudes. In summary, we observed a tendency to decrease the alpha-bacterial diversity indices with the altitude increase (Table 1).

The altitudes ASVs profiles were clustered, as illustrated in Figure 1, and three groups were obtained for bacteria and fungi. The 800 m bacterial profile was very distant and different from the other altitudes. The 1,400 and 1,000 m profiles were grouped for bacteria and fungi, meaning they were the most similar. On the other hand, the fungal cluster showed that the 1,200 m profile was different from the other altitudes and close to the 800 m profile.



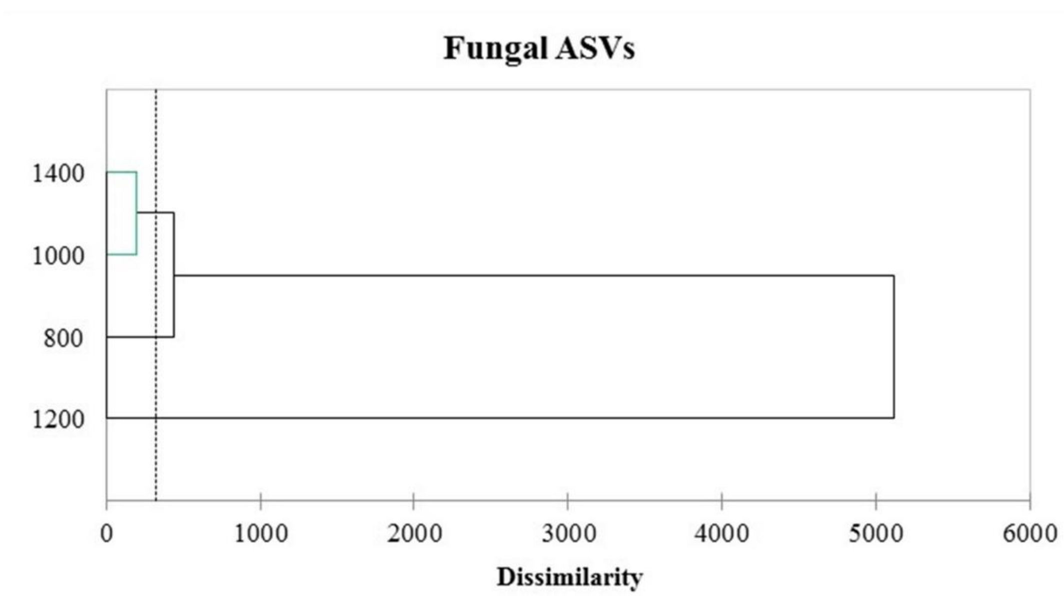
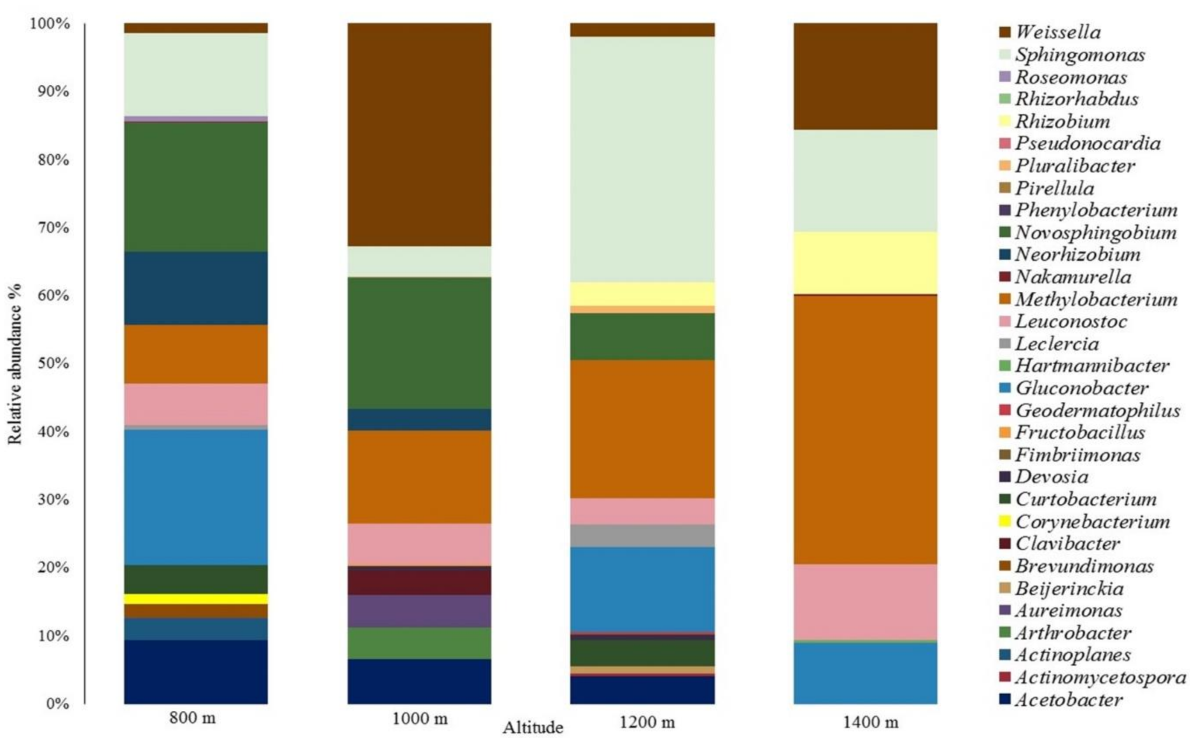


Figure 1. Cluttering of ASVs profile from amplicon sequence of the 16S and ITS region.

A total of 31 genera were assigned in the bacterial community, as shown in Figure 2A. Most sequences in the 800 m sample were assigned to genera *Gluconobacter* (19.8%), *Novosphingobium* (18.9%), and *Sphingomonas* (12.2%). As for the other altitudes, genera *Weissella* (32.7%; 1,000 m), *Sphingomonas* (36.2%; 1,200 m), and *Methylobacterium* (39.4%; 1,400 m) had the highest abundances.

The genera *Actinoplanes*, *Brevundimonas*, *Corynebacterium*, *Roseomonas*, *Phenylobacterium*, *Pseudonocardia*, and *Rhizorhabdus*, were only identified at 800 m, *Arthrobacter*, *Clavibacter*, *Fructobacillus*, and *Pirellula* were only identified at 1,000 m, *Beijerinckia*, *Pluralibacter*, *Actinomycetospora*, *Geodermatophilus*, and *Fimbriimonas* were only identified at 1,200 m, and *Nakamurella* and *Hartmannibacte*r at 1,400 m. *Sphingomonas*, *Methylobacterium*, *Leuconotoc*, and *Weissella* were found in all altitudes. Genus *Gluconobacter* was only identified in samples at 800, 1,200, and 1,400 m with relative abundances of 19.8%, 12.5%, and 9%, respectively.

A



B

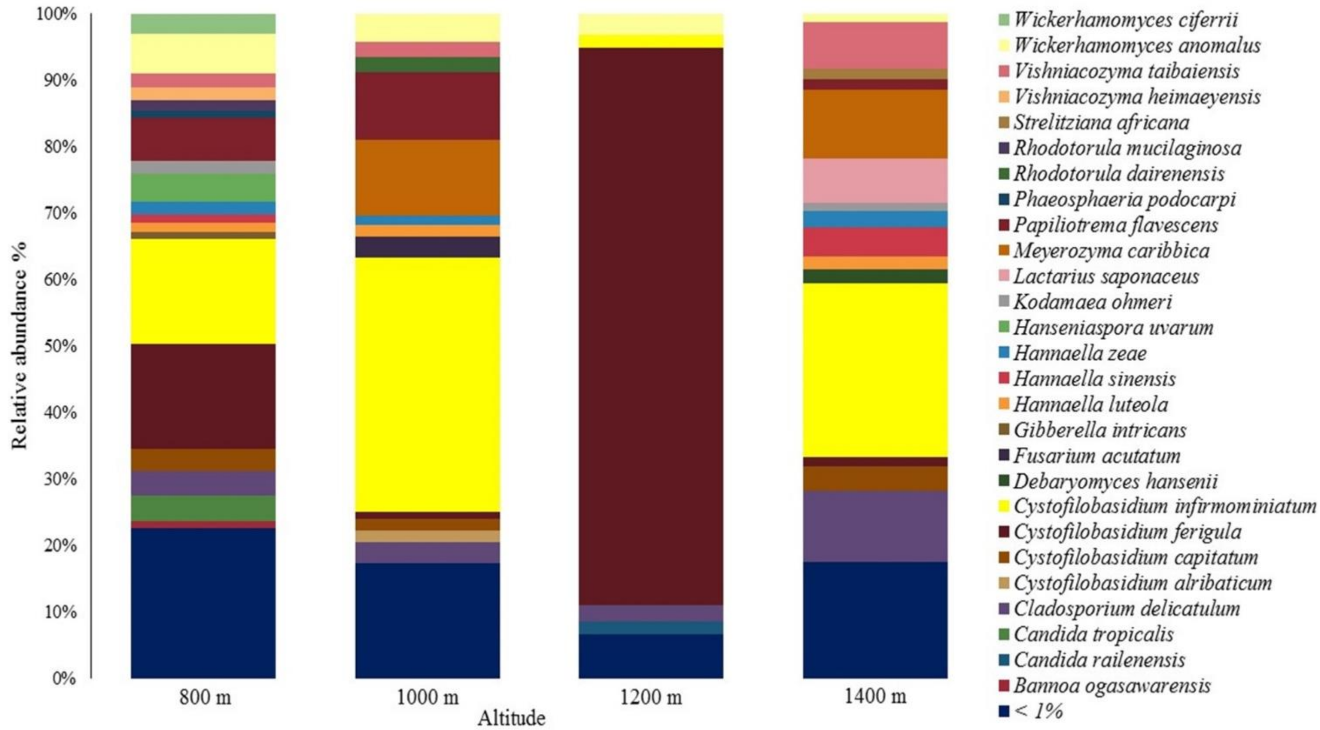


Figure 2. Relative abundance of the microbial communities at different altitudes. **A.** Bacterial community. **B.** Fungal community.

Regarding the fungal community, a total of 223 species were assigned, showing a yeast predominance (Figure 2B). The most abundant species were *Cystofilobasidium infirmominiatum* (15.831%), *Cystofilobasidium ferigula* (15.700%), and *Papiliotrema flavescens* (6.571%) at 800 m. Following, *Cystofilobasidium infirmominiatum* (38.218%), *Meyerozyma caribbica* (11.445%), and *Papiliotrema flavescens* (10.271%) at 1,000 m, *Cystofilobasidium ferigula* (83.857%), *Wickerhamomyces anomalus* (3.216%), and *Cladosporium delicatulum* (2.539%) at 1,200 m, and *Cystofilobasidium infirmominiatum* (26.187%), *Cladosporium delicatulum* (10.817%), and *Meyerozyma caribbica* (10.216%) at 1,400 m. The species that were below 1% relative abundance are available in Supplementary Material 1.

Each altitude had a broad range of distinctive fungal species, including Candida *sake*, *Sampaiozyma vanillica*, *Apiotrichum laibachii*, and *Citeromyces matritensis* for 800 m. *Fellomyces borneensis*, *Rhodotorula babjevae*, *Rhodotorula taiwanensis*, *Papiliotrema laurentii*, *Candida blattae*, *Wickerhamomyces pijperi*, *Cryptococcus saitoi*, *Rhynchogastrema complexa*, and *Cutaneotrichosporon terricola* for 1,000 m. *Papiliotrema perniciosus*, *Wickerhamomyces* *sydowiorum*, *Nakazawaea holstii*, and *Eupenidiella venezuelensis* for 1,200 m, and *Neoascochyta paspali*, *Euteratosphaeria verrucosiafricana*, *Sporobolomyces johnsonii*, and *Rhodosporidiobolus ruineniae* for 1,400 m. The rest of the distinctive species identified are available in Supplementary Material 2.

We also identified frequently described species grouped in the species below 1% of abundance (Supplementary Material 1). Those include *Saccharomyces cerevisiae*, which was only identified in altitudes 1,000 (0.006%) and 1,200 m (0.003%), *Candida parapsilosis* in the same altitudes with 0.022% and 0.015%, and *Torulaspora delbrueckii* in altitudes 800, 1,000, and 1,200 m with 0.062, 0.036, and 0.005%. There were other yeasts like *Meyerozyma guilliermondii* (identified at 800 and 1,000 m: 0.904 and 0.130%), *Candida tropicalis* (lower abundances at 1,000, 1,200, and 1,400 m: 0.182, 0.162, and 0.156%), *Debaryomyces hansenii* (lower abundances at 800, 1,000, and 1,200 m: 0.282, 0.398, and 0.869%), *Pichia kluyveri*, *Debaryomyces nepalensis*, *Rhodotorula mucilaginosa* (only in altitudes 1,000, 1,200, and 1,400), *Candida orthopsilosis*, *Candida quercitrusa*, *Fellomyces mexicanus*, *Derxomyces anomalus*, and *Wickerhamomyces lynferdii*.

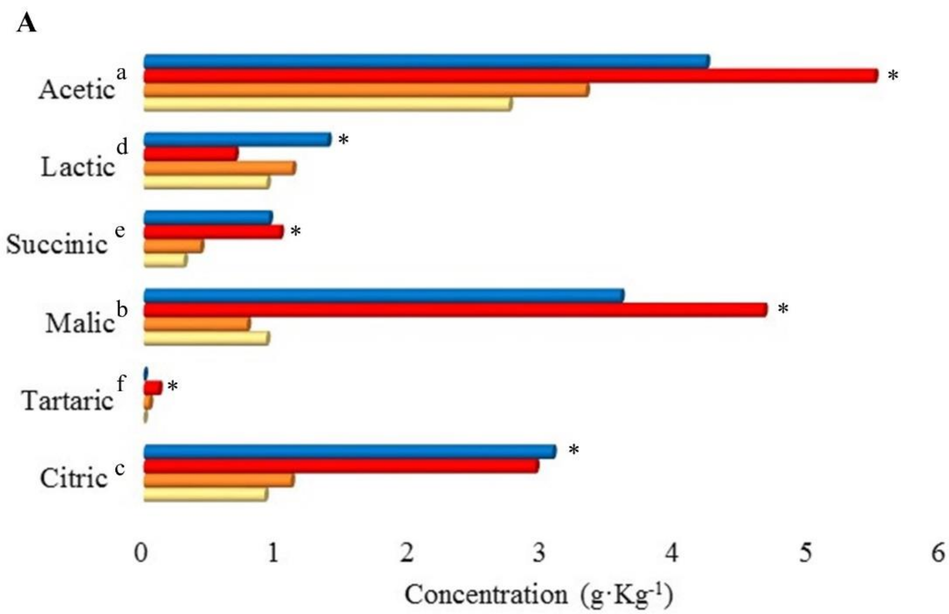
Filamentous fungi species such as *Aspergillus westerdijkiae*, *Alternaria argyroxiphii*, *Botrytis caroliniana*, *Cladosporium aphidis*, *Cladosporium halotolerans*, *Colletotrichum annellatum*, *Colletotrichum theobromicola*, *Fusarium asiaticum*, *Fusarium delphinoides*, *Fusarium proliferatum*, *Gibberella intricans*, *Lecanicillium antillanum*, *Penicillium kongii*, and *Penicillium solitum* were also identified.

3.3 Organic acids

3.3.1 Effect of altitude on acids content

Acetic, malic, citric, lactic, succinic, and tartaric acid concentrations were statistically different among the altitudes (Figure 3A). Acetic, malic, and citric acid concentrations at 1,400 and 1,200 m were higher than 1,000 and 800 m. When concentrations from 1,400 and 1,200 m were compared with the other altitudes in each acid, there were differences of 0.90 - 2.75 (acetic), 2.66 – 3.88 (malic), and 1.84 – 2.16 (citric) g. Kg^{-1}. As for altitudes 1,000 and 800 m, acetic (g. Kg^{-1}: 3.32 and 2.74), lactic (g. Kg^{-1}: 1.12 and 0.92), citric (g. Kg^{-1}: 1.10 and 0.91), and malic acid (g. Kg^{-1}: 0.78 and 0.92) were found in higher concentrations than succinic and tartaric acid. Within the acetic acid results, 1,200 m altitude presented the highest content (5.49 g. Kg^{-1}), and altitude 800 m presented the lowest content with 2.74 g. Kg^{-1}. Malic and succinic acid were significantly higher at 1,200 m (g. Kg^{-1}: 4.66 and 1.02). Citric and lactic acid were higher at 1,400 m (3.07 g. Kg^{-1} and 1.38 g. Kg^{-1}, respectively). Tartaric acid was only detected at 1,000 and 1,200 m with concentrations of 0.04 and 0.11g. Kg^{-1}, respectively.

The PCA showed that 1,200 m and 1,400 m altitudes were correlated with citric, succinic, malic, and acetic acid (Figure 3B). 1,400 m altitude was characterized by lactic acid, while 1,200 m altitude was characterized by tartaric acid (Figure 3B).



B

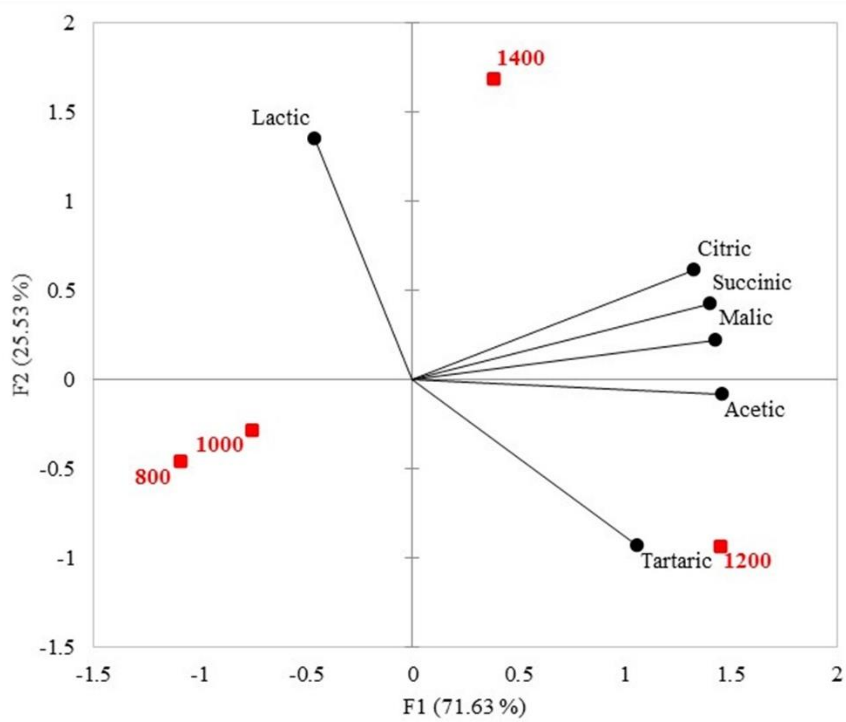


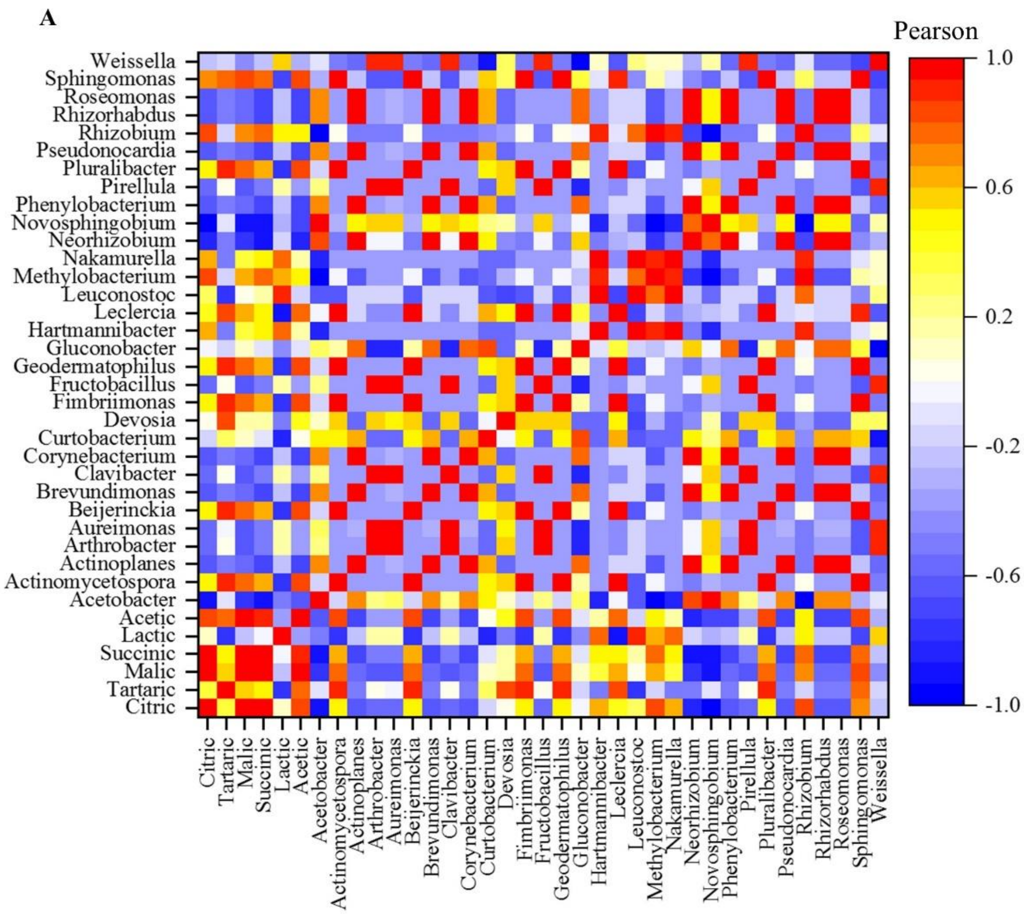
Figure 3. A. Organic acid concentrations at 48 h of fermentation on different altitudes. Each bar represents an altitude: 800, 1000, 1200, and 1400 m. Significant values ($p \leq 0.05$) are represented in letters, from the highest amount to the lowest. * Altitude that was statistically significant ($p \leq 0.05$) and higher in contrast to the other altitudes. Citric, tartaric, malic, succinic, lactic, and acetic standard deviations: 800 m (0.19, not detected, 0.09, 0.01, 0.43, 0.44), 1,000 m (0.46, 0, 0.37, 0.10, 0.73, 1.57), 1,200 m (1.52, 0, 0.87, 0.41, 0.17, 1.95), 1,400 m (2.55, not detected, 1.97, 0.65, 1.11, 1.65) **B.** Principal component analysis (PCA) plot of organic acids and altitudes.

3.3.2 Acids correlation with bacterial community and dissimilarity with fungal community

The Pearson correlation between acid content and bacterial community is depicted in Figure 4A. *Leuconostoc* showed a high positive correlation (0.93) with lactic acid content. Malic acid had the highest positive correlation (0.87) with the *Sphingomonas* genus. Acetic acid was positively correlated (0.87) with *Sphingomonas* and negatively correlated with *Acetobacter* (-0.64). The genera *Pluralibacter*, *Geodermatophilus*, *Fimbriimonas*, *Beijerinckia*, and *Actinomycetospora*, were highly positively correlated with tartaric acid (0.93 for all).

Additionally, succinic and citric acid were highly positively correlated with *Rizhobium* (0.78, 0.87) and *Methylobacterium* with citric acid (0.83).

The NMDS plot (Figure 4B) showed that similar acetic, malic, citric, lactic, and succinic acid contents are shared between the fungal community, mainly in greater abundance. Most species with an abundance below 1% were close and different from the high abundance species and did not affect the acids in high concentrations.



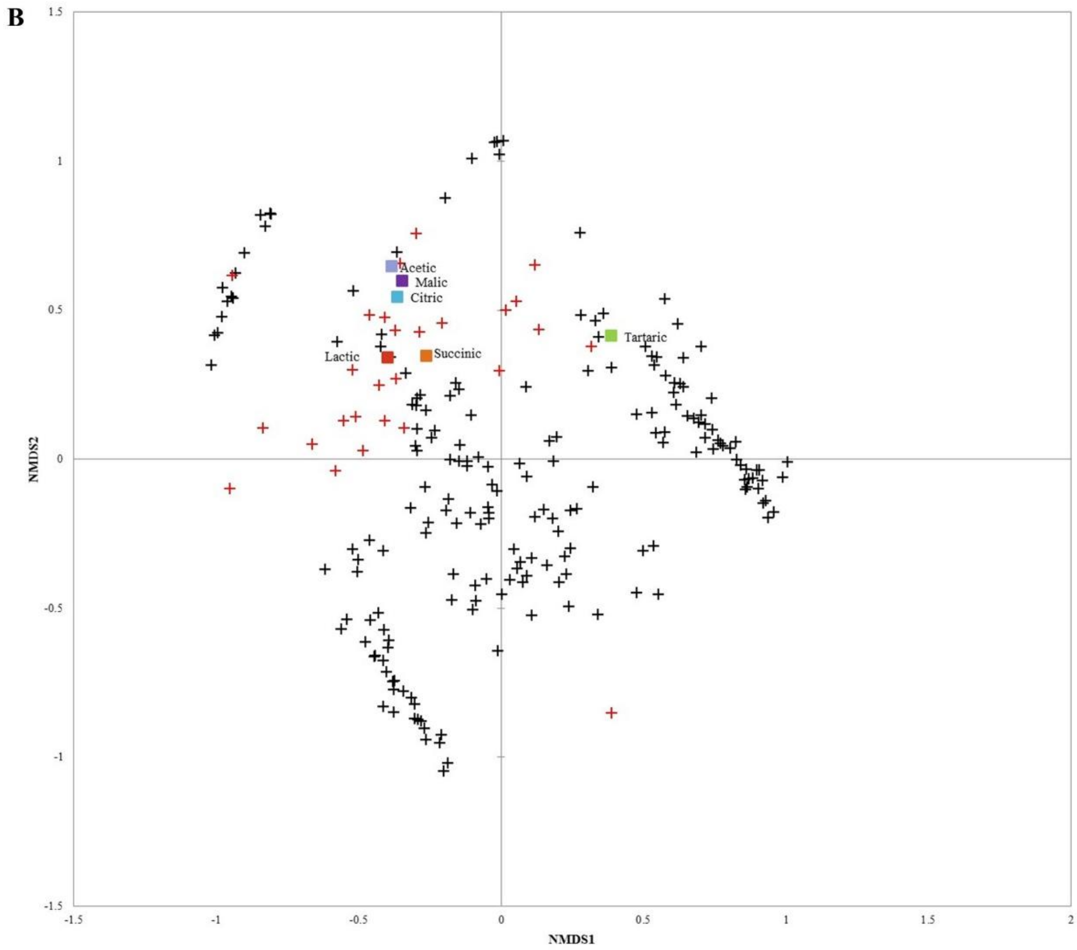


Figure 4. A. Pearson correlation matrix of bacterial genera and acids. **B.** Non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity for eukaryotic species and organic acids**.** + species below 1% and + abundant species above 1%.

3.4 Volatile compounds

3.4.1 Effect of altitude on volatiles

A total of 67 volatile compounds were detected. These compounds were classified as alcohols (19), esters (13), aldehydes (10), ketones (6), furans (5), phenols (4), pyrans (3), acids (3), alkanes (2), lactones (1), and pyrazines (1).

The total relative concentration of each chemical group was statistically significant. The following chemical groups had the most abundant relative concentrations: alcohols, phenols, aldehydes, lactones, and ketones. The alcohols 2-heptanol and 1,6-octadien-3-ol, 3,7-dimethyl- were the most significant among the other compounds with relative concentrations varying from 70.6 to 26.3 mg. g^{-1} and 37.5 to 11.8 mg. g^{-1} (Figure 5A). The relative concentration of these alcohols was higher at 1,400 and 800 m compared to the other altitudes, respectively. Moreover, methyl salicylate and benzoic acid, 2-hydroxy-, ethyl esters were the most abundant within the phenols group and both in altitudes at 800 m (27.4 mg. g^{-1} and 3.3 mg. g^{-1}). Aside from the previous groups, other compounds such as benzeneacetaldehyde, benzaldehyde, 2(3H)-furanone, dihydro-3,5-dimethyl-, acetoin, and 2-propanone, 1-hydroxy- were the most abundant within the aldehydes, lactones, and ketones groups, at altitudes 1,400 (relative concentration: 16.1 mg. g^{-1} and 10.1 mg. g^{-1}), 1,200 (10.8 mg. g^{-1}), 1,000 (20.1 mg. g^{-1}), and 800 (6.9 mg. g^{-1}) m. Some compounds were detected only in certain altitudes: 2-propanone, 1-hydroxy- and phenol, 4-ethyl-2-methoxy- at 800m, 1-propanol, 2-methyl- and 2-decenal, (E)- at 1000 m, 2(3H)-furanone, dihydro-3,5-dimethyl- at 1200 m, and nonanoic acid at 1400 m. Other compounds like (S)-3-ethyl-4-methylpentanol, benzyl alcohol, phenylethyl alcohol, 2,3-butanediol, [R-(R*, R*)]-, and benzyl acetate were detected.

The PCA graph in Figure 5B showed that around 36% (7) of those alcohols characterized 1,000 and 800 m altitudes from the total volatile alcohols. 1,200 m altitude was characterized by the only lactone 2(3H)-furanone, dihydro-3,5-dimethyl-. 1,400 m altitude was primarily characterized by esters (70%-7 from the total).

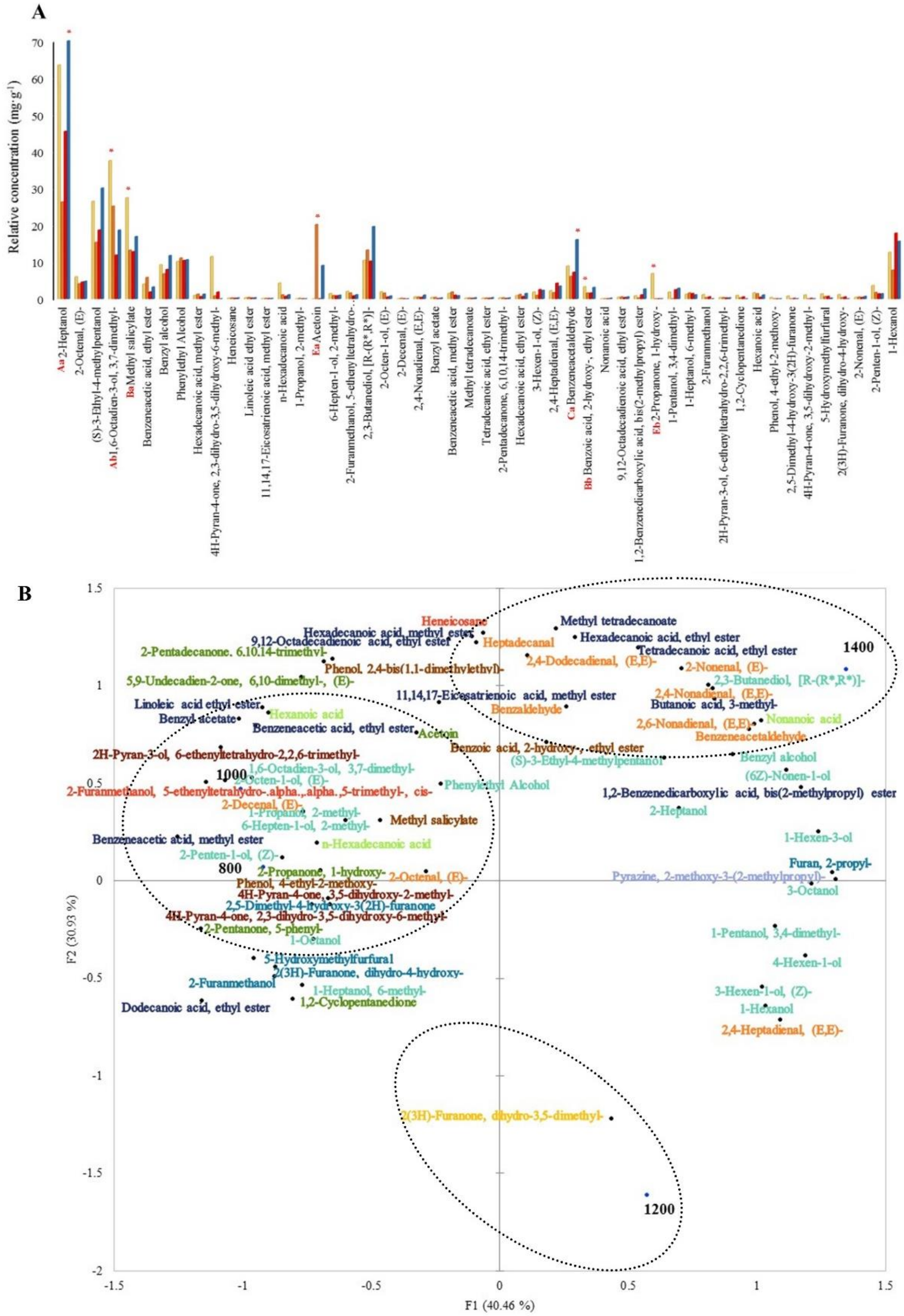


Figure 5. A. Volatile compounds relative concentrations at 48 h of fermentation on different altitudes. Each bar represents an altitude: 800, 1000, 1200, and 1400 m. Significant values ($p \leq 0.05$) are represented in letters. From the highest amount to lowest, different uppercase letters represent the most predominant groups (A: alcohols, B: phenols, C: aldehydes, D: lactones, and E: ketones), and different lowercase letters represent the two most predominant compounds within the groups. * Altitude that was statistically significant ($p \leq 0.05$) and higher in

contrast to the other altitudes. **B.** Principal component analysis (PCA) plot of volatile compounds and altitudes. Volatile groups: ■ acids,■ alcohols, ■ aldehydes, ■ alkanes, ■ esters, ■ furans, ■ ketones, ■ lactones, ■ phenols, ■ pyrans, ■ pyrazines.

3.4.2 Volatiles correlation with bacterial community and dissimilarity with fungal community

The Pearson correlation between the volatile compounds and bacterial profile is depicted in Figure 6A. Methyl salicylate and 2-propanone, 1-hydroxy- were positively correlated (1) with the genera only found at 800 m altitudes (*Actinoplanes*, *Brevundimonas*, *Corynebacterium*, *Roseomonas*, *Phenylobacterium*, *Pseudonocardia*, and *Rhizorhabdus*). 2(3H)-furanone, dihydro-3,5-dimethyl- was positively correlated (1) with the genera only found at 1,200 m altitude (*Pluralibacter*, *Geodermatophilus*, *Fimbriimonas*, *Beijerinckia*, and *Actinomycetospora*). The genus *Weisella* had a strong positive correlation (0.99) with the ketone acetoin, 11,14,17-eicosatrienoic acid, methyl ester (0.98), and phenylethyl alcohol (0.97). The highest correlation (0.96) for benzeneacetaldehyde was with the species only found at 1,400 m altitude (*Nakamurella* and *Hartmannibacter*).

The NMDS plot (Figure 6B) showed that the species with the highest abundance within the fungal community might be producing similar contents of detected volatiles. Though not all the high abundance species affect the same volatile groups, some affect alcohols contents instead of aldehydes contents as observed in the plot. Most fungal species below 1% abundance are close and different from high abundance species and are grouped with low content volatiles (from 0-0.4 mg. g^{-1}) phenol, 2,4-bis(1,1-dimethylethyl)-, phenol, 4-ethyl-2-methoxy-, 11,14,17-eicosatrienoic acid, methyl ester, dodecanoic acid, ethyl ester, 2-decenal, (E)-, 1-propanol, 2-methyl-, and (6Z)-nonen-1-ol.

A

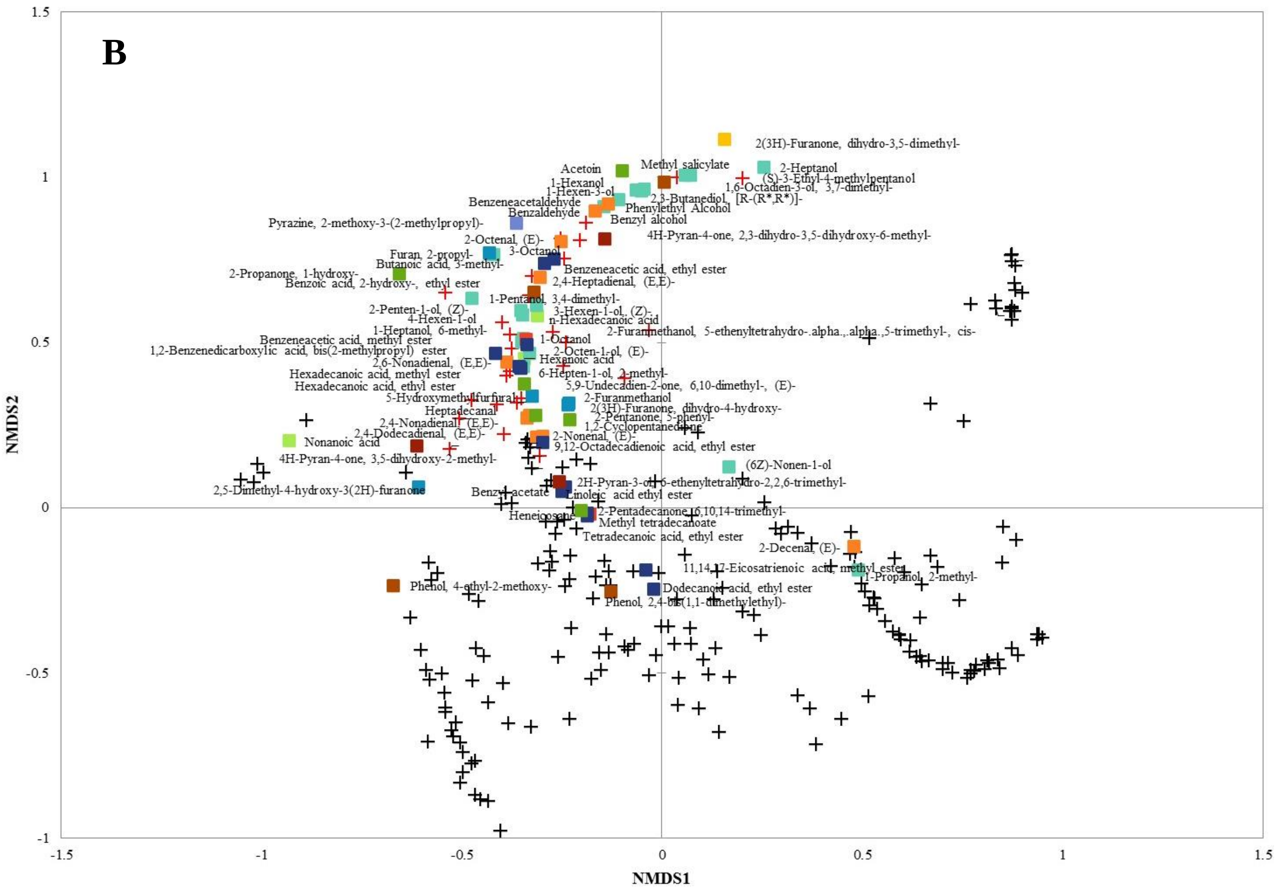


Figure 6. A. Down-stream Pearson correlation matrix of bacterial genera and volatile compounds. **B.** Non-metric scaling (NMDS) using Bray-Curtis dissimilarity for eukaryotic species and volatiles. Volatile groups: ■ acids, ■ alcohols, ■ aldehydes, ■ alkanes, ■ esters, ■ furans, ■ ketones, ■ lactones, ■ phenols, ■ pyrans, ■ pyrazines. + species below 1% and + abundant species above 1%.

3.5 Effect of altitude on caffeine, chlorogenic acids, trigonelline, total phenolics concentration, and antioxidant activity

Caffeine total concentration was higher (44.5%) than those of trigonelline (29.9%) and chlorogenic acids (25.6%), mainly at 1,200 m with a significant value of 11.64 g. Kg^{-1} (Figure 7A). Among the altitudes, trigonelline concentration was higher at 1,000 m (6.63 g. Kg^{-1}), and chlorogenic acid was higher at 800 m (9.70 g. Kg^{-1}). Total phenolics concentration was higher at 1,200 m with 203.90 mg. g^{-1}, followed by 1,400 m (160.12 mg. g^{-1}). Regarding the antioxidant activity, after the ABTS assay, samples at 1,000 and 1,200 m had the highest activity (331.72 and 340.16 µM TE. g^{-1}) compared to other altitudes, and after the DPPH assay, the highest value was reported at 1,000 m (95.01 µM TE. g^{-1}).

The PCA on Figure 7B displays the correlation between altitudes, antioxidants, and their activity. 1,200 m altitude was characterized by ABTS activity, caffeine, and total phenolics due to the high concentrations detected at that altitude. A similar characterization was seen for 1,000 m, however, with DPPH activity. Total phenolics were grouped with caffeine and chlorogenic acids, and trigonelline was grouped with ABTS activity.

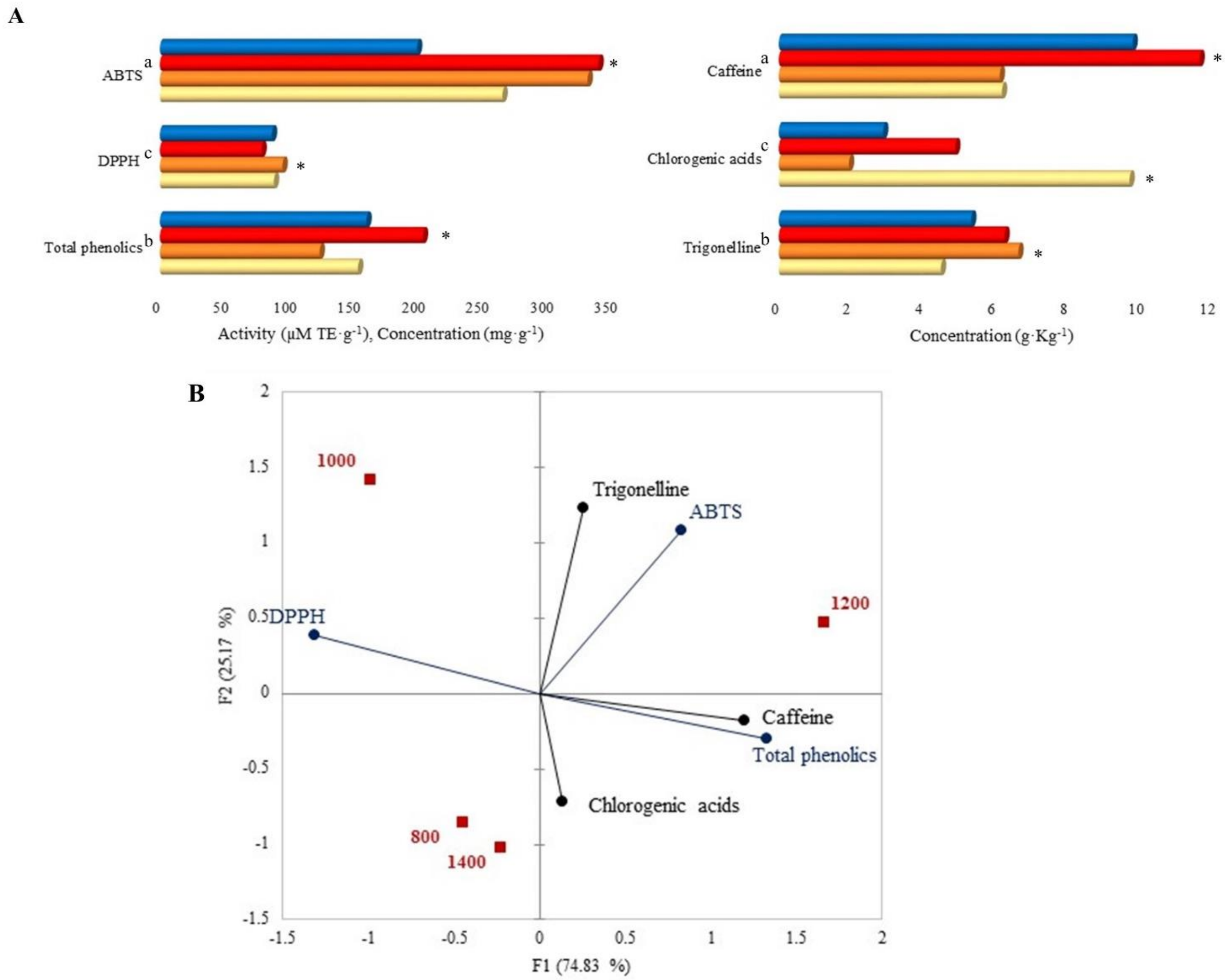


Figure 7. A. Caffeine, chlorogenic acids, trigonelline, total phenolics concentrations, and antioxidant activity by ABTS and DPPH assays. Each bar represents an altitude: 800, 1000, 1200, and 1400 m. Statistically significant values ($p \leq 0.05$) are represented in letters, from the highest amount to the lowest. * Altitude that was statistically significant ($p \leq 0.05$) and higher in contrast to the other altitudes. Trigonelline, chlorogenic acids, caffeine, total phenolics, DPPH, and ABTS standard deviations: 800 m (1.62, 8.33, 5.21, 0.21, 0, 0.03), 1,000 m (0.78, 0.06, 1.17, 3.72, 0, 21.84), 1,200 m (0.03, 0.01, 0, 3.15, 0, 17.10), 1,400 m (0.08, 0.01, 0.02, 6.60, 0, 0.15). **B.** Principal component analysis (PCA) plot of antioxidants, antioxidant activity, and altitudes.

4. Discussion

The aim was to characterize the microbial community and compounds profiles associated with fermented natural coffee from different altitudes. Out of the detected fungal community, yeast species were the most abundant possibly to the region's high relative moisture and temperatures, which were within our ranges. Furthermore, this region has rainy summer (November to January) and a cold and dry winter (June to August). The average annual rainfall ranges from 1,000 to 1,500 mm, and the average annual temperature ranges from 19 to 22ºC (Campanha et al., 2017). Other factors that influenced yeast occurrence were the temperature inside our mass that varied from 21.6 to 25 ºC and SIAF conditions, becoming beneficial for their growth.

The coffee's microbiota in this work varied at different altitudes. Lower altitudes favored bacterial richness, meaning that altitude is a factor that affects this microbial group. The high *Gluconobacter* abundances found in this work were also reported in a fermented natural processed coffee from Ecuador (De Bruyn et al., 2017). Therefore, the high abundances depend on the processing instead of the altitude. Acetic acid bacteria (AAB) are known to be strictly aerobic and capable of oxidizing alcohols, aldehydes, and sugars into carboxylic acids (Gomes et al., 2018). However, there was no correlation between *Gluconobacter* and acetic acid production in our work, but possibly other AAB, such as *Acetobacter,* were responsible for the high acetic acid production. A food fermentation with similar microbial dynamics as in coffee is cocoa (Schwan et al., 2015). Ho et al. (2018) have confirmed AAB's role in cocoa fermentation, which involves acetic acid production (primary acid involved in cocoa fermentation), pH increase, and volatiles production. A bacterial genus capable of producing acetic acid is *Weissella*, which might have aided with the acetic acid production in this study since the genus was present at all altitudes. However, *Weissella* belongs to the lactic acid bacteria (LAB) group and is heterofermentative (producing acetic acid and lactic acid) (Lorenzo et al., 2018). According to Martins et al. (2020), among the isolated LAB from the Caparaó region, *Weissella paramesenteroides* were more abundant in natural coffees. Moreover, this genus has been found in coffee fruits from Taiwan (Leong et al., 2014), a wet-processed coffee from Colombia (Junqueira et al., 2019), and an Ecuadorian natural processed coffee (De Bruyn et al., 2017).

The LAB *Leuconostoc* was detected at all altitudes and showed a strong positive correlation with lactic acid, suggesting that this genus may be responsible for its production. Prior works testing the *Leuconostoc* genus' potential in coffee have shown that they are incapable of producing pectinolytic enzymes (Avallone et al., 2002). However, they produce lactic acid as a primary compound during fermentation (De Bruyne et al., 2007). In coffee, species of *Leuconostoc* have been isolated from Ethiopian coffee fermentation (De Bruyne et al., 2007) and were abundant in a coffee fermentation performed at 1,329 m in Ecuador (De Bruyn et al., 2017).

For the first time, we report a high abundance of the Proteobacteria *Methylobacterium* in a natural processed coffee fermentation at 1,400 m. Limited information on its function during fermentation is provided, yet they are known as plant growth-promoting bacteria (Ponnusamy et al., 2017). There is no current information about their correlation with citric acid or the contribution to coffee fermentation. Yet, there was a positive correlation between *Methylobacterium* and citric acid, and where the highest content of this acid was detected, this genus was most abundant, possibly to its overproduction during Krebs Cycle. However, further studies must be done to understand their relation.

Similarly, it is the first time *Sphingomonas* (most abundant in 1,200 m fermented coffee), *Roseomonas*, *Fructobacillus*, and *Nakamurella* are reported in the natural coffee processed fermentations. Still, some have already been identified on a wet-processed coffee fermentation (De Carvalho Neto et al., 2018).

According to Martinez et al. (2019), bacteria are the primary acid producers in wet fermentation. Therefore, in this work, the bacterial community from dry-processed coffees was correlated with acids. Acetic acid significantly predominated coffees from the Caparaó region. Bressani et al. (2018) reported similar results in a different region: coffee was fermented with fewer hours and another variety Catuaí at an altitude of 750-800 m. Citric acid is expected to be significant because it is a primary compound produced by any microorganism and enhances fruity flavors. Overall, coffees from 1,200 and 1,400 m favor acetic, malic, and citric acid content (Figure 3). Therefore, altitude affects their concentration, and they are expected in higher concentrations because they positively contribute to the beverage acidity (Buffo and Cardelli-freire, 2004). No detection of butyric and propionic acid indicates that 48 h is a proper time for SIAF fermentation and guarantees nonproduction of off-flavors (Silva, 2015; Haile and Kang, 2019a). As observed in our results, 1,200 m favored tartaric acid production and was correlated with the bacteria genera only found at that altitude and not in the other altitudes, which means that they might be responsible for the tartaric acid production or stimulated the other genera to produce. Until now, no reports have shown their capacity to produce tartaric acid. Detection of tartaric acid in coffee is positively favorable since it produces fruity flavors like wine (Dziezak, 2016).

The bacterial communities were also correlated with volatile compounds because they contribute to their production. In this work, *Weisella* was correlated with Acetoin, and both were abundant at 1,000 m. Also, Acetoin was only detected at that altitude, suggesting that this genus may induce its production or other microorganisms. The same behavior was observed for compounds Methyl salicylate, 2-Propanone, 1-hydroxy-, and Benzeneacetaldehyde in the other altitudes with their respective genera.

Filamentous fungi and yeasts also compose the microbial communities of coffee during fermentation. The fungal diversity varied depending on the altitude and diversity index. Genus *Cystofilobasidium* (yeast) was the most abundant in all altitudes during SIAF conditions. Among the genus, *Cystofilobasidium ferigula* occurrence was at all altitudes with different relative abundances. This specie was formerly designated as *Cryptococcus ferigula* and has been previously isolated from leaves submerged in a stream from a natural park in Portugal (Sampaio et al., 2007). C*ystofilobasidium infirmominiatum* is naturally found in cold habitats (Hu et al., 2014), suggesting that its predominance is due to the region's characteristics and capability to resist low temperatures harvesting.

37.7% fungi were not present at all coffee growing altitudes, from which 17.16% represented the species that were only identified in coffee from 800 m, and 25.31%, 12.29%, and 5.74% in coffees from 1,000, 1,200, and 1,400 m. Therefore, even if coffee belongs to the same region, the altitude's influence on the niches was evident. Since most abundant fungi species were yeast, those who are culturable can be isolated, studied, and use as inoculants for future fermentations in the Caparaó region. For this purpose, yeasts in high abundance such as *Meyerozyma caribbica* and *Wickerhamomyces anomalus* can be further used. The capacity to produce polygalacturonase and pectin lyase enzymes from *Wickerhamomyces anomalus* has already been demonstrated (Haile and Kang 2019b). *Saccharomyces cerevisiae* was dominant in other coffee-producing regions (Silva et al., 2000; Evangelista et al., 2014b; Bressani et al., 2018), but not in this work.

The species below 1% abundance were clustered together, meaning they were not as influential as higher abundance species. Consequently, the NMSD plots showed that high abundance microbiota influences acids and volatiles contents, which was also confirmed in the Pearson correlations. The same behavior was seen for tartaric acid but with low abundance species.

The relative abundance of most filamentous fungi was within the 1%, which was expected since their populations usually dominate after several drying days due to reduced water activity (Silva et al., 2008b).

As expected, the alcohol group in this work had the highest number of compounds and content, possibly due to the high yeast abundance. Yeast uses the nitrogen compounds from amino acids to produce a pool of volatile alcohols (Dzialo et al., 2017), including phenylethyl alcohol, one of the alcohols detected in all altitudes. Coffees processed via the natural method in Evangelista et al. (2014b) and Bressani et al. (2020) had alcohols as the leading group during fermentation, and most were related to fruity odors. Like Bressani et al. (2020), high contents of 1-hexanol, 2-heptanol, benzyl alcohol, and benzaldehyde were also detected here. These volatiles compounds are essential for tea aroma (Ho et al., 2015).

In coffee, either alcohols or esters are significant because they confer the most sensed odor descriptors. In this study, low altitudes and microbiota are strongly associated with volatile alcohols; these were also the altitudes with the highest bacterial and fungal richness and probably influenced the alcohol quantity. Simultaneously, high altitudes and their microbiota are strongly associated with high contents of aldehydes and esters.

Caffeine, chlorogenic acids, and trigonelline concentrations in our work were in the same range as those previously detected at 800 m in Bressani et al. (2018). Caffeine is crucial because it confers bitterness to the beverage (Sunarharum et al., 2014). As for chlorogenic acids, they are responsible for pigmentation, astringency, and the production of volatile phenols (Duarte et al., 2010; Sunarharum et al., 2014). Trigonelline is responsible for the overall sensory perception. Most importantly, they all exert antioxidant properties. After fermentation, the coffees from higher altitudes contained higher concentrations of caffeine. Total phenolics are mainly composed of tannins and partly chlorogenic compounds (Farah and Donangelo, 2006). With the obtained results, it was observed that the concentration of chlorogenic acids was only a tiny part of total phenolics concentration, being supported when correlated (Figure 7).

Hence, the antioxidant activity depends on time, temperature, nature of the substance, and concentration of antioxidants or other compounds (Yashin et al., 2013). Concerning our fermented coffees, the altitude that contained the highest content of total phenolics (*i.e.*, 1,200 m) was the altitude with the highest antioxidant activity when measured by ABTS.

Conclusion

This work microbial and chemical characterization revealed a new perspective of why coffee from the Caparaó region is different from other Brazilian regions. The altitude and other region characteristics drive shifts in the microbiota profile and abundance, favoring yeast communities during fermentation. Moreover, altitude and high abundance of microbiota affect acetic and citric acid concentration and volatile compounds. 800 m coffee favors bacterial richness, and 1,000 m favors fungal richness during fermentation under SIAF conditions. Yeast that resists low temperatures dominates the Caparaó region coffee's (mainly from genus *Cystofilobasidium*). Dominant microbiota from different altitudes and controlled conditions by SIAF fermentations are the main drivers of biochemical compounds. Coffee from lower altitudes has higher contents of volatile alcohols, while high altitudes have higher esters, aldehydes, and total phenolic contents. Besides, the AAB function in coffee is still unknown; future approaches implementing AAB as inoculants need to be studied.

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Supplementary Material 1

Species less than 1% of abundance

(grey)	Not identified
> 1%	Species illustrated in Figure 3

Specie	Altitude 800	1000	1200	1400
Acremonium furcatum	0.170	0.020	0.006	
Acremonium hennebertii		0.019		0.017
Acrocalymma fici	0.007	0.027		
Acrocalymma walkeri	0.014	0.056	0.002	0.017
Alfaria terrestris		0.005		
Alternaria argyroxiphii	0.043	0.027	0.003	
Antennariella placitae	0.045			
Apiotrichum laibachii	0.069			
Aplosporella yalgorensis	0.050			
Articulospora proliferata	0.311	0.019	0.009	0.095
Aspergillus westerdijkiae	0.134		0.007	
Aureobasidium pullulans	0.033			
Bannoa ogasawarensis	**> 1%**	0.003	0.009	0.382
Barnettozyma californica		0.005		
Biatriospora mackinnonii			0.002	
Blastobotrys buckinghamii		0.011		
Boeremia exigua	0.557	0.370	0.131	0.147
Botrytis caroliniana	0.091	0.056	0.090	0.590
Brachyphoris oviparasitica		0.036		
Bulleromyces albus	0.036	0.017	0.003	0.017
Candida blattae		0.019		
Candida orthopsilosis		0.293	0.009	0.364
Candida parapsilosis		0.022	0.015	
Candida quercitrusa		0.039	0.065	0.199
Candida railenensis	0.763	0.072	**> 1%**	0.182
Candida sake	0.244			
Candida tropicalis	**> 1%**	0.182	0.162	0.156
Capitofimbria compacta	0.010			
Capnodium coffeae		0.006	0.007	
Catenulostroma hermanusense			0.005	
Citeromyces matritensis	0.017			
Cladosporium aphidis	0.724	0.450	0.161	0.616
Cladosporium dominicanum	0.158	0.005	0.006	

Cladosporium flabelliforme	0.074			
Cladosporium halotolerans	0.225	0.011	0.015	
Cladosporium sphaerospermum	0.648	0.670	0.036	0.442
Claviceps maximensis		0.011		
Clavispora lusitaniae		0.044		0.182
Clonostachys compactiuscula		0.008		
Clonostachys miodochialis			0.007	
Clonostachys rosea		0.033		
Clonostachys wenpingii		0.027		
Colletotrichum annellatum	0.033	0.024	0.025	0.017
Colletotrichum lupini	0.033		0.003	0.069
Colletotrichum theobromicola	0.057	0.138	0.033	0.087
Coniothyrium sidae	0.096	0.047	0.018	0.269
Cryptococcus dimennae	0.375	0.174		0.278
Cryptococcus saitoi		0.006		
Curvibasidium cygneicollum	0.124	0.008	0.005	
Curvularia americana	0.122			
Cutaneotrichosporon jirovecii			0.003	
Cutaneotrichosporon moniliiforme	0.060			
Cutaneotrichosporon terricola		0.075		
Cyberlindnera fabianii	0.519	0.033	0.008	
Cyphellophora eucalypti	0.091			
Cyphellophora europaea	0.301	0.024	0.010	0.147
Cyphellophora fusarioides	0.038			
Cyphellophora laciniata	0.031			
Cyphellophora vermispora	0.100		0.003	
Cystobasidium oligophagum		0.005		
Cystofilobasidium alribaticum	0.084	**> 1%**	0.392	0.052
Cystofilobasidium capitatum	**> 1%**	**> 1%**	0.135	**> 1%**
Cystofilobasidium intermedium		0.031		0.121
Debaryomyces hansenii	0.282	0.398	0.869	**> 1%**
Debaryomyces nepalensis	0.112	0.061	0.016	
Deltopyxis triangulispora	0.074	0.003		
Derxomyces anomalus	0.438	0.096	0.008	0.243
Didymella calidophila		0.053		
Didymella coffeae-arabicae	0.045	0.220	0.100	
Didymella nigricans		0.067		
Dimennazyma cistialbidi	0.069	0.019	0.070	0.295
Dioszegia var._yunnanensis	0.022	0.050	0.012	0.078
Diutina catenulata	0.227	0.044		0.139

Epicoccum draconis	0.208	0.184	0.019	
Epicoccum nigrum	0.617	0.347	0.098	0.902
Erythrobasidium hasegawianum	0.148	0.033	0.022	0.815
Eupenidiella venezuelensis			0.002	
Euteratosphaeria verrucosiafricana				0.087
Exophiala castellanii		0.099		
Exophiala phaeomuriformis		0.016		
Exophiala salmonis	0.053	0.238	0.002	
Fellomyces borneensis		0.077		
Fellomyces mexicanus	0.086			0.269
Filobasidium chernovii	0.045			
Filobasidium floriforme	0.048	0.041	0.008	0.043
Fusarium acutatum	0.220	**> 1%**	0.040	
Fusarium asiaticum	0.048	0.130		
Fusarium delphinoides	0.067	0.036		
Fusarium penzigii		0.008		0.026
Fusarium proliferatum	0.504	0.292	0.039	
Fusarium solani		0.158		
Gibberella intricans	**> 1%**	0.373	0.076	0.095
Hannaella kunmingensis	0.232	0.125		0.364
Hannaella luteola	**> 1%**	**> 1%**	0.070	**> 1%**
Hannaella oryzae	0.277	0.110	0.009	0.408
Hannaella siamensis		0.133		
Hannaella sinensis	**> 1%**	0.949	0.077	**> 1%**
Hannaella zeae	**> 1%**	**> 1%**	0.103	**> 1%**
Hanseniaspora uvarum	**> 1%**	0.661	0.206	0.356
Hansfordia pulvinata		0.025		
Holtermanniella wattica	0.084	0.011	0.012	
Kazachstania exigua		0.019	0.001	
Kazachstania gamospora		0.009		
Knufia tsunedae	0.055			
Kodamaea ohmeri	**> 1%**	0.362	0.417	**> 1%**
Lactarius saponaceus	0.822		0.134	**> 1%**
Lecanicillium antillanum	0.007	0.008		
Lectera colletotrichoides	0.036	0.006	0.009	
Leptoxyphium madagascariense		0.045		
Lodderomyces elongisporus	0.368		0.007	
Lophiotrema rubi		0.141		0.061
Macroventuria anomochaeta		0.105		0.078
Meyerozyma caribbica	0.873	**> 1%**	0.179	**> 1%**

Meyerozyma guilliermondii	0.904	0.130		
Mortierella ambigua			0.006	
Musicillium theobromae		0.082		
Mycosphaerella ellipsoidea	0.146	0.055		
Myrmaecium fulvopruinatum		0.006		
Myxospora aptrootii		0.011		
Naganishia albida	0.112	0.833	0.022	0.295
Naganishia diffluens		0.042		
Naganishia randhawae		0.442	0.041	0.278
Nakazawaea holstii			0.002	
Nectria balansae		0.005		
Neoascochyta paspali				0.225
Neodevriesia modesta	0.024			
Neonectria major	0.151	0.042		
Nigrospora oryzae	0.019	0.198	0.022	0.425
Occultifur externus	0.122			0.208
Papiliotrema flavescens	**> 1%**	**> 1%**	0.739	**> 1%**
Papiliotrema laurentii		0.130		
Papiliotrema perniciosus			0.019	
Paraconiothyrium archidendri		0.056	0.002	0.035
Paraconiothyrium fungicola	0.019	0.143	0.020	0.043
Paraconiothyrium variabile		0.009		
Penicillium kongii		0.067	0.007	0.052
Penicillium solitum		0.035	0.003	
Peniophora albobadia			0.005	
Peniophora laxitexta				0.061
Periconia byssoides	0.110	0.033	0.003	
Periconia cookei	0.007	0.006	0.002	
Periconia macrospinosa		0.009	0.003	
Phacidiella eucalypti	0.010	0.030		
Phaeosphaeria caricis		0.013		
Phaeosphaeria podocarpi	**> 1%**	0.869	0.140	0.885
Phialemoniopsis ocularis		0.011		
Phoma omnivirens	0.055	0.019		
Pichia kluyveri	0.148	0.011	0.015	
Pilidium concavum	0.167			
Plectosphaerella cucumerina		0.027		0.113
Pleurotus pulmonarius	0.103		0.040	0.078
Polyporus tricholoma			0.004	
Psathyrella luteopallida	0.029			
Pseudocercospora bixae	0.225	0.044	0.014	0.885

Pseudomerulius curtisii			0.010	
Pseudophaeomoniella oleae	0.084	0.030	0.011	0.199
Pseudoplectania affinis		0.045	0.004	
Pseudorobillarda phragmitis	0.120	0.006	0.003	
Pseudoteratosphaeria ohnowa			0.007	
Pyrenochaetopsis leptospora	0.163	0.201		0.061
Rachicladosporium cboliae	0.081	0.031	0.038	
Rachicladosporium paucitum			0.029	0.026
Resinicium friabile		0.008		
Rhodosporidiobolus fluvialis	0.373	0.144	0.014	0.173
Rhodosporidiobolus lusitaniae	0.074		0.023	
Rhodosporidiobolus odoratus	0.555	0.050	0.009	
Rhodosporidiobolus ruineniae				0.069
Rhodotorula araucariae		0.045	0.013	
Rhodotorula babjevae		0.042		
Rhodotorula dairenensis	0.423	**> 1%**	0.042	0.278
Rhodotorula diobovata	0.308	0.064	0.013	0.026
Rhodotorula mucilaginosa	**> 1%**	0.359	0.033	0.130
Rhodotorula taiwanensis		0.017		
Rhynchogastrema complexa		0.091		
Rhynchogastrema nanyangensis	0.897	0.323	0.035	0.165
Roussoella solani		0.031		
Saccharomyces cerevisiae		0.006	0.003	
Saitozyma flava	0.026	0.011		
Saitozyma paraflava	0.026	0.127	0.007	
Saitozyma podzolica		0.003	0.083	
Sampaiozyma vanillica	0.010			
Schizophyllum commune	0.072		0.003	
Scolecobasidium terreum		0.014		
Selenophoma mahoniae	0.022	0.085	0.025	
Septoria cretae	0.147	0.040	0.117	0.052
Setophoma chromolaenae	0.012	0.187	0.007	0.182
Setophoma terrestris		0.025		
Sirobasidium brefeldianum			0.002	
Solicoccozyma terrea		0.009		0.347
Sphaeropsis citrigena		0.024		
Sporobolomyces johnsonii				0.078
Sporobolomyces koalae	0.655	0.331	0.039	0.130
Strelitziana africana	0.703	0.579	0.122	**> 1%**
Strelitziana eucalypti	0.014	0.042	0.006	0.104
Symmetrospora coprosmae	0.065	0.014		0.078

Symmetrospora vermiculata	0.077		0.014	0.139
Taphrina inositophila	0.019			
Torulaspora delbrueckii	0.062	0.036	0.005	
Toxicocladosporium irritans	0.222	0.052	0.001	0.052
Toxicocladosporium strelitziae	0.206	0.041		
Trametes hirsuta	0.124			
Trichomerium foliicola	0.115			
Trichosporon asahii	0.050		0.004	
Trichosporon coremiiforme	0.284	0.020	0.012	0.052
Udeniomyces pyricola	0.746	0.684	0.048	0.410
Vishniacozyma dimennae	0.148	0.267	0.014	0.902
Vishniacozyma foliicola	0.115	0.218	0.091	0.945
Vishniacozyma heimaeyensis	**> 1%**	0.045	0.053	
Vishniacozyma taibaiensis	**> 1%**	**> 1%**	0.240	**> 1%**
Vishniacozyma victoriae	0.031		0.029	
Volutella consors		0.121		
Wallemia hederae			0.006	
Wickerhamomyces ciferrii	**> 1%**	0.130	0.035	0.087
Wickerhamomyces lynferdii	0.602	0.347	0.200	0.460
Wickerhamomyces pijperi		0.011		
Wickerhamomyces sydowiorum			0.009	
Wickerhamomyces xylosica		0.022	0.008	
Xeromyces bisporus	0.057	0.102		0.035
Zymoseptoria verkleyi	0.017			

Supplementary Material 2

800 m	1000 m	1200 m	1400 m
Candida sake	*Fusarium solani*	*Papiliotrema perniciosus*	Neoascochyta paspali
Pilidium concavum	*Hannaella siamensis*	*Pseudomerulius curtisii*	Euteratosphaeria verrucosiafricana
Trametes hirsuta	*Papiliotrema laurentii*	*Wickerhamomyces sydowiorum*	Sporobolomyces johnsonii
Curvularia americana	*Volutella consors*	*Clonostachys miodochialis*	Rhodosporidiobolus ruineniae
Trichomerium foliicola	*Exophiala castellanii*	*Pseudoteratosphaeria ohnowa*	Peniophora laxitexta
Cyphellophora eucalypti	*Rhynchogastrema complexa*	*Mortierella ambigua*	
Cladosporium flabelliforme	*Musicillium theobromae*	*Wallemia hederae*	
Apiotrichum laibachii	*Fellomyces borneensis*	*Catenulostroma hermanusense*	
Cutaneotrichosporon moniliiforme	*Cutaneotrichosporon terricola*	*Peniophora albobadia*	
Knufia tsunedae	*Didymella nigricans*	*Polyporus tricholoma*	
Aplosporella yalgorensis	*Didymella calidophila*	*Cutaneotrichosporon jirovecii*	
Antennariella placitae	*Leptoxyphium madagascariense*	*Nakazawaea holstii*	
Filobasidium chernovii	*Naganishia diffluens*	*Biatriospora mackinnonii*	
Cyphellophora fusarioides	*Rhodotorula babjevae*	*Eupenidiella venezuelensis*	
Aureobasidium pullulans	*Brachyphoris oviparasitica*	*Sirobasidium brefeldianum*	
Cyphellophora laciniata	*Clonostachys rosea*		
Psathyrella luteopallida	*Roussoella solani*		
Neodevriesia modesta	*Clonostachys wenpingii*		
Taphrina inositophila	*Hansfordia pulvinata*		
Citeromyces matritensis	*Setophoma terrestris*		
Zymoseptoria verkleyi	*Sphaeropsis citrigena*		
Capitofimbria compacta	*Candida blattae*		
Sampaiozyma vanillica	*Rhodotorula taiwanensis*		
	Exophiala phaeomuriformis		
	Scolecobasidium terreum		
	Phaeosphaeria caricis		
	Blastobotrys buckinghamii		
	Claviceps maximensis		
	Myxospora aptrootii		

Phialemoniopsis ocularis
Wickerhamomyces pijperi
Kazachstania gamospora
Paraconiothyrium variabile
Clonostachys compactiuscula
Resinicium friabile
Cryptococcus saitoi
Myrmaecium fulvopruinatum
Alfaria terrestris
Barnettozyma californica
Cystobasidium oligophagum
Nectria balansae

ARTICLE 2 - A biostudy of pulped natural fermented coffees from different altitudes: the dominant microbial communities and biochemical profile

Article within the guidelines of the Food Microbiology Journal

Abstract

Altitude changes the coffee fruits and beans composition before and after harvesting. We aimed to evaluate the effect of altitude in the microbial community structure associated with coffee fruits under self-induced anaerobic fermentation (SIAF) and on their acids, volatiles, and antioxidants biochemical profiles. The most abundant bacterial genera were *Gluconobacter* (800 m), *Weissella* (1,000 m), and *Leclercia* (1,200 and 1,400 m). Yeasts dominated the pulped natural fermentations within the fungal species, containing high abundances of *Cystofilobasidium infirmominiatum*, *Wickerhamomyces anomalus*, and *Meyerozyma caribbica*. Citric, alcohols, and caffeine were the most dominant compounds in SIAF among acids, volatiles, chemical group, and antioxidants, respectively. High altitude coffees favor alcohols, aldehydes, and esters groups, while low altitude coffees favored phenols.

Keywords: coffee fermentation, anaerobic, Illumina sequencing, organic acids, antioxidants, volatiles

1. Introduction

Since the coffee discovery in Ethiopia, its spread worldwide followed new cultivation systems and consumption forms (Guimarães et al., 2019). *Coffea arabica* L. cultivation requires temperatures between 19 and 22 ºC, well-distributed rainfalls from 1,200 to 1,800 mm, and soils with a medium texture and higher capacity to retain moisture (DaMatta et al., 2007; Guimarães et al., 2019). Besides those conditions, an adequate altitude is also a determinant factor of coffee quality. For instance, higher altitudes are frequently associated with coffees containing higher acidity and better aroma characteristics (Alpizar and Bertrand, 2004; Guimarães et al., 2019), apart from influencing the bean size, acid, caffeine, fats, and trigonelline contents (Guyot et al., 1996; Bertrand et al., 2006). According to da Mota et al. (2020), high lactic acid contents are associated with low altitude coffees processed via natural.

Fermentation is a biochemical process conducted by natural microorganisms where carbohydrates or any organic compound are transformed into other compounds while energy is liberated. The practice of fermenting coffee enriches the available coffee compounds, increases attribute variability, and improves sensory scores and quality (Evangelista et al., 2014a; Evangelista et al., 2015; Ribeiro et al., 2017b). Several fermentation methods have emerged through the years, and those are yeast inoculation through spraying, open and closed batch (Evangelista et al., 2014a; Martinez et al., 2017; Bressani et al., 2018; Martins et al., 2020; da Mota et al., 2020). A new method called Self-Induced Anaerobic Fermentation (SIAF) was introduced within the closed batch fermentation, favoring anaerobic conditions and gas production by inoculated yeasts in bioreactors. The SIAF method ensures the best microbial performances and control, favoring pyrroles and furans groups in roasted coffee beans (da Mota et al., 2020). Conversely, open batch fermentations favor other chemical groups in roasted beans, such as pyrazines and pyridines (Martinez et al., 2017). They are indicating that depending on the applied fermentation method, and the coffee compounds profile changes.

The processing method also contributes to the coffee profile variations and its microbiota. After harvesting, coffee fruits are processed via three methods: natural (known as dry), wet, and pulped natural (known as semidry) (Schwan et al., 2012; Batista et al., 2016a). As the coffee collection in Brazil is carried out, mainly with machinery, the fruits are collected at different maturation stages. Consequently, the pulped natural method was innovated and became an alternative to avoid critical selection, which facilitated processes and lowered losses. The method turned into an intermediate process between the natural and wet method, where fruits are depulped as in the wet method and after beans are spread in cement or suspended platforms for aerobic fermentation and drying as in the natural method (Schwan et al., 2012; Evangelista et al., 2014b; Batista et al., 2016a).

A variety of microbial communities dominate in the different processing methods. For example, in open batch fermentations with coffees processed via pulped natural, bacteria dominate at first, followed by yeasts (Vilela et al., 2010; Martinez et al., 2017). The bacteria constantly identified in this process belong to *Bacillus*, *Acinetobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Serratia*. As for yeasts, the genera belong to *Arxula*, *Candida*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces*, and *Torulaspora*. Similarly, the same dynamic was observed in close batch fermentation lasting 72 h, with bacterial and fungal genera belonging to *Acinetobacter*, *Enterobacter*, *Erwinia*, *Gluconobacter*, *Leuconostoc*, *Micrococcus*, *Pantoea*, *Serratia*, *Sphingomonas*, *Staphylococcus*, *Tatumella*, *Cystofilobasidium*, *Debaryomyces*, *Hanseniaspora*, *Lodderomyces*, *Meyerozyma*, *Pichia*, *Rhodotorula*, and *Wickerhamomyces* (Martins et al., 2020).

Although some genera are shared due to the processing method, others are characteristic of the region (Martinez et al., 2021). Changes in the microbial communities influence the sensory profile and attribute type and dominance (Evangelista et al., 2014b; Martins et al., 2020; Bressani et al., 2021). Therefore, it is important to study the dominant communities of fermentation carried out under different conditions. This work aims to study the dominant communities from fermentations with coffees collected at different altitudes and processed via pulped natural using Illumina high-throughput sequencing. Study the effect of altitude and microbial communities on the biochemical compounds profile (acids, volatiles, and antioxidants) during the dominant fermentative period.

2. Material and Methods

2.1 Coffee processing and SIAF

Fruits of *Coffea arabica* cv Catuaí Vermelho IAC-44 were manually collected from different altitudes: 800, 1,000, 1,200, and 1,400 m at the Caparaó region (Brazil). They were then processed via the pulped natural method. After depulping the fruits, the beans for fermentation are obtained. Later they are transferred into 20 L polypropylene bioreactors with lids, then closed for SIAF. The fermentations were performed in triplicate.

All coffee fermentative processes were carried out simultaneously in closed batches at a farm located at 1,200 m to avoid any environmental interference. The bioreactors were placed under an open storage house for fermentation. Portable data loggers (INKBIRD) were placed in the bioreactors to register the mass temperature during fermentation. Fermentation lasted 72 h, and sub-samples of approximately 100 g were taken after 48 h of fermentation for dominant microbiota profiling and metabolites evaluation. Fruits' initial sugar content (Brix degree-°Bx) was measured with a refractometer (Sigma-Aldrich, Germany). We also measured the bean weight before fermentation and the farm environmental temperature during fermentation.

2.2 Composition and abundance of bacteria and fungi communities

2.2.1 DNA extraction

Total DNA was extracted from 48 h fermented coffee beans belonging to 800, 1,000, 1,200, 1,400 m of altitude. 100 g of beans were vortexed in 50 ml sterilized Milli-Q water for 10 min. Then the resulted suspension was transferred to another tube and centrifuged (12,745 x g for 10 min at 4 ºC). 30 mg of the pellet was used for DNA extraction with the QIAamp DNA Mini Kit, following the "DNA Purification from Tissues" protocol (Qiagen, Hilden, Germany). The extracted DNA purity was checked with a Nanodrop Lite spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) (260/280 nm ratio), quantified by Qubit® 4.0 fluorometer using the dsDNA HS Assay kit (Invitrogen™), and its integrity was confirmed by electrophoresis in a 0.8 % agarose gel with 1 X TAE buffer.

2.2.2 Illumina high-throughput sequencing of bacterial/archaeal 16S rRNA genes and fungal internal transcribed spacer (ITS)

The V3-V4 regions of the 16S rRNA gene of bacteria/archaea and the ITS1 and ITS2 regions of fungi were amplified from the total DNA extracted. The primers used for sequencing were 341F (5'- CCTACGGGNGGCWGCAG -3') and 806R (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013) for bacteria/archaea, and the ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA -3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (Gardes and Bruns, 1993; Smith and Peay, 2014) for fungi.

Samples were paired-ended sequenced (2x 250 bp) on an Illumina MiSeq platform, using the V2 kit (Illumina Inc), at the NGS Soluções Genômicas in Piracicaba-Sao Paulo, Brazil.

2.2.3 Illumina high-throughput sequencing data processing

The raw .fastq files were processed and used to build a table of amplicon sequence variants (ASVs) with dada2 version 1.12 (Callahan et al., 2016). Briefly, the raw data quality was evaluated, filtered, and trimmed. The filtering parameters were maxN= 0, truncQ= 2, rm.phix= TRUE, maxEE=(2,2) and truncLen (235, 230). The truncLen parameter was not applied for ITS1, and ITS2 reads since the expected sequence length is variable for fungi. Then, the forward and reverse reads were merged to obtain a full denoised sequence, and a higher-resolution table of amplicon sequence variants (ASVs) was constructed. Only ASVs with total abundances higher than 0.1% are reported. Chimeric sequences were detected and removed. Taxonomy was assigned to each ASV using the RDP ribosomal RNA gene database (version 11.5) for the 16S rRNA gene and with UNITE database (version 8.2) for fungal ITS. Sequences were matched the reference sequence with 100% identity.

2.3 Metabolites analysis

2.3.1 Organic acids evaluation

The organic acids in the beans were evaluated after 48 h of fermentation. Three grams of beans were homogenized in Falcon tubes containing 20 mL of 16 mM perchloric acid and Milli-Q water at room temperature for 10 min. The extracts were centrifuged at 10,000 x g for 10 min, at 4 ºC. The pH of the supernatant was adjusted to 2.11 using perchloric acid and recentrifuged under the same conditions. Then, the supernatant was filtered through a 0.22 μm cellulose acetate membrane. 20 μL of the filtered supernatants were analyzed using a high-performance liquid chromatography (HPLC) system (Shimadzu Corp., Japan) equipped with a detection system consisting of a UV–Vis detector (SPD 10Ai) and a Shimpack SCR-101H (7.9 mm 30 cm) column, operating at 50 ºC, to achieve chromatographic separation of water-soluble acids at a flow rate of 0.6 mL min^{-1}. The acids were identified by comparison with the retention times of authentic standards, and the quantification was performed using calibration curves constructed with standard compounds. Malic and citric acid were purchased from Merck (Germany), lactic and tartaric acid was purchased from Sigma-Chemical (Saint Louis, MO, USA), acetic and succinic acids were purchased from Sigma-Aldrich, isobutyric and butyric acid were purchased from Riedel-de Haen (Germany). All analyses were performed in duplicate.

2.3.2 Caffeine, Trigonelline, and Chlorogenic acids by HPLC

Caffeine, chlorogenic acid [5-CGA], and trigonelline were identified using a Shimadzu liquid chromatography system (Shimadzu Corp., Japan) equipped with a C18 column, according to Malta and Chagas (2009). 0.5 g of grounded coffee beans were transferred to tubes containing 50 mL Milli-Q water and boiled for 3 min for extraction. Identification and quantitative analysis were performed using caffeine calibration curves, trigonelline, and 5-CGA (Sigma-Aldrich, Saint Luis, EUA). All analyses were performed in duplicate.

2.3.3 Total polyphenols and antioxidant activity

Coffee samples were defatted according to Batista et al. (2016b). Four grams of each coffee sample was grounded with liquid nitrogen. Then, 20 mL of n-hexane (Merck) was added, homogenized for 5 min, and centrifuged at 4,200 x g at 4 ºC for 10 min., to eliminate lipids. This step was performed three times. Then the lipid-free samples were air-dried for 24 hours to evaporate the residual organic solvent.

The polyphenol and antioxidants were extracted according to Kim et al. (2018), with minor modifications. The amount of ground coffee used was 2.75 g in 50 mL of distilled water, at 90 °C, following the recommendations of the Specialty Coffee Association (SCA, 2018). The extract was left by standing at room temperature for 20 min., followed by filtration through a Whatman No. 2 filter paper.

2.3.3.1 Determination of total polyphenol content (TPC)

Total polyphenol contents (TPC) were determined by spectrophotometric assays (UV-VIS Spectrum SP-2000 UV, Biosystems), following the Follin – Ciocalteau methodology (Singleton and Rossi, 1965). 500 µL of coffee extract, 2.5 mL of Folin–Ciocalteau reagent (10%), and 2.0 mL of Na_2CO_3 (4% w/v) were homogenized and incubated at room temperature in the dark for 120 min. The sample absorbance was measured at 750 nm. TPC concentrations were calculated based on the standard curve of gallic acid (ranging from 10 to 100 µg mL^{-1}) and expressed as milligrams of gallic acid equivalents per gram of ground coffee (mg GAE g^{-1}). All analyses were performed in triplicate.

2.3.3.2 Antioxidant Activity Assays

Two different methodologies were applied to measure the antioxidant activity of coffee extracts. In the first one, the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging assay was performed as follows: 0.1 mL of coffee extract was added to 3.9 mL of the DPPH radical solution (0.06 mM) and incubated at room temperature, in the dark for 120 min, then the absorbance was measured at 515 nm. Trolox was used as a standard. A calibration curve ($y = -0.0004x + 0.6636$) was assembled using a range of 10, 20, 30, 40, 50 and 60 μM Trolox with linearity R2 = 0.9999 (Batista et al., 2016b). The results were expressed as μM Trolox Equivalents (TE) per gram of ground coffee (μM TE g^{-1}).

The second assay was performed with a 2,2′-azinobis (3-ethylbenzothiazoline-6-suslfonic acid) (ABTS) stock solution reaction (7 mM) with potassium persulphate (140 mM). After, the mixture was left in the dark at room temperature for 16 h before use. The ABTS solution was diluted in ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. Thirty microliters of the coffee extracts were added to 3.0 mL of the ABTS radical solution and after 6 min. The absorbance was measured. Trolox was used as a standard. A calibration curve ($y = -0.0003x + 0.6802$) was assembled using a range of 100, 500, 1,000, 1,500 and 2,000 μM Trolox with linearity of $R^2 = 0.9983$. The results were expressed as μM Trolox Equivalents (TE) per gram of ground coffee (μM TE g^{-1}).

2.3.4 Volatile compounds

Volatile compounds were extracted from 48 h fermented beans using a headspace solid-phase microextraction (HS-SPME). 2 g of beans were macerated with liquid nitrogen and placed in a 15 ml hermetically sealed vial. After equilibration at 60 °C for 15 min, the volatile compounds were extracted at 60 °C for 30 min, and the column desorption time was 2 min.

A Shimadzu QP2010 GC with a silica capillary Carbo-Wax 20M (30 m × 0.25 mm × 0.25 mm) column, equipped with mass spectrometry (MS), was used to separate and identified the compounds, respectively. The operation conditions were the following: the oven temperature was maintained at 50 °C for 5 min., then raised to 200 °C at 8 °C $min.^{-1}$ and maintained for 15 min.

The injector and detector were kept at 230 and 200 °C, respectively, and He carrier gas was maintained at a flow rate of 1.9 ml $min.^{-1}$. The volatile compounds were identified by comparing their mass spectra against those available in the NIST11 library. The retention index (RI) for each compound was calculated using an alkane series (C10–C40) compared with those found in the literature.

2.4 Statistical analysis

Alpha and beta diversity analyzes were estimated for the evaluated microbial communities. The microbial richness and abundance in each altitude were used to calculate the bacterial and fungal Shannon and Simpson diversity indices. The relative abundances were calculated using the XLSTAT software (Addinsoft, version 2020.1.3). Bray-Curtis-based non-metric multidimensional scaling (NMDS) was used to evaluate the dissimilarities between the fungal community and organic acids and volatile compounds with the XLSTAT software (Addinsoft, version 2020.1.3).

The raw data normal distribution was evaluated with the Shapiro-Wilk and Anderson-Darling tests. All values in the figures are expressed as averages. Standard deviations were calculated using the XLSTAT software (Addinsoft, version 2020.1.3). The Tukey test was run with $p \leq 0.05$ to evaluate the difference in acid concentration and antioxidants concentration and activity in the SISVAR software (Ferreira, 2014). The principal component analysis was run between the bacterial communities and acids and volatile groups using the XLSTAT software (Addinsoft, version 2020.1.3). The volatile groups' heat map was constructed using the Origin software (version 2021) and the Venn diagram with a free online program available for academic use (https://web.rniapps.net/netsets/).

3. Results

3.1 Sugar content, weight of beans and coffee mass temperature

Coffee beans initial ºBrix varied from 16 to 20, with the highest content in beans from altitude 1,200 (Table 1). Bean weight increased with altitude, from 0.30 to 0.41 g (Table 1). At the beginning of fermentation, the coffee mass temperature was 18 ºC for all altitudes, increasing between 2 - 4 ºC at 24 and 48 h (Table 1). The highest temperature (22.5 ºC) was observed for

1,000 m coffee with 48 h. The farm environmental temperature oscillated from 8 ºC to 23.1 ºC during fermentation.

Table 1. Beans characteristics, coffee mass temperature, and microbial diversity indices. Data is expressed as Mean ± SD.

Coffee Altitude (m)	Initial Brix (ºBx)	Bean weight (g)	Coffee mass temperature (ºC)			Bacterial Diversity Indices		Eukaryotic Diversity Indices	
			0 h	24 h	48 h	Shannon	Simpson	Shannon	Simpson
800	18 ± 2	0.30 ± 0.1	18 ± 0	21.5 ± 0.7	22 ± 0	1.391 ± 0.2	3.442 ± 0.7	3.403 ± 0.1	13.459 ± 2.4
1,000	18 ± 0	0.32 ± 0.1	18 ± 0	20.5 ± 0.7	22.5 ± 0.7	1.205 ± 0.5	2.707 ± 1.0	3.341 ± 0.1	14.800 ± 4.3
1,200	20 ± 1	0.35 ± 0.1	18 ± 0	21.5 ± 0.7	21.5 ± 0.7	1.082 ± 0.2	2.295 ± 0.6	2.046 ± 0.1	2.838 ± 0.5
1,400	16 ± 1	0.41 ± 0.2	18 ± 0	21 ± 1.4	22 ± 1.4	1.367 ± 0.2	3.395 ± 1.4	3.388 ± 0.1	14.780 ± 2.1

SD: Standard deviation

3.2 Effect of altitude on dominant bacterial and fungal communities

At altitudes 800, 1,000, 1,200, and 1,400 m we obtained a total of 43.146, 61.038, 41.794, and 51.939 filtered 16S rRNA partial gene sequences and 168.182, 152.259, 174.959, and 103.160 filtered partial ITS sequences, respectively.

Coffee from altitude 800 m had the highest bacterial richness, with 7 genera assigned, while those from 1,000 m had the highest fungal richness, with 126 species. The alpha-diversity indices for bacteria decrease as altitude increases up until 1,200 m. However, similar values were estimated for 800 m and 1,400 m (Table 1).

A total of 12 genera were assigned to the bacterial community (Figure 1). At 800, 1,000, 1,200, and 1,400 m most sequences were assigned to *Gluconobacter* (43.7%), *Weissella* (54.1%), *Leclercia* (61.1%), and *Leclercia* (38.1%), respectively. The following species were distinctive of each altitude: *Methylobacterium*, *Mangrovibacter*, and *Azotobacter* (at 800 m), *Ochrobactrum* (at 1,000 m), *Rosenbergiella*, and *Yersinia* (at 1,200 m), and *Curtobacterium* (at 1.400 m). *Leclercia* and *Weissella* were found in all altitudes. *Gluconobacter* was only found in altitudes 800, 1,000 (14.8%), and 1,200 (12.2%). *Leuconostoc* was only found in altitudes 800 (18.3%), 1,000 (22.0%), and 1,400 (7.5%).

A total of 203 species were assigned to the fungal community. The species with a relative abundance (RA) above 1% are exhibited in Figure 1. The most abundant species at altitudes 800, 1,000, and 1,200 m were Cystofilobasidium infirmominiatum with 20.819%, 16.841%, and 58.282% (Figure 1). The yeast *Wickerhamomyces anomalus* was the second most abundant species at 800 m (10.99%), *Meyerozyma caribbica* (10.346%) at 1,000, and *Papiliotrema flavescens* (3.719%) at 1,200 m. *Cladosporium delicatulum* was the most abundant species (18.74%) at 1,400 m, followed by *C. infirmominiatum* (10.42%) (Figure 1). Species with RA below 1% are available in Supplementary Material 1.

Out of the total species, only 49(24.14%) were detected in all altitudes. Some of those species belong to *Candida railenensis*, *C. tropicalis*, *Cystofilobasidium capitatum*, *C. ferigula*, *Debaryomyces hansenii*, *D. nepalensis*, *Hannaella sinensis*, *H. zeae*, *Hanseniaspora uvarum*, *Rhodotorula mucilaginosa*, *Torulaspora delbrueckii*, *Wickerhamomyces anomalus*, *W. ciferrii*, and others (Supplementary Material 1).

Other species were altitude-specific. From the 203 species, only 21 (10.34%) were only detected in fermented beans from 800 m altitude, 32 species (15.76%) from 1,000 m, and 17 (8.37%) and 16 (7.88%) species from 1,200 and 1,400 m coffee, respectively. For more detailed information, see Supplementary Material 1.

The yeast *Candida parapsilosis* and *Saccharomyces cerevisiae*, commonly found in coffee, were detected in lower abundance than other species and altitudes. *C. parapsilosis* was present in fermented coffee beans from 800 (RA: 0.73%) and 1,000 m (RA: 0.08%), and *S. cerevisiae* was present in beans from 1,000 (RA: 0.01%) and 1,200 m (RA: 0.02%).

Filamentous fungi were also detected, including *Alternaria argyroxiphii*, *Aspergillus westerdijkiae*, *Colletotrichum theobromicola*, *Cladosporium aphidis*, *Cladosporium halotolerans*, *Fusarium acutatum*, and others (Supplementary Material 1).

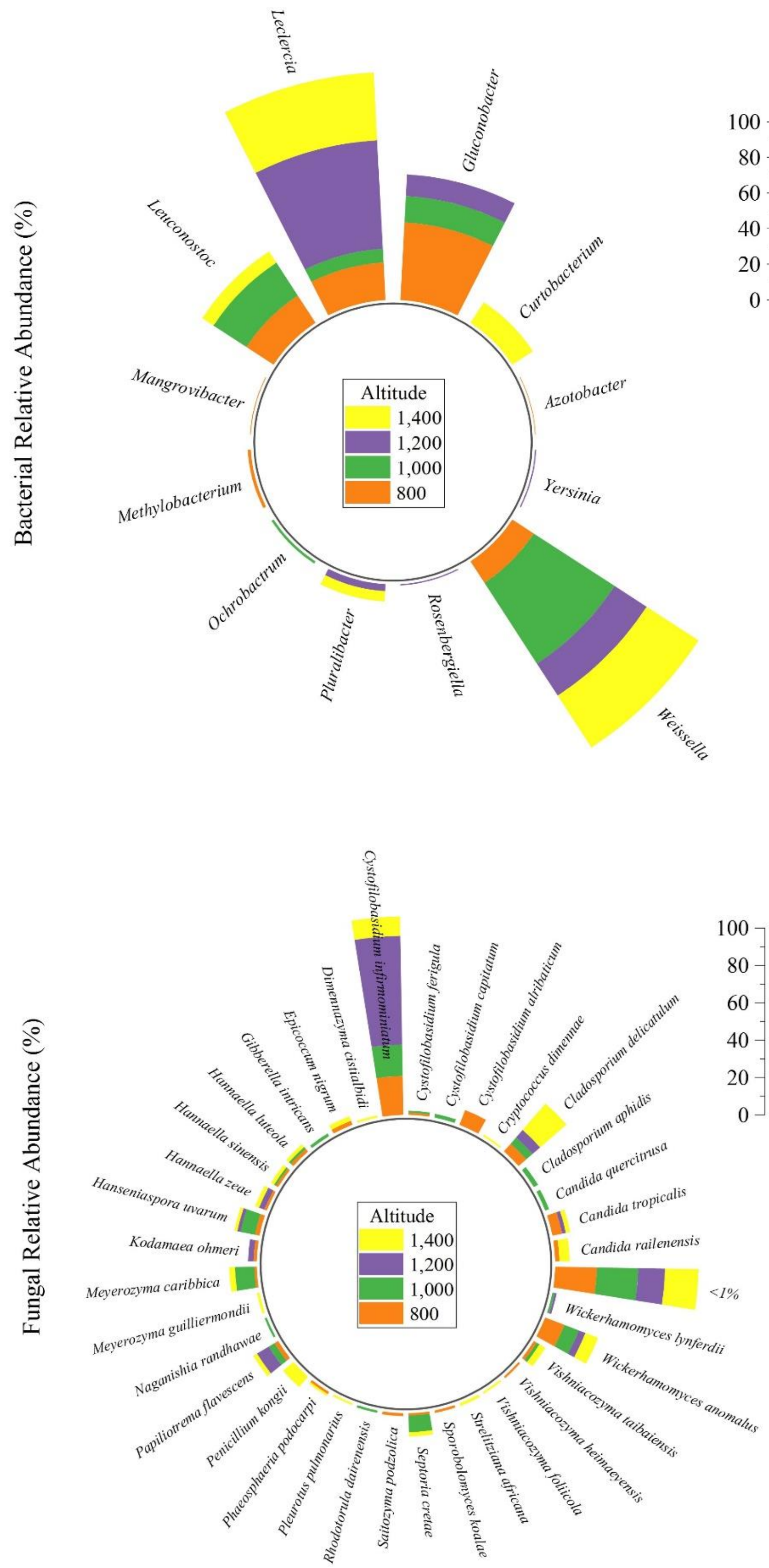


Figure 1. Relative abundances of the bacterial and fungal communities at different altitudes.

3.3 Effect of altitude on biochemical compounds

3.3.1 Organic acids

The acids (citric, tartaric, malic, succinic, lactic, and acetic) detected were different and varied from altitude (Figure 2A). Citric acid had the highest content at 800 (0.57 g·Kg^{-1} ± 0.15), 1,200 (1.37 g·Kg^{-1} ± 0.71), and 1,400 m (2.27 g·Kg^{-1} ± 0.39). Tartaric acid was only found at 1,200 m, with 0.16 g·Kg^{-1} ± 0. Malic acid content was high at high altitudes, with 0.94 ± 0.61 (1,200 m) and 1.43 ± 0.26 (1,400 m) g·Kg^{-1}. Similarly, higher altitude coffees had the highest acetic acid contents, with 1.08 ± 0.21 (1,200 m) and 0.98 ± 0.33 (1,400 m) g·Kg^{-1}. The highest succinic acid content was found at 1,400 m, with 0.51 g·Kg^{-1} ± 0.08, followed by 1,200 m (0.37 g·Kg^{-1} ± 0.18). Higher and equal contents of lactic acid were found at 1,000 and 1,400 m.

The PCA in Figure 2B shows that acids from 800 and 1,000 had similar profiles, while acids from 1,200 and 1,400 m had different profiles from the other altitudes.

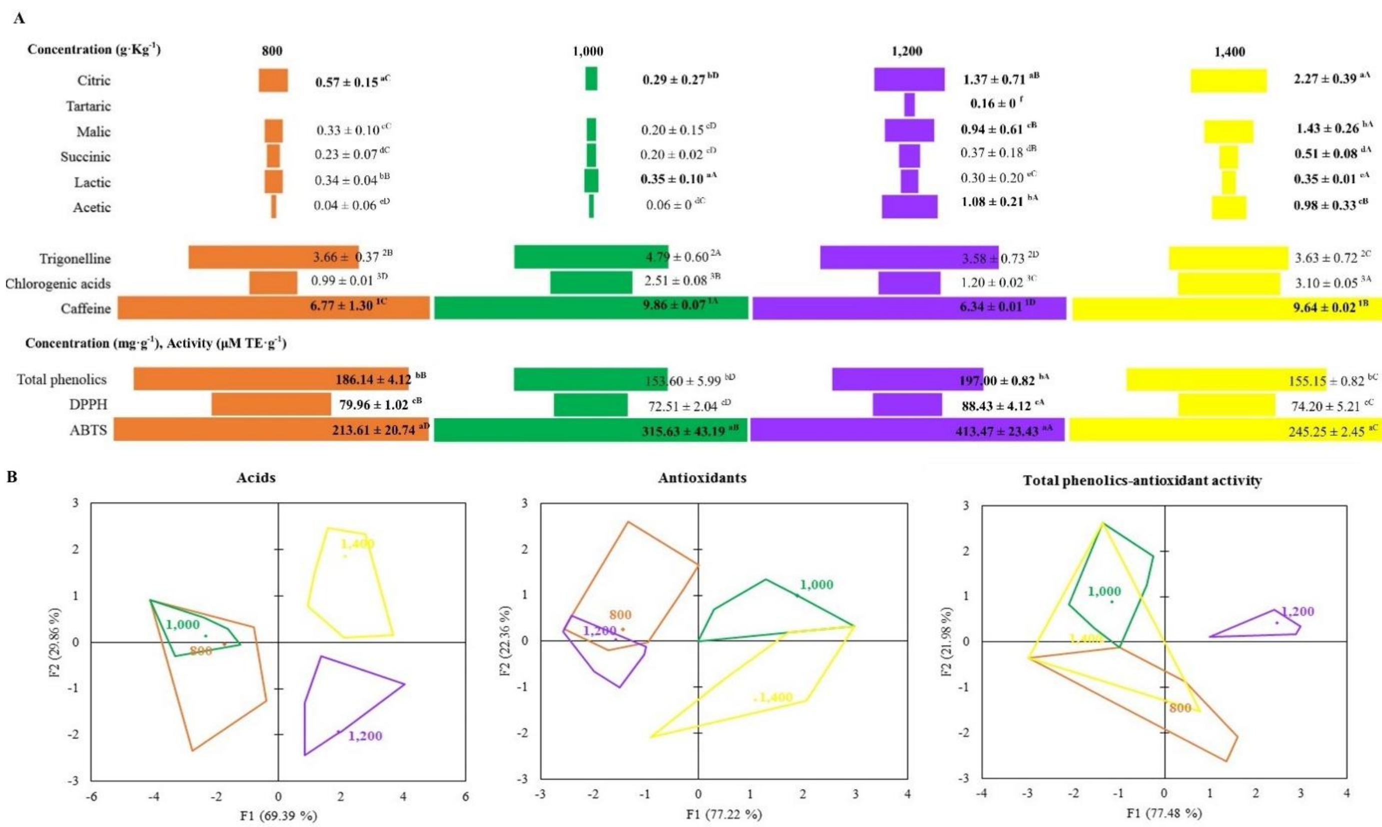


Figure 2A. Funnel diagrams representing the organic acids, antioxidant compounds, and total phenolics concentration, and antioxidant activity at different altitudes. Values are expressed as mean ± standard deviation. Significant values ($p \leq 0.05$) are represented in letters and numbers. In organic acids different lowercase letters indicate the difference among them within each altitude and uppercase letters indicate the difference among the altitudes within each acid. In antioxidants different numbers indicate the difference among them within each altitude and uppercase letters indicate the difference among the altitudes within each antioxidant. In total phenolics and antioxidant activity different lowercase letters indicate the difference among them within each altitude and uppercase letters indicate the difference among the altitudes within total phenolics and activity. **2B.** Confidence bootstraps of acids, antioxidants, total phenolics, and antioxidant activity.

3.3.2 Antioxidants: trigonelline, chlorogenic acids, and caffeine

Caffeine was the compound with the highest content in the different altitudes, followed by trigonelline and chlorogenic acids (Figure 2A). The highest caffeine contents were at 1,000 and 1,400 m, with 9.86 and 9.64 $g \cdot Kg^{-1}$, respectively. Lower altitude coffees had significantly high contents of trigonelline ($g \cdot Kg^{-1}$: 3.66- 800 m and 4.79- 1,000 m) than high altitude coffees ($g \cdot Kg^{-1}$: 3.58- 1,200 m and 3.63- 1,400 m). The highest content of chlorogenic acids was observed at 1,400 m, with 3.10 $g \cdot Kg^{-1}$.

The antioxidants at 800 and 1,200 m have similar profiles. However, the antioxidants profiles at 1,000 and 1,400 m are close but different from the other altitudes (Figure 2B).

3.3.3 Total phenolics and antioxidant activity

The altitude with the highest total phenolics content was 1,200 m, with 197.00 $mg \cdot g^{-1}$, followed by 800 m, with 186.14 $mg \cdot g^{-1}$. The exact altitudes had the highest DPPH activity, with 88.43 $\mu M\ TE \cdot g^{-1}$. In contrast, ABTS activity increase with altitude, up to 1,200 m.

Altitudes 800, 1,000, and 1400 m showed similar phenolics and antioxidant activity profiles (Figure 2B); only the 1,200 m profile was different.

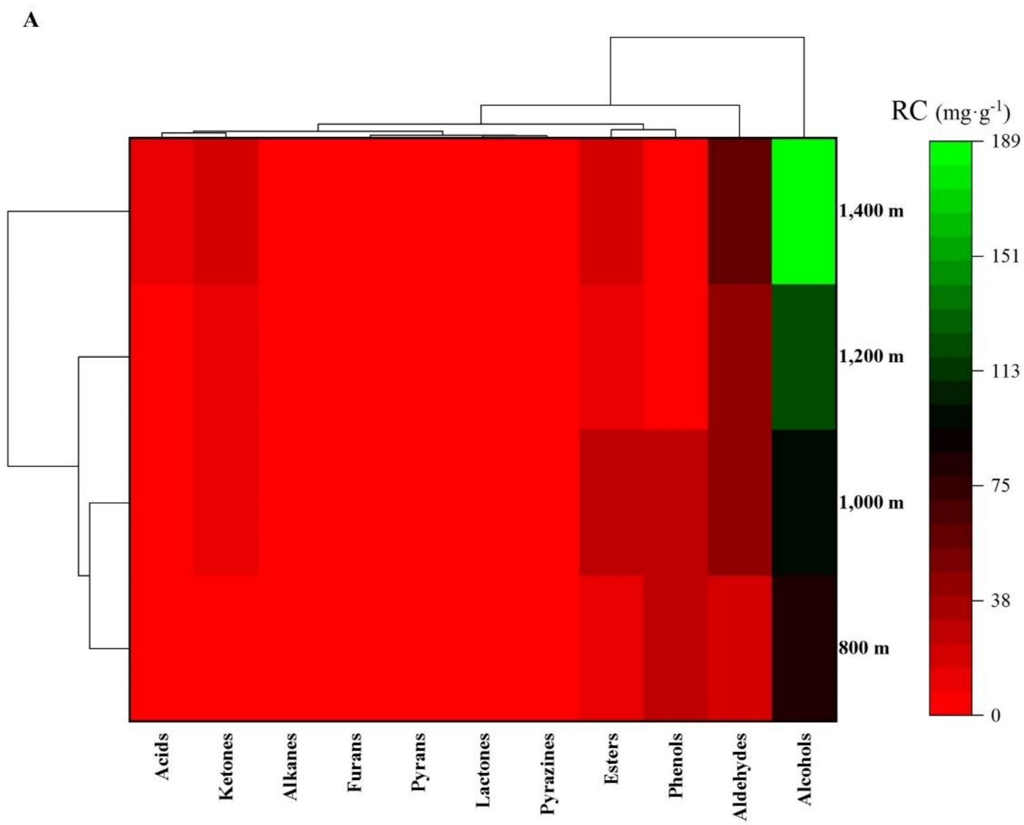
3.3.4 Volatiles

51 volatile compounds were identified and grouped into 11 chemical groups: acids, alkanes, furans, pyrans, lactones, pyrazines, ketones, esters, phenols, aldehydes, and alcohols. The chemical group with the highest relative concentration in all altitudes was alcohols, followed by aldehydes (Figure 3A). Alcohol's concentration increases with altitude, being highest at 1,200 and 1,400 m, with 120 and 188 $mg \cdot g^{-1}$, respectively. Aldehydes content also increased with

altitude, however phenols concentration decreased while altitude increase ($mg{\cdot}g^{-1}$: 23.8- 800, 28.4- 1,000, 6.5- 1,200, and 6.7- 1,400 m). Similarly, lactones concentration decreased with altitude increase. Ketones content increase with altitude, starting at 5.7 (800 m) and ending at 17.7 $mg{\cdot}g^{-1}$ (1,400 m). High acids concentration (14.3 $mg{\cdot}g^{-1}$) was only recorded at 1,400 m altitude, and the other altitudes had concentrations below 2 $mg{\cdot}g^{-1}$. Ester's concentration was high at 1,000 (25.2 $mg{\cdot}g^{-1}$) and 1,400 m coffees (20.5 $mg{\cdot}g^{-1}$). Pyrazines group was detected in all altitudes except at 1,200 m. Pyrans, furans, and alkanes were the less dominant groups in all altitudes with concentrations below 1.5 $mg{\cdot}g^{-1}$.

Out of the 51 volatile compounds, only 41 were shared in all altitudes (Figure 3B and Supplementary Material 2). Three compounds were exclusively detected at 1,000 m altitude samples belonging to dodecanoic acid, ethyl ester, 2,4-dodecadienal, (E,E)-, and ethyl 9-hexadecenoate. Only 1 compound [2-penten-1-ol, (Z)-] was shared between 800 and 1,000 m, 1 (1-pentanol, 3,4-dimethyl-) with 800 and 1,200 m, 1 with 1,000 and 1,200 m, 1 with altitudes 1,000 and 1,400 m, and 1 with 1,200 and 1,400 m (see the other compounds in the supplementary material 2). Pyrazine, 2-methoxy-3-(2-methylpropyl)- and acetic acid, phenylmethyl ester were common in three altitudes except for 1,200 and 1,400 m, respectively.

Within the alcohols group, 2-heptanol, 2,3-butanediol, [R-(R*, R*)]-, and phenylethyl alcohol was the most dominant (Supplementary Material 2). The highest contents of 2-heptanol and 2,3-butanediol, [R-(R*, R*)]- were found at higher altitude coffees with 33.9 $mg{\cdot}g^{-1}$ (1,200 m) and 74.9 $mg{\cdot}g^{-1}$ (1,400 m) respectively. The altitude with the highest phenylethyl alcohol content was 1,400 m, with 29.6 $mg{\cdot}g^{-1}$. Among the aldehydes, benzeneacetaldehyde presented the highest contents and increase while altitude increase ($mg{\cdot}g^{-1}$: 16.9, 29.8, 29.1, and 33.9 at 800, 1,000, 1,200, and 1,400 m, respectively). Methyl salicylate presented the highest contents in the phenol group and at lower altitudes, with 23.5 (800 m) and 28.1 $mg{\cdot}g^{-1}$ (1,000 m). A complete list of the volatiles detected is displayed on Supplementary Material 2.


A
RC (mg·g-1)
189
151
113
75
38
0
1,400 m
1,200 m
1,000 m
800 m
Acids
Ketones
Alkanes
Furans
Pyrans
Lactones
Pyrazines
Esters
Phenols
Aldehydes
Alcohols

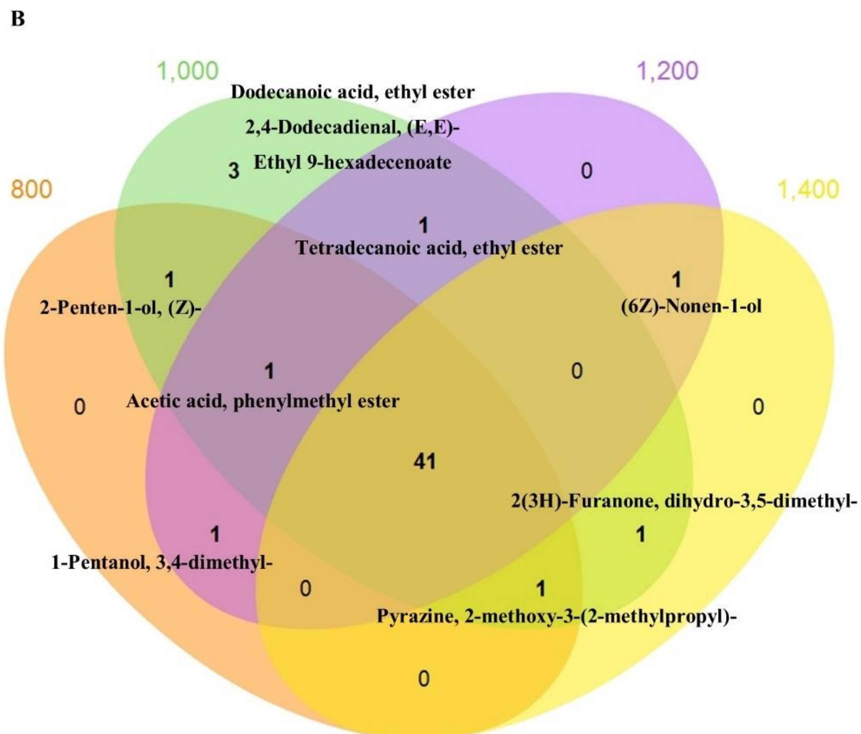


Figure 3A. Heat map representing the volatiles groups relative concentration at the different altitudes. **3B.** Venn diagram of the volatile compounds found in each altitude.

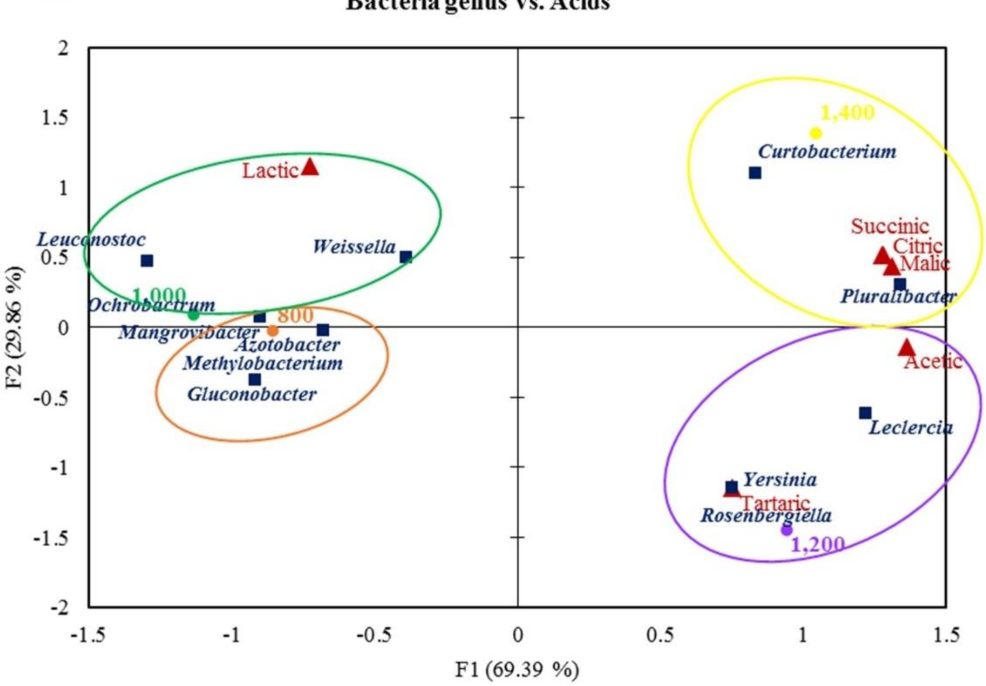
3.4 Relation between the microbial community and biochemical compounds

3.4.1 Bacterial and fungal community versus acids contents

Lactic acid was positively correlated with genera *Leuconostoc*, *Weissella*, and *Ochrobactrum* in the altitude, where both were the most abundant (Figure 4A). Citric, malic, and succinic acid were positively correlated with the most abundant genera (*Curtobacterium* and *Pluralibacter*) at 1,400 m. Acetic acid was positively correlated with *Leclercia* at 1,200 m. In addition, tartaric acid was grouped with the genera *Yersinia* and *Rosenbergiella*, only found at 1,200 m.

Among the fungal community, yeasts may influence the acid contents equally, especially those in high abundances (Figure 4B). *Cystofilobasidium infirmominiatum*, *Wickerhamomyces anomalus*, and *Meyerozyma caribbica* did not correlate with acids contents, as the other abundant yeasts. Most species with abundance below 1% were distant and different from the high abundance species and did not correlate with acid's contents.

A



B

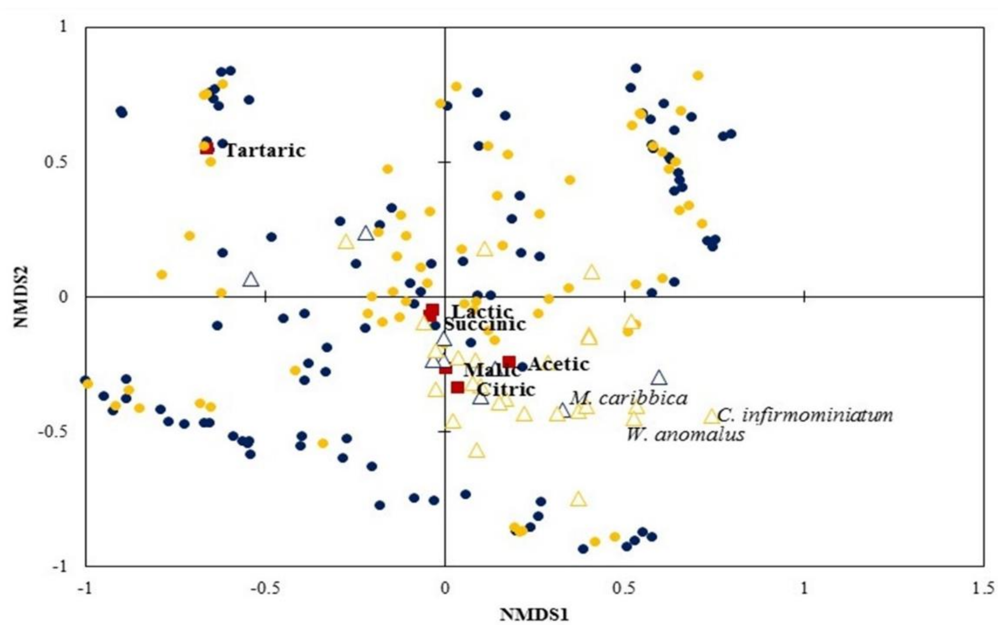
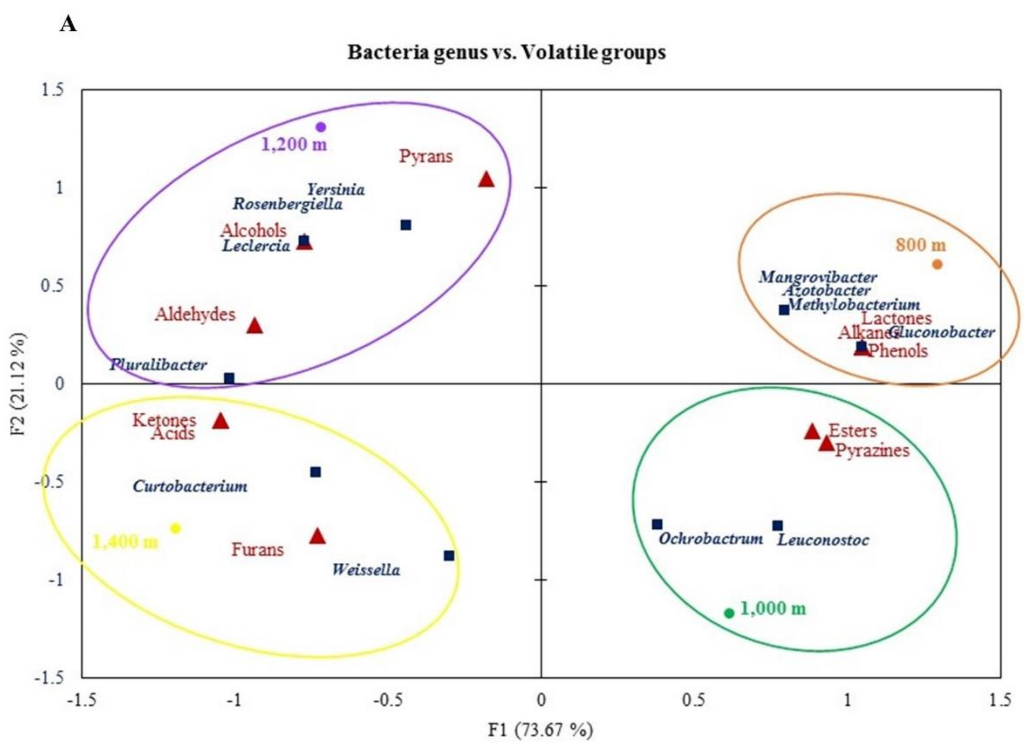


Figure 4A. Principal component analysis (PCA) plot of bacterial community vs. organic acids at the different altitudes. **4B.** Non-metric scaling (NMDS) using Bray-Curtis dissimilarity for fungal species vs. acids. ● non-dominant filamentous fungi species, ● non-dominant yeasts species, △ dominant filamentous fungi species, and △ dominant yeasts.

3.4.2 Bacterial and fungal community versus volatiles contents

The alcohols group was positively correlated with *Leclercia* in the 1,200 m altitude, where both were the most abundant (Figure 5A). This group and pyrans were also positively correlated with the genera that were only found at that altitude. Acids and ketones were grouped with *Curtobacterium*, a genus only found at 1,400 m. At 800 m, *Gluconobacter* and the genera only detected at that altitude may be influencing the contents of lactones, alkanes, and phenols. At 1,000 m, *Leuconostoc and Ochrobactrum* (the latter only found at 1,000 m) may be influencing the esters and pyrazines contents.

Within the fungal community, the most abundant species, especially yeasts, may exert a strong influence on the contents of the most volatile groups (Figure 5B). *Cystofilobasidium infirmominiatum*, *Wickerhamomyces anomalus*, and *Meyerozyma caribbica* greatly influence the volatiles groups found in higher contents (alcohols, aldehydes, phenols, and esters). The species with abundances below 1% were distant and different from the high abundance species and did not directly influence the volatile contents.



B

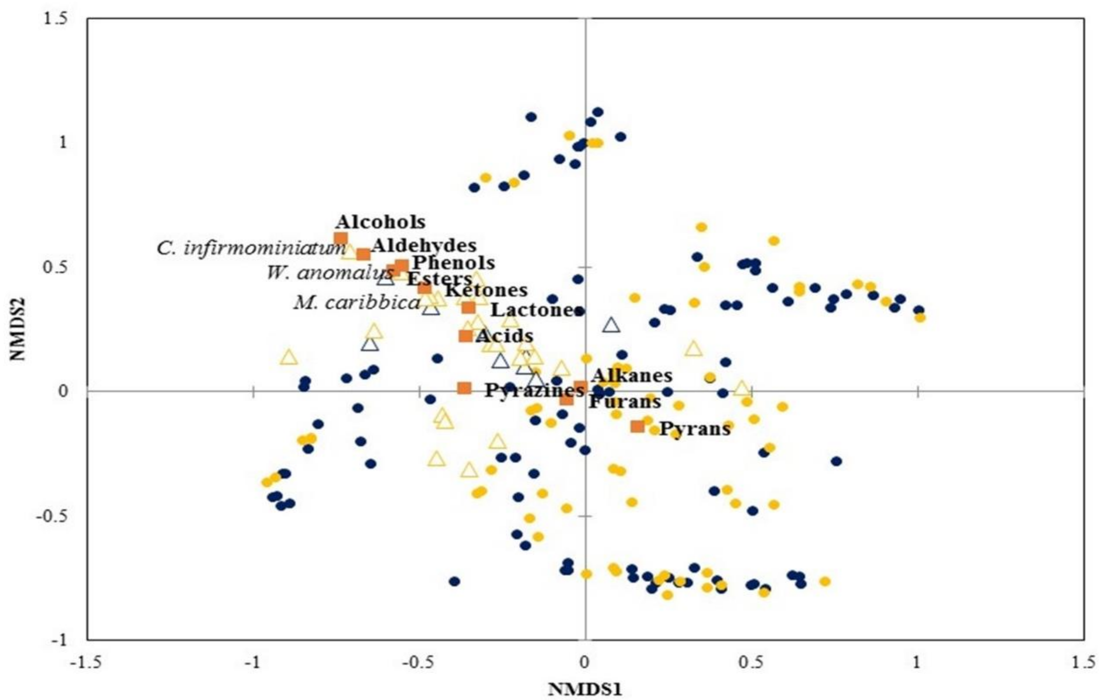


Figure 5A. Principal component analysis (PCA) plot of bacterial community vs. volatile groups at the different altitudes. **5B.** Non-metric scaling (NMDS) using Bray-Curtis dissimilarity for fungal species vs. volatile groups. ● non-dominant filamentoys fungi species that are not yeasts, ● non-dominant yeasts species, △ dominant filamentus fungi species, and △ dominant yeasts.

4. Discussion

The composition of beans varies depending on the harvesting conditions. Although we used the same variety and machine to depulped fruits, altitude influenced the sugar content in our study. Similarly, altitude was a determinative factor of bean weight, showing bean weight increase. The same results were found in Alpizar and Bertrand (2005) and Tolessa et al. (2017). Heavier beans affect slow maturation occurring at higher altitudes and low temperatures; a slow bean maturation conduces to a better bean filling than lipid accumulation (Tolessa et al., 2017).

At the different altitudes, the coffee microbiota varied. Coffee processed via pulped natural and from lower altitudes favored bacterial and fungal richness during SIAF. The detection of various bacterial genera per altitude was low due to the processing type since during processing, the mucilage which contains the sugars for microbial growth and the excess of bacteria cells are removed.

With 48 h of fermentation, the lactic acid bacteria (LAB) *Leuconostoc* and *Weissella* presence was evident and abundant. The LAB group has always been part of the pulped natural process in different fermentation periods, as in Vilela et al. (2010), Martinez et al. (2017), and Martins et al. (2020). In coffee, this group aids during mucilage degradation, bioactive compounds generation, and flavor-forming (de Melo Pereira et al., 2020). They probably interact with yeasts as in cocoa fermentation, where yeasts release nutrients for LAB, and in return, LAB produces acid creating a favorable growth environment (Schwan and Wheals, 2004). The production of lactic acid is one of the traits of this group. In this study, there was a correlation between this acid and LAB *Leuconostoc* and *Weissella*, and they are possibly the main ones responsible for its production.

As in this work, the genus *Leuconostoc* have been previously reported in fermented coffees processed via pulped natural at different altitudes (750-1,400 m) using other DNA-based techniques (Vilela et al. 2010; Evangelista et al., 2014b; Martins et al., 2020); a common specie identified in those works include *L. mesenteroides*. *Weissella* contributes to lactic and acetic acid production and was also reported in pulped natural coffees from Evangelista et al. (2014b) and Martins et al. (2020). However, this genus has been found in fermented coffees processed via wet (De Bruyn et al., 2017; Junqueira et al., 2019).

Other genera like *Gluconobacter* have been found in coffees from 750-800 and 1,200 m (Evangelista et al., 2014b; Martins et al., 2020). Although we expected this genus to be correlated with acetic acid, that was not the case. Maybe other bacteria are producing or encouraging other bacteria to produce, reminding that those microorganisms only produce acetic acid in coffee, as observed in Martinez et al. (2019). Yet *Gluconobacter* was correlated with volatiles groups lactones, alkanes, and phenols. There are no studies available regarding their relation.

On the contrary, acetic acid was correlated with genus *Leclercia*. Limited information is unknown about this genus and the role it has on coffee. What is well known is that *Leclercia* is a psychrotolerant coliform formerly part of the genus *Escherichia* frequently found in milk and dairy plants (Masiello et al., 2016). *Ochrobactrum* is associated with the nodulation of legumes and has been found in the fermented food koji (Ma et al., 2018). Moreover, it was detected at one altitude; therefore, the coffee composition and microbiota at 1,000 m were different.

The fungal diversity varied from altitude, and the species predominating were yeasts. The occurrence of species tolerant to low temperatures was evident; for example, *C. infirmominiatum* was abundant at altitudes above 1,000 m showing tolerance to low temperatures, except when reaching a 1,400 m altitude. It is the first time this species has been reported as abundant in coffees processed via pulped natural.

The presence of fungal species such as *W. anomalus, T. delbrueckii*, and some species from *Pichia* are significant in coffee for mucilage degradation according to Masoud and Jespersen (2006), Silva et al. (2013), and Haile and Kang (2019). *W. anomalus* was found in cereal grains, fruits, maize silage, wine, and natural processed coffees (Kurtzman and Fell, 1988; Martins et al., 2020) and was reported as a killer toxin producer against spoilage yeasts (Comitini et al., 2004). *M. caribbica*, apart from controlling phytopathogen fungi (Bautista-Rosales et al., 2013), raises the coffee mass temperature during fermentation, as observed in Bressani et al. (2021). A temperature increase is significant to produce desirable volatile compounds. In this work, the highest temperature value was seen at the altitude (1,000 m) where *M. caribbica* was most abundant, and consequently, the same altitude had the highest number of volatile compounds. Also, this yeast has been found in different Brazilian regions, coffee processes, and coffee species (Evangelista et al., 2015; Martins et al., 2020; Bressani et al., 2021; da Silva et al., 2021).

Either *C. infirmominiatum*, *W. anomalus,* and *M. caribbica* did not significantly influence the acids content as the other abundant yeast. However, they highly influenced the volatiles groups found in higher contents expected since yeast are the leading volatiles producers.

Natural organic acids are important for flavor, contributing to beverage acidity (Ribeiro et al., 2017a). Most acids are used to maintain low pH during coffee fermentation. When acids are esterified with natural alcohols, they generate esters (Schaft, 2015). Generally, fermented coffees from high altitudes favored organic acids contents, specifically citric, malic, acetic, and succinic acid. The dominance and high concentration of citric acid in all altitudes are probably due to the high yields produced through the tricarboxylic acid (TCA or Krebs cycle) by the microbiological processes or inside the beans (Vandenberghe et al., 2018). The bigger the beans, the higher the citric acid concentrations are. The citric and malic acid concentration at altitude 800 m was within the values (0-0.5 $g \cdot Kg^{-1}$) found in Martinez et al. (2017) at 750-800 m using the pulped natural process. Unlike what da Mota et al. (2020) demonstrated, succinic acid in this work was predominant at high altitudes, not at lower altitudes; these changes might also be related to the region evaluated. Tartaric acid was probably due to the specific microorganisms from that altitude *Yersinia*, *Rosenbergiella*, and yeast *Papiliotrema laurentii*, showing a strong correlation. Meaning they are producing the acid or stimulate other microorganisms to produce.

Somehow caffeine values are always higher than trigonelline; as seen in Martinez et al. (2017), the caffeine concentration at 800 m was below the concentrations found in the mentioned work. Those high values may directly be involved in the beverage bitterness (Sunarharum et al., 2014). Based on our results, caffeine shifts are not affected by growing altitude but other factors, which is opposite to what Girma et al. (2020) found that caffeine decreases while altitude increases. Similarly, altitude does not affect trigonelline and chlorogenic acids. Trigonelline is an alkaloid present in green coffee beans that yields coffee odorants such as pyridines and pyrroles (Lee et al., 2015). Coffee from lower altitudes had higher trigonelline contents, and this evidence is different from what Nugroho et al. (2020) obtained; that is, there is an increase of this compound with an altitude increase resulting in higher values at higher altitudes. Chlorogenic acids are important compounds because they contribute to flavor by providing astringency, acidity, and bitterness. They are produced in plants to protect them from abiotic stress (temperature, water content, UV exposure, and nutrients deficiency) and pathogens attack (Girma et al., 2020). Some suggestion as to why chlorogenic acids are not influenced by altitude is that they are likely to be influenced by the differences in the genotype or daily temperature during seed formation (Joët et al., 2015; Tolessa et al., 2019).

The antioxidant activity depends on the number of total phenolics or compounds that have antioxidant capacity. 1,200 m had a different profile than the other altitudes because it presented the highest antioxidant activity and phenolics contents. The high activity might be because of trigonelline and caffeine, which were used and consequently lowered to the values obtained.

Coffee beans contain a pool of important compounds interacting with them during the biochemical reactions occurring along the processing chain. Those reactions are possible through Maillard and Strecker degradation, amino acid breakdown, trigonelline, pigments, and lipids degradation (Buffo and Cardelli-Freire, 2004). Maillard reactions are responsible for generating various chemical groups such pyrazines, pyrroles, thiols, furanones, pyridines, and thiophenes (Lee et al., 2015). Strecker reactions yield aldehydes and sulfur compounds (de Melo Pereira et al., 2019). The volatile groups (alcohols, aldehyde, phenols, and esters) dominating the pulped natural process in this work have also been reported in coffees from 750-800 and 1,100-1,250 m (Martinez et al., 2017; Bressani et al., 2020).

High altitudes favor alcohols, aldehydes, and ketones groups. Alcohols predominance was strongly associated with the abundant yeasts species illustrated on the NMSD figure, which was expected since yeast synthesize various higher alcohols. Among them, phenylethyl alcohol was detected in high abundance. The presence of this volatile coffee indicates that microorganisms are metabolically active (Martinez et al., 2019). Phenylethyl alcohol and 2-heptanol were detected in Bressani et al. (2020), and according to the authors, both compounds might be a yeast product and are related to esters formation through esterification. Aldehydes are important precursors of higher alcohols and esters; for example, acetaldehyde is released during yeast cells apoptosis, then diffuses into the beans, affecting the fruity and floral attributes. Esters are mainly derived from yeasts and contribute to fruity flavors, the most abundant compound from this group methyl salicylate, but that is not the case for this compound because it was previously reported as a compound generated by bacteria during wet fermentation (Martinez et al., 2019), furthermore is a plant hormone generated for defense (Kalaivani et al., 2016).

Conclusion

Altitude affects the dominant bacterial and fungal communities in coffees processed via pulped natural, favoring the richness of both communities at low altitudes. Fungal species tolerant to low temperatures were the most abundant (*C. infirmominiatum*). Also, altitude is a dependent factor of the abundant organic acids and volatile compounds. Higher altitude coffees under SIAF favored citric, malic, succinic, and acetic contents. Altitude was not a determinant factor of antioxidant activity and compounds with antioxidant capacity. Out of the abundant fungal species, yeast affected more the abundant volatile groups. High altitude coffees favor alcohols, aldehydes, and esters groups, while low altitude coffees favored phenols. Although coffee came from different altitudes, by using SIAF, external factors were avoided, and their natural microbiota was kept. Therefore, this fermentation method may help farmers obtained a more controlled process and consequently increase in quality.

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Supplementary Material

Supplementary Material 1

RA% all altitudes

Not detected

Species in bold are species found in all altitudes

Specie	Cd 800	Cd 1000	Cd 1200	Cd 1400
Acremonium fusidioides		0.044		
Acrocalymma walkeri				0.197
Alternaria argyroxiphii	0.168			0.111
Alternaria novae-guineensis			0.048	
Antennariella placitae		0.008		
Apiotrichum domesticum		0.081		
Articulospora proliferata	0.267			0.008
Aspergillus westerdijkiae	0.261	0.014	0.028	0.267
Aureobasidium pullulans	0.032	0.036	0.034	
Bannoa ogasawarensis	0.141	0.016		
Barnettozyma californica	0.077	0.044		
Bipolaris sorokiniana		0.065		
Boeremia exigua	0.205	0.449	0.682	0.988
Bulleribasidium foliicola		0.069		
Bulleromyces albus		0.004		
Candida blattae		0.059		
Candida orthopsilosis		0.218	0.013	0.338
Candida parapsilosis	0.727	0.085		
Candida quercitrusa		2.176	0.055	0.127
Candida railenensis	2.261	0.117	0.234	5.533
Candida solani		0.016		
Candida tropicalis	5.307	0.295	2.029	2.449
Candida vaughaniae	0.012	0.063		
Capitofimbria compacta	0.007			0.059
Catenulostroma hermanusense			0.176	
Catenulostroma protearum				0.105
Ceriporia alachuana			0.041	
Cladosporium aphidis	0.978	2.615	0.083	0.537
Cladosporium delicatulum	5.509	4.476	5.310	18.747
Cladosporium dominicanum	0.190	0.073	0.017	
Cladosporium flabelliforme	0.059	0.049		

Cladosporium fusiforme	0.171			
Cladosporium halotolerans	0.344	0.079	0.024	
Cladosporium sphaerospermum	0.604	0.459	0.204	0.481
Clonostachys miodochialis				0.016
Colletotrichum annellatum	0.321		0.766	0.111
Colletotrichum lupini	0.007		0.158	0.132
Colletotrichum theobromicola	0.428	0.380	0.098	0.105
Coniothyrium sidae	0.004			0.861
Coriolopsis gallica		0.010		
Cryptococcus dimennae	0.055	0.180		1.050
Cryptococcus uniguttulatus		0.038		
Curvibasidium cygneicollum	0.167	0.020	0.046	0.278
Curvularia americana	0.022			
Curvularia caricae-papayae				0.019
Cutaneotrichosporon jirovecii		0.160		
Cyberlindnera fabianii	0.180	0.030	0.184	0.203
Cyphellophora europaea	0.345			0.008
Cyphellophora vermispora	0.110			
Cystofilobasidium alribaticum	8.470	0.208		0.086
Cystofilobasidium capitatum	0.956	2.097	0.598	0.335
Cystofilobasidium ferigula	1.211	1.016	0.429	0.786
Cystofilobasidium infirmominiatum	20.819	16.841	58.282	10.418
Cystofilobasidium intermedium		0.014		
Cystofilobasidium macerans		0.028		
Debaryomyces hansenii	0.102	0.485	0.035	0.348
Debaryomyces nepalensis	0.167	0.095	0.128	0.030
Derxomyces anomalus				0.024
Diaporthe nothofagi			0.010	
Didymella calidophila				0.662
Didymella coffeae-arabicae		0.135	0.131	0.232
Didymella nigricans		0.935	0.319	
Dimennazyma cistialbidi			0.641	1.193
Dioszegia var._yunnanensis	0.564	0.194	0.091	0.170
Diutina catenulata		0.246		
Epicoccum draconis	0.473	0.192	0.053	0.246
Epicoccum nigrum	2.245	0.725	0.555	2.651
Erythrobasidium hasegawianum	0.586	0.048	0.030	0.200
Fellomyces mexicanus	0.141			
Fibrodontia alba				0.035
Filobasidium chernovii	0.016			

Filobasidium floriforme	0.064	0.081		0.113
Filobasidium wieringae				0.116
Fusarium acutatum	0.195	0.974	0.025	0.124
Fusarium anguioides			0.013	
Fusarium asiaticum		0.065	0.066	0.232
Fusarium delphinoides	0.007			
Fusarium penzigii		0.097		
Fusarium proliferatum	0.576	0.677		0.527
Fusarium solani		0.022	0.014	
Fusculina eucalypti			0.003	
Genolevuria amylolytica				0.019
Gibberella intricans	0.843	1.713	0.245	
Golovinomyces sonchicola			0.023	
Hannaella kunmingensis	0.231	0.044		0.103
Hannaella luteola	1.793	1.410	0.229	1.866
Hannaella oryzae		0.188	0.052	0.032
Hannaella siamensis			0.025	
Hannaella sinensis	1.454	1.057	0.501	1.976
Hannaella zeae	1.373	0.808	3.022	2.263
Hanseniaspora uvarum	2.465	7.878	1.761	1.685
Hanseniaspora vineae	0.029		0.007	
Heterochaete shearii		0.073		
Holtermanniella wattica	0.306	0.160		
Hyphopichia burtonii		0.034		
Hypoxylon trugodes	0.012			
Irpex hydnoides			0.023	
Issatchenkia orientalis	0.162			
Kazachstania exigua		0.020	0.052	
Kodamaea ohmeri	1.793	0.685	2.891	0.324
Kwoniella dendrophila			0.021	
Lachancea lanzarotensis			0.020	
Lactarius saponaceus			0.326	
Lectera colletotrichoides	0.550			
Leptoxyphium madagascariense	0.062			0.208
Loramyces macrosporus		0.016		
Meyerozyma caribbica	1.297	10.346	0.261	3.085
Meyerozyma guilliermondii				1.496
Mortierella ambigua	0.554			
Mycosphaerella ellipsoidea	0.286			
Mycosphaerella pseudocryptica	0.012	0.008		
Myrmecridium schulzeri	0.013			

Naganishia albida	0.109	0.756	0.115	0.437
Naganishia diffluens		0.105		0.089
Naganishia randhawae		1.182	0.103	0.035
Neoascochyta paspali		0.024	0.070	
Nigrospora oryzae	0.322	0.293	0.243	0.068
Nothophoma arachidis-hypogaeae		0.445		
Ochroconis cordanae	0.067			
Papiliotrema flavescens	2.258	3.719	7.276	2.314
Papiliotrema laurentii			0.146	
Paraconiothyrium fungicola	0.157	0.022	0.068	
Paramyrothecium foliicola	0.054			
Penicillium kongii		0.372	0.090	7.355
Penicillium solitum			0.063	0.084
Peniophora albobadia			0.027	
Peniophora laxitexta		0.044		0.043
Periconia byssoides		0.083	0.105	0.081
Periconia cookei		0.018		
Periconia macrospinosa		0.107		
Periconia prolifica				0.726
Phaeosphaeria podocarpi	1.371	0.408	0.250	1.123
Phanerochaete laevis	0.004			0.113
Phlebia brevispora		0.053		
Phoma multirostrata		0.119		
Phoma omnivirens		0.063		0.076
Pichia kluyveri		0.851	0.020	
Pichia myanmarensis		0.075		
Plectosphaerella cucumerina	0.003			0.292
Pleurotus pulmonarius	0.502	0.356	0.273	1.007
Polyporus tricholoma	0.133		0.004	0.065
Pseudocercospora bixae	0.746		0.011	
Pseudomerulius curtisii	0.653			
Pseudophaeomoniella oleae	0.059		0.096	0.410
Pseudoplectania affinis		0.386		
Pseudorobillarda phragmitis		0.376		
Pseudoteratosphaeria ohnowa	0.776			
Pyrenochaetopsis leptospora		0.053		
Rachicladosporium cboliae	0.126		0.127	0.313
Rachicladosporium paucitum			0.100	
Ramularia pratensis			0.048	0.321
Resinicium saccharicola				0.038
Rhodosporidiobolus fluvialis	0.303	0.091	0.195	0.159

Rhodosporidiobolus lusitaniae	0.451	0.093	0.018	
Rhodosporidiobolus odoratus	0.213		0.053	
Rhodosporidiobolus ruineniae				0.143
Rhodotorula araucariae		0.364		0.324
Rhodotorula babjevae		0.030		
Rhodotorula dairenensis	0.608	1.251	0.246	0.108
Rhodotorula diobovata	0.135	0.653	0.024	0.000
Rhodotorula mucilaginosa	0.110	0.780	0.184	0.338
Rhodotorula taiwanensis		0.055		
Rhynchogastrema complexa	0.071		0.008	0.092
Rhynchogastrema nanyangensis	0.232	0.459	0.134	0.030
Rhytidhysteron rufulum	0.051			
Riopa pudens				0.014
Saccharomyces cerevisiae		0.014	0.017	
Saitozyma paraflava	0.461	0.097		0.078
Saitozyma podzolica	1.589	0.119		
Sampaiozyma vanillica	0.025			
Schizophyllum commune	0.022	0.059	0.018	0.176
Selenophoma mahoniae	0.038	0.073	0.148	0.205
Septoria cretae	1.253	8.922	0.600	2.252
Setophoma chromolaenae	0.078	0.022	0.046	0.057
Sirobasidium brefeldianum		0.156		
Sistotremastrum guttuliferum		0.008		
Sporobolomyces bannaensis	0.007			
Sporobolomyces johnsonii	0.029			
Sporobolomyces koalae	1.304	0.600	0.564	0.521
Strelitziana africana	0.682	0.289	0.149	1.596
Strelitziana eucalypti	0.051	0.075	0.007	0.316
Symmetrospora coprosmae	0.020		0.042	
Symmetrospora vermiculata			0.212	
Torulaspora delbrueckii	0.048	0.028	0.010	0.046
Toxicocladosporium irritans	0.086			0.078
Toxicocladosporium strelitziae		0.032		
Trichosporon asahii	0.077	0.222		0.043
Trichosporon coremiiforme	0.173	0.109	0.021	0.186
Udeniomyces pyricola	0.861	0.497	0.153	0.915
Vishniacozyma dimennae		0.430		0.270
Vishniacozyma foliicola		0.594	0.631	1.134
Vishniacozyma heimaeyensis	1.079	0.079	0.063	
Vishniacozyma taibaiensis	1.783	1.754	0.534	3.710
Vishniacozyma tephrensis				0.030

Vishniacozyma victoriae				0.116
Wallemia tropicalis	0.020			
Wickerhamomyces anomalus	10.995	8.276	3.846	7.150
Wickerhamomyces ciferrii	0.744	0.184	0.510	0.138
Wickerhamomyces lynferdii		1.170	1.142	0.419
Wickerhamomyces pijperi	0.274			0.038
Wickerhamomyces sydowiorum		0.162	0.072	
Wickerhamomyces xylosica		0.299		0.014
Xeromyces bisporus			0.003	

Distinctive species in each altitude

800 m	**1,000 m**	**1,200 m**	**1,400 m**
Cladosporium fusiforme	*Acremonium fusidioides*	*Alternaria novae-guineensis*	*Acrocalymma walkeri*
Curvularia americana	*Antennariella placitae*	*Catenulostroma hermanusense*	*Catenulostroma protearum*
Cyphellophora vermispora	*Apiotrichum domesticum*	*Ceriporia alachuana*	*Clonostachys miodochialis*
Fellomyces mexicanus	*Bipolaris sorokiniana*	*Diaporthe nothofagi*	*Curvularia caricae-papayae*
Filobasidium chernovii	*Bulleribasidium foliicola*	*Fusarium anguioides*	*Derxomyces anomalus*
Fusarium delphinoides	*Bulleromyces albus*	*Fusculina eucalypti*	*Didymella calidophila*
Hypoxylon trugodes	*Candida blattae*	*Golovinomyces sonchicola*	*Fibrodontia alba*
Issatchenkia orientalis	*Candida solani*	*Hannaella siamensis*	*Filobasidium wieringae*
Lectera colletotrichoides	*Coriolopsis gallica*	*Irpex hydnoides*	*Genolevuria amylolytica*
Mortierella ambigua	*Cryptococcus uniguttulatus*	*Kwoniella dendrophila*	*Meyerozyma guilliermondii*
Mycosphaerella ellipsoidea	*Cutaneotrichosporon jirovecii*	*Lachancea lanzarotensis*	*Periconia prolifica*
Myrmecridium schulzeri	*Cystofilobasidium intermedium*	*Lactarius saponaceus*	*Resinicium saccharicola*
Ochroconis cordanae	*Cystofilobasidium macerans*	*Papiliotrema laurentii*	*Rhodosporidiobolus ruineniae*
Paramyrothecium foliicola	*Diutina catenulata*	*Peniophora albobadia*	*Riopa pudens*
Pseudomerulius curtisii	*Fusarium penzigii*	*Rachicladosporium paucitum*	*Vishniacozyma tephrensis*
Pseudoteratosphaeria ohnowa	*Heterochaete shearii*	*Symmetrospora vermiculata*	*Vishniacozyma victoriae*
Rhytidhysteron rufulum	*Hyphopichia burtonii*	*Xeromyces bisporus*	
Sampaiozyma vanillica	*Loramyces macrosporus*		
Sporobolomyces bannaensis	*Nothophoma arachidis-hypogaeae*		
Sporobolomyces johnsonii	*Periconia cookei*		

Wallemia tropicalis	*Periconia macrospinosa*
	Phlebia brevispora
	Phoma multirostrata
	Pichia myanmarensis
	Pseudoplectania affinis
	Pseudorobillarda phragmitis
	Pyrenochaetopsis leptospora
	Rhodotorula babjevae
	Rhodotorula taiwanensis
	Sirobasidium brefeldianum
	Sistotremastrum guttuliferum
	Toxicocladosporium strelitziae

Supplementary Material 2

List volatile compounds

Group	**Compound (mg. g-1)**	**800 m**	**1000 m**	**1200 m**	**1400 m**
Alcohols	2-Heptanol	24.7	22.2	33.9	74.9
Aldehydes	Benzeneacetaldehyde	16.9	29.8	29.1	33.9
Alcohols	Phenylethyl Alcohol	12.8	20.5	15.6	29.6
Alcohols	2,3-Butanediol, [R-(R*,R*)]-	13.1	21.3	35.6	24.6
Aldehydes	Benzaldehyde	5.2	9.8	8.8	18.6
Alcohols	1-Hexanol	4.5	6.0	8.8	17.0
Ketones	1-Hexen-3-ol	5.0	8.5	7.9	16.4
Esters	Butanoic acid, 3-methyl-	7.0	8.9	9.7	13.5
Acids	n-Hexadecanoic acid	0.3	0.4	0.4	12.7
Alcohols	1,6-Octadien-3-ol, 3,7-dimethyl-	9.4	10.7	8.4	12.2
Alcohols	3-Octanol	1.9	2.1	2.0	7.4
Phenols	Methyl salicylate	23.5	28.1	6.3	6.2
Alcohols	(S)-3-Ethyl-4-methylpentanol	4.4	4.8	4.4	5.2
Aldehydes	2,6-Nonadienal, (E,E)-	0.2	0.5	0.7	4.1
Alcohols	Benzyl alcohol	3.4	4.1	4.9	3.8
Esters	Benzeneacetic acid, ethyl ester	5.0	11.9	3.5	3.7
Pyrazines	Pyrazine, 2-methoxy-3-(2-methylpropyl)-	3.7	4.5	0	3.2
Alcohols	2(3H)-Furanone, dihydro-3,5-dimethyl-	0	2.9	0	3.1
Alcohols	3-Hexen-1-ol, (Z)-	1.0	1.2	0.8	2.5
Alcohols	4-Hexen-1-ol	1.4	0.9	1.3	2.4
Aldehydes	Heptadecanal	0.3	1.1	0.9	1.9
Lactones	2-Pentanone, 5-phenyl-	6.6	6.2	3.8	1.7
Alcohols	1-Octanol	0.6	0.9	1.3	1.6
Alcohols	1-Heptanol, 6-methyl-	0.6	0.9	1.3	1.6
Aldehydes	2-Nonenal, (E)-	0.3	0.6	0.7	1.5

Aldehydes	2-Octenal, (E)-	0.6	0.9	0.8	1.4
Alcohols	6-Hepten-1-ol, 2-methyl-	0.7	0.9	0.8	1.2
Furans	Furan, 2-propyl-	0.3	0.7	0.4	1.1
Esters	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.6	0.4	0.2	1.1
Ketones	5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	0.6	0.9	0.9	1.0
Esters	Hexadecanoic acid, ethyl ester	0.3	1.3	0.8	1.0
Acids	Hexanoic acid	0.5	0.6	0.8	0.8
Esters	Benzeneacetic acid, methyl ester	0.9	1.6	0.7	0.8
Acids	Hexadecanoic acid, methyl ester	0.2	0.4	0.4	0.7
Alcohols	2-Octen-1-ol, (E)-	0.3	0.3	0.3	0.6
Aldehydes	2,4-Nonadienal, (E,E)-	0.2	0.3	0.3	0.5
Alcohols	(6Z)-Nonen-1-ol	0	0	0.2	0.5
Phenols	Phenol, 2,4-bis(1,1-dimethylethyl)-	0.3	0.3	0.2	0.4
Alkanes	Heneicosane	0.1	0.2	0.2	0.4
Esters	9,12-Octadecadienoic acid, ethyl ester	0.1	0.4	0.3	0.3
Ketones	2-Pentadecanone, 6,10,14-trimethyl-	0.1	0.2	0.1	0.3
Alkanes	2-Furanmethanol, 5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-, cis-	0.5	0.5	0.4	0.3
Pyrans	2H-Pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl-	0.1	0.1	0.1	0.2
Esters	Linoleic acid ethyl ester	0.1	0.1	0.1	0.2
Alcohols	1-Pentanol, 3,4-dimethyl-	0.4	0	0.5	0
Esters	Acetic acid, phenylmethyl ester; Benzyl acetate	0.4	0.3	0.2	0
Esters	Tetradecanoic acid, ethyl ester	0	0.2	0.2	0
Alcohols	2-Penten-1-ol, (Z)-	0.8	0.5	0	0
Esters	Dodecanoic acid, ethyl ester	0	0.2	0	0
Aldehydes	2,4-Dodecadienal, (E,E)-	0	0.2	0	0
Alcohols	Ethyl 9-hexadecenoate	0	0.2	0	0

Venn diagram

Common in 800, 1,000,1,200 and 1,400 (41 compounds):	**Exclusive in 1,000 (3 compounds):**	**Common in 800 and 1,000 (1 compound) :**	**Common in 1,000 and 1,200 (1 compound):**	**Common in 800, 1,000 and 1,200 (1 compound):**	**Common in 1,200 and 1,400 (1 compound):**	**Common in 1,000 and 1,400 (1 compound):**	**Common in 800, 1,000 and 1,400:**	**Common in 800 and 1,200:**
(S)-3-Ethyl-4-methylpentanol	Dodecanoic acid, ethyl ester	2-Penten-1-ol, (Z)-	Tetradecanoic acid, ethyl ester	Acetic acid, phenylmethyl ester	(6Z)-Nonen-1-ol	2(3H)-Furanone, dihydro-3,5-dimethyl-	Pyrazine, 2-methoxy-3-(2-methylpropyl)-	1-Pentanol, 3,4-dimethyl-

1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	2,4-Dodecadienal, (E,E)-
1,6-Octadien-3-ol, 3,7-dimethyl-	Ethyl 9-hexadecenoate
1-Heptanol, 6-methyl-	
1-Hexanol	
1-Hexen-3-ol	
1-Octanol	
2,3-Butanediol, [R-(R*,R*)]-	
2,4-Nonadienal, (E,E)-	
2,6-Nonadienal, (E,E)-	
2-Furanmethanol, 5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-, cis-	
2-Heptanol	
2H-Pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl-	
2-Nonenal, (E)-	
2-Octen-1-ol, (E)-	
2-Octenal, (E)-	
2-Pentadecanone, 6,10,14-trimethyl-	
2-Pentanone, 5-phenyl-	
3-Hexen-1-ol,	

(Z)-
3-Octanol
4-Hexen-1-ol
5,9-
Undecadien-
2-one, 6,10-
dimethyl-,
(E)-
6-Hepten-1-ol,
2-methyl-
9,12-
Octadecadieno
ic acid, ethyl
ester
Benzaldehyde
Benzeneacetal
dehyde
Benzeneacetic
acid, ethyl
ester
Benzeneacetic
acid, methyl
ester
Benzyl
alcohol
Butanoic acid,
3-methyl-
Furan, 2-
propyl-
Heneicosane
Heptadecanal
Hexadecanoic
acid, ethyl
ester
Hexadecanoic
acid, methyl
ester
Hexanoic acid
Linoleic acid
ethyl ester
Methyl
salicylate
n-
Hexadecanoic
acid
Phenol, 2,4-
bis(1,1-
dimethylethyl)
-

Phenylethyl
Alcohol

FINAL CONSIDERATIONS

More studies regarding the different factors that affect the microbial communities are still lacking, mainly of altitude and producing regions. In this work, the aims stated were reached, such as evaluating the effect of altitude on the dominant microbial communities and biochemical compounds. The results showed that shifts are produced due to the altitude in either processing methods (natural and pulped natural) under anaerobiosis.

Future studies would be meaningful to conduct analysis involving metatranscriptome to evaluate what the microbial communities are really expressing and consequently producing during fermentation and correlate those results with the respective pathways since there are answers yet to be found.