SILVIA JULIANA MARTINEZ GELVEZ

IMPROVEMENT CHARACTERISTICS OF SEMI-DRY COFFEE FERMENTATION WITH STARTER YEASTS

LAVRAS – MG
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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Gelvez, Silvia Juliana Martinez.
Improvement characteristics of semi-dry coffee fermentation with starter yeasts / Silvia Juliana Martinez Gelvez. - 2017.
56 p.

Orientador(a): Rosane Freitas Schwan.
.
Dissertação (mestrado acadêmico) - Universidade Federal de Lavras, 2017.
Bibliografia.

1. Qualidade de café. 2. qPCR. 3. Compostos voláteis. I. Schwan, Rosane Freitas. . II. Título.

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MELHORAMENTO DAS CARACTERÍSTICAS NA FERMENTAÇAO DO CAFÉ SEMI-SECO COM LEVEDURAS INICIADORAS

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Mestre.

APROVADA em 14 de fevereiro de 2017.

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LAVRAS – MG
2017

To my parents Nubia and Jaime, for their love, advices, mentoring and support

To my sister's, Monica and Maria Paula, for their care and love

Dedico

AGRADECIMENTOS

To God, which guide me through this adventurous road and health.

To my best and dearest advisor Dr. Rosane Freitas Schwan, for her orientation, teaching, trust and opportunity.

To the Universidade Federal de Lavras (UFLA) and Programa de Microbiologia Agricola, for the opportunity to use their instalations during my master project.

To the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico of Brasil (CNPQ), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

To Gabi, for her guidance and help during the execution of my project and the writing of the article.

To all technicians and lab friends, for their help, knowledge and laughs, especially to Ana Paula, Cidinha and Rose.

To all the people I left without mentioning but where a part of my life and in which during this phase made things easy and possible.

Many thanks!

RESUMO

Brasil é conhecido mundialmente por ser um dos maiores produtores e exportadores de café. Antes de produzir a bebida, os grãos de café devem passar por método de processamento, que pode ser o seco, semisseco e o úmido. A escolha do tipo de processamento vai depender do produtor ou do agricultor. Durante o processo, os frutos ou grãos são fermentados espontaneamente durante a secagem até atingir a umidade de, aproximadamente, 11%. Algumas vezes, as culturas iniciadoras são utilizadas para ajudar na fermentação, resultando em cafés especiais. Os microrganismos naturais do café envolvem leveduras, fungos filamentosos, bactérias aeróbicas e facultativas que, ao longo da fermentação, consomem açúcares, produzindo ácidos e, consequentemente, baixando o pH. Cafés de alta qualidade têm sabor e aroma característicos e a maioria tem compostos benéficos para a saúde. No presente estudo, o objetivo foi avaliar o comportamento de três leveduras iniciadoras (*Saccharomyces cerevisiae* CCMA 0543, *Candida parapsilosis* CCMA 0544 e *Torulaspora delbrueckii* CCMA 0684) inoculadas em café processado via semisseca, implementando dois métodos de inoculação, inoculação direta e inoculação em balde. A população total de leveduras, bactérias lácticas e mesófilas foi avaliada por plaqueamento. Além disso, a população das culturas iniciadoras foi monitorada por PCR em tempo real (qPCR). Os metabólitos produzidos e consumidos nos grãos verdes e torrados durante a fermentação foram avaliados por cromatografia líquida (HPLC) e cromatografia gasosa (GC-MS). Finalmente, foi feito o teste de xícara para avaliação da bebida final. O resultado de contagem em placa mostrou que o método em balde manteve alta população de leveduras, bactérias lácticas e mesófilas no final da fermentação (secagem). A sacarose foi consumida em todos os tratamentos avaliados. Os ácidos cítrico e succínico foram detectados durante todos os tempos de fermentação. Após torra, a média de ácido clorogênico foi maior no método em balde, e o mesmo foi observado para as concentrações de trigonelina e cafeína, exceto no tratamento com *T. delbrueckii* CCMA0684. Nos grãos verdes, álcoois e ácidos, e nos grãos torrados, pirazinas e piridinas foram os principais compostos voláteis detectados por GC-MS. As notas do teste de xícara foram acima de 80 para os dois métodos de inoculação, demonstrando que a inoculação teve efeito positivo e produziu bebidas de boa qualidade.

Palavras-chave: Qualidade de café. qPCR. Compostos voláteis. Fermentação de café.

ABSTRACT

Brazil is known worldwide for being one of the highest producers and exporters of coffee. Before beverage production, beans go through different processing methods: dry, semi-dry and wet, choosing one will depend on the producer or farmer. During the process, cherries or beans are fermented spontaneously while drying until reaching approximately 11% of moisture. Sometimes starter cultures are used to help fermentation that results in special coffees. Natural organisms of coffee involve yeasts, filamentous fungi, aerobic bacteria and facultative bacteria that consume sugars, produce acids that subsequently low pH. Coffees of high qualities have characteristic aromas and flavors and most have beneficial health compounds. This work aimed at evaluating the behavior of three-yeast previously tested starters (*Saccharomyces cerevisiae* CCMA 0543, *Candida parapsilosis* CCMA 0544 and *Torulaspora delbrueckii* CCMA 0684) inoculated in coffee process via semi-dry method, implementing two inoculation methods: direct inoculation and bucket inoculation. Total population of yeast, lactic acid and mesophilic bacteria was evaluated by plating. Posteriorly, the population of starters was monitored by real-time polymerase chain reaction (qPCR). Metabolites consumed and produced during fermentation in both inoculation methods were evaluated using liquid chromatography (HPLC) and gas chromatography (GC-MS) for green and roasted beans. Finally, a cup test was carried out as sensorial analysis. As a result, plate counting showed that bucket method maintained a higher population of yeast, lactic acid and mesophilic bacteria at the end of fermentation (drying). Sucrose was consumed in all tested treatments. Citric and succinic acid were detected during all fermentation processes. After roasting, the average levels of chlorogenic acid were higher for the bucket method and the same for trigonelline and caffeine concentration, except for the *T. delbrueckii* CCMA0684 assay. Group of acids and alcohols in green beans and pyrazines and pyridines in roasted beans were the main volatile compounds. Scores from both inoculation methods in the cup test were above 80, proving that the inoculation had a positive effect and produced beverages of good quality.

Keywords: Coffee quality. qPCR. Volatile compounds. Coffee fermentation.

RESUMEN

Mundialmente Brasil es conocido por ser uno de los mayores productores y exportadores de café. Para producir la bebida, sus granos pasan por diferentes métodos de procesamiento, pudiendo ser estos seco, semi-seco y húmedo. Su selección dependerá del productor o agricultor. Durante el proceso, los frutos o granos son fermentados espontáneamente mientras son secados hasta alcanzar aproximadamente un 11% de humedad. Algunas veces se utilizan culturas iniciadoras para ayudar en la fermentación, resultando en cafés especiales. Los organismos naturales del café comprenden levaduras, hongos filamentosos, bacterias aeróbicas y facultativas que a lo largo de la fermentación consumen azucares y producen ácidos que consecuentemente bajan el pH. Los cafés de alta calidad tienen sabores y aromas característicos, y la mayoría contienen compuestos benéficos para la salud. En este estudio, el objetivo fue evaluar el comportamiento de tres levaduras iniciadoras previamente ensayadas (*Saccharomyces cerevisiae* CCMA 0543, *Candida parapsilosis* CCMA 0544 y *Torulaspora delbrueckii* CCMA 0684) e inoculadas en un café procesado por el método semi-seco, implementando dos métodos de inoculación: inoculación directa e inoculación en balde. La población total de levaduras, bacterias lácticas y mesófilas fue evaluada por cultivo en placa. Luego, la población de las levaduras iniciadoras fue monitoreada por PCR en tiempo-real (qPCR). Los metabolitos consumidos y producidos durante la fermentación en los granos verdes y tostados para ambos métodos de inoculación, fueron evaluados utilizando cromatografía liquida (HPLC) y cromatografía gaseosa (GC-MS). Finalmente, para el análisis sensorial se realizó una prueba de taza. Como resultado del recuento en placa, se observó que al final de la fermentación (secado) el método en balde mantuvo una mayor población de levaduras, bacterias lácticas y mesófilas. En todos los tratamientos evaluados la sacarosa fue consumida. Los ácidos cítrico y succínico fueron detectados en todos los tiempos de fermentación. Después de la torrefacción, el promedio de ácido clorogênico fue mayor en el método en balde, siendo observado de igual manera para las concentraciones de trigonelina y cafeína, excepto en el tratamiento con *T. delbrueckii* CCMA0684. En los granos verdes los ácidos y alcoholes, y en los granos tostados las pirazinas y piridinas, fueron los principales compuestos volátiles. Los valores para ambos métodos en la prueba de taza fueron mayores a 80, demostrando que la inoculación posiblemente tuvo un efecto positivo y se produjeron bebidas de buena calidad.

Palabras-clave: Calidad de café. qPCR. Compuestos volátiles. Fermentación de café.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUCTION

Coffee is a dark brown-slightly bitter beverage made from ground and roasted beans that is served hot or iced. It is also considered one of the most popular beverages around the world because of its valuable properties and beans composition (BALLESTEROS; TEIXEIRA; MUSSATTO, 2014). The main production is located in South and Central America, The Caribbean, Africa and Asia (RESTUCCIA et al., 2015), being Brazil, Paraguay, Venezuela, Colombia, Indonesia, Ethiopia, India and Mexico the main coffee producers (LEONG et al., 2014). Brazil, apart from being one of the largest producers in the world, is also the largest exporter of coffee. Total production for crop in 2016 was around 151,624 bags; for the commercial Arabica specie, the production was of approximately 95,204 bags compared to the Robusta specie with 56.419 thousand bags (INTERNATIONAL COFFEE ORGANIZATION - ICO, 2017).

After harvesting, coffee can be processed by different methods:: dry, wet and semi-dry process (SILVA et al., 2013). In the dry method, the whole cherry is dried under the sun or in a mechanical dryer after being washed. During this process, the most important step is the drying operation because it determines the quality of coffee. In the wet method, which requires a big amount of water, the pulp is eliminated by a pulper and beans are placed in fermentation tanks and submerged under water for 48 h to remove mucilage. The semi-dry or pulped natural method is an intermediate of both dry and wet; at the beginning, cherries are depulped by a wet mechanical process and beans are dried with its mucilage in patios (DUARTE; PEREIRA; FARAH, 2010; SILVA et al., 2013).

Therefore, the type of process should be carefully chosen because sometimes it changes concentrations of several biochemical compounds such as acetic acid, lactic acid, caffeine, chlorogenic acid and others. Furthermore, it also contributes to the formation of new compounds, either give coffee its flavor. In addition, a few of non-volatile or volatile compounds have health benefits such as, antioxidant activity, antidiabetic activity and reduce cholesterol levels (BELGUIDOUM et al., 2014). In this sense, determination of these compounds before and after processing becomes important because they are influenced by coffee variety, geographical origin, roasting conditions and microbiota during fermentation.

Fermentation of coffee fruits occurs naturally regardless the processing method. The objective of fermentation is to remove the mucilaginous layer, which is rich in

polysaccharides (pectins), and to reduce the water content (SILVA et al., 2013). Most microorganisms are responsible for fermentation, and these are indigenous species that originate as natural microbiota of coffee, such as bacteria (positive and negative), yeast and filamentous fungi. Population of each microbial group varies and depends on the processing method and extent of water loss (SILVA et al., 2000). Species of yeasts most commonly found during coffee fermentation are *Pichia guilliermondi*, *Pichia anomala, Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Debaryomyces hansenii* and *Torulaspora delbrueckii*. Bacteria of the genera *Erwinia*, *Klebsiella*, *Aerobacter*, *Escherichia* and *Bacillus* can also be found (AVALLONE et al., 2001; SILVA et al., 2008, 2013). It has been described that fermented coffee has a better quality and its control minimizes the production of poor beverages. The incomplete fermentation results in a second fermentation during drying and storage, and over-fermentation or bad fermentation produces butyric and propionic acids (AVALLONE et al., 2002).

One option to optimize and enhance coffee fermentation is the usage of starter cultures. Besides this advantage, they can also prevent the growth of filamentous fungi that produces ochratoxins (EVANGELISTA et al., 2014; SILVA et al., 2013). The implementation of initial cultures can increase beverage value without increasing the cost. Moreover, the selection of microorganisms should be based on pectinase production, acidic and other compounds since they interfere in the final product (SILVA et al., 2013). Yeasts are a good example of having antagonistic effects against fungi and a strong pectinolytic activity, such as *S. cerevisiae* (MASOUD et al., 2004); in other words, they are potential tools.

The behavior of such starter cultures during fermentation and other present microbiota can be trace with culture dependent and independent methods since both can give a complete scene of the microbial diversity.This means that in some cases the low population of species considered viable but non-culturable (VBNC) does not always growth in agar and are identified by molecular methods (VILELA et al., 2010). The qPCR methods have showed to be useful for quantitative analysis of microorganisms in food fermentations (BRINKMAN et al., 2003), for example in the recent research of semi-dry coffee by Ribeiro et al. (2017) and other foods such as a cocoa fermentation in Batista et al. (2015).

In summary, this study aims to evaluate the dynamic behavior of inoculated yeasts implemented in semi-dry coffee fermentation by qPCR, metabolites production by HPLC and GC-MS and cupping test analysis of the beverage.

2 LITERATURE REVIEW

2.1 General characteristics of coffee

The botanist Linnaeus first described the coffee specie *Coffea arabica* L. in 1753. The genus *Coffea* L. belongs to the family Rubiaceae subfamily Ixoroideae, tribe Coffeeae DC. The genus *Coffea* L. currently comprises 104 species classified into the two subgenera: *Coffea* subgen. *Coffea* with 95 species and *Coffea* subgen. *Baracoffea* with nine species (DAVIS et al., 2006).

Three species used for beverage coffee production belong to *Coffea* subgen. *Coffea*: *C. Arabica* L. (arabica coffee), *C. canephora* Pierre ex A. Froehner (robusta coffee) and *C. liberica* Bull ex Hiern (liberica or excels coffee). *C. arabica* L. and *C. canephora* account for almost all the coffee produced and consumed worldwide (DAVIS et al., 2006, 2007).

In terms of natural origin, genus *Coffea* L. species was exclusively found in Africa, Madagascar and the Madagascar Islands. The cultivated species *C. arabica* L. probably originated from the west side of the Great Rift Valley in southern Ethiopia, where subspontaneous population still grows. Coffee was first explored by the Arabians who may have introduced plants from Ethiopia to Yemen as early as 575. Two varieties of arabica coffee, called Typica and Bourbon, spread from Yemen (SAKIYAMA; GAVA, 2015).

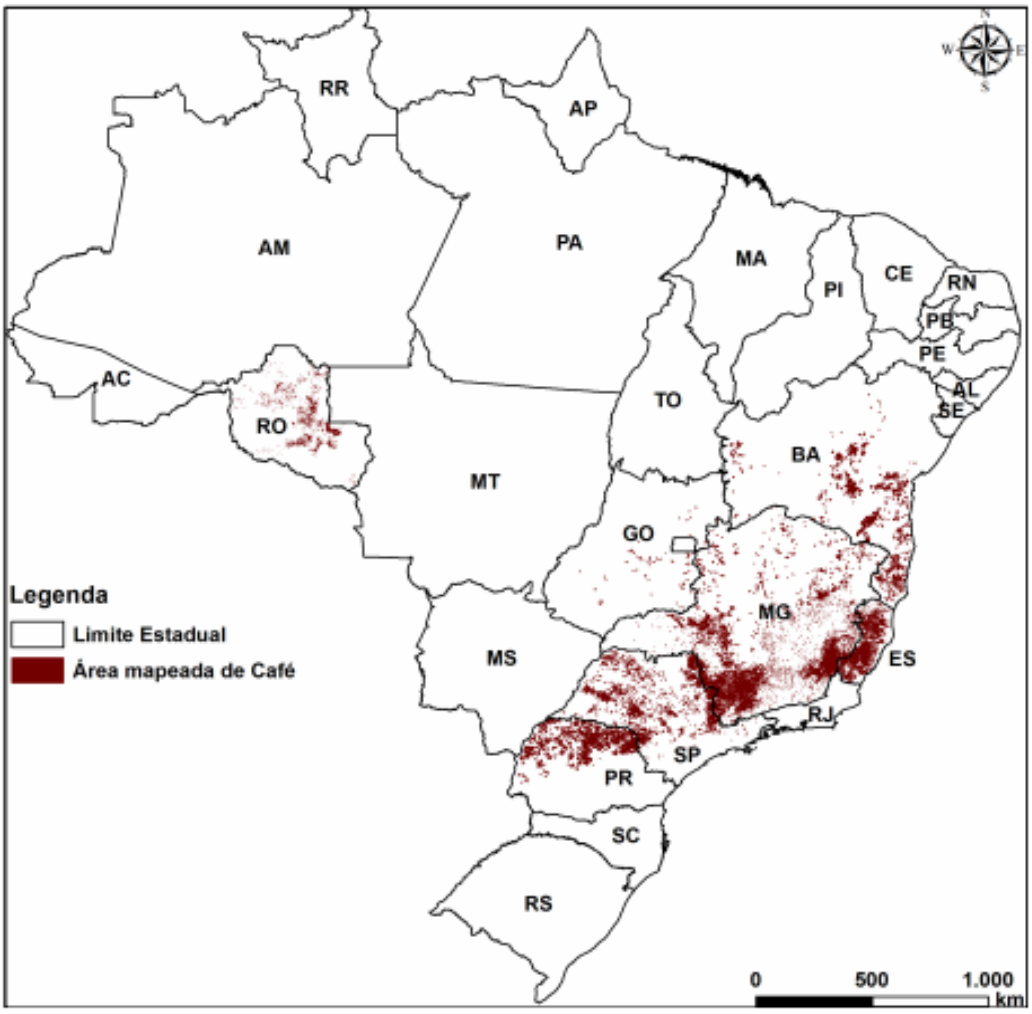
2.2 Coffee production in Brazil

Brazil is so far the most important arabica coffee producer in the world and grows cultivars essentially derived from Typica and Bourbon. Cultivation of arabica coffee in Brazil began with the introduction of the first seeds and seedlings from Guyana in 1727, being first introduced to the Typica cultivar. The second arabica coffee introduced into Brazil was the Bourbon Vermelho cultivar from the Reunion Island in 1852, since this cultivar had greater productivity than the original Typica cultivar. Natural mutations in the Typica cultivar produced the Amarelo de Botucatu cultivar, in 1871. The third arabica coffee introduced was the Sumatra cultivar (cultivar of Typica variety) from the Sumatra Island in 1896, which presented good vegetative vigor, beverage quality and its productivity was below expectations. Selection of natural hybrid between the cultivars Bourbon Vermelho and Amarelo de Botucatu originated the Bourbon Amarelo cultivar, in 1930. Dwarf cultivars Caturra Vermelho and Caturra Amarelo were selected from Bourbon Vermelho, in 1937 (SAKIYAMA; GAVA, 2015).

Nowadays, the two most important cultivars grown in Brazil are Mundo Novo and Catuaí (Catuaí Vermelho and Catuaí Amarelo). Natural hybridization between Sumatra with Bourbon Vermelho in 1943 generated the Mundo Novo cultivar. The hybridization between Mundo Novo and Caturra Amarelo in 1949 originatedthe Catuaí Amarelo and Catuaí Vermelho cultivars. The Catuaí Amarelo cultivar was backcrossed with Mundo Novo in order to improve the vegetative vigor and gave the following new dwarf cultivars: Rubi, Topázio, Ouro verde, Ouro Amarelo, Ouro Bronze and Travessia (SAKIYAMA; GAVA, 2015).

In Brazil, the main states producing coffee are Minas Gerais, Espírito Santo, São Paulo, Bahia, Paraná, Rondônia and Goiás (Figure 1), with 98.6 % of total production. Arabica coffee is the most produced in all the country (75.1% out of the total). The Minas Gerais state cultivates approximately 98.87% of specie arabica in their growing areas (COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB, 2013).

Figure 1 - Map of main states producing coffee in Brazil.



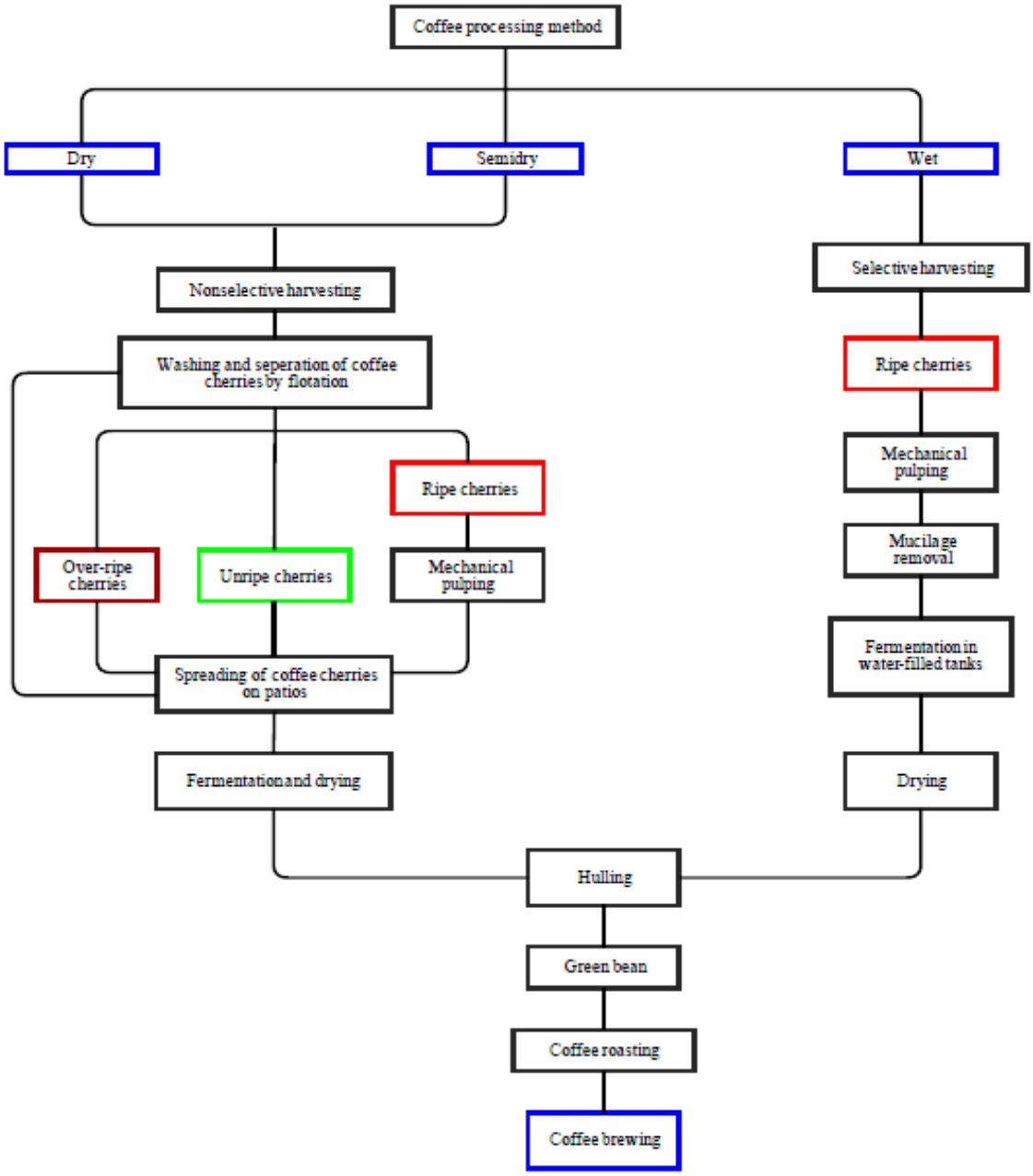
Source: CONAB (2013).

2.3 Coffee processes

The processing of coffee initiates after harvesting the coffee cherries, followed by the removal of both the pulp and hulk, which can be by either dry, wet or semi-dry methods (MUSSATTO et al., 2011) (Figure 2).

Coffee that is processed by the wet method is called washed or parchment coffee; it requires reliable pulping equipment and adequate supply of clean water (MURTHY; NAIDU, 2012). In this process, the objective is, before drying, to remove both the pulp and the mucilage, covering the seeds in an environmental friendly way. For initiation, only ripe cherries will be harvest and used, and depending on the product harvested, separation may vary (mechanically or not); when mechanically, cherries may be pulped in a water flow. Then, mucilage is removed by fermentation (can be placed in tanks), followed by washing or machines processes (BRANDO; BRANDO, 2015).

Figure 2 - Coffee processing methods.



Source: Dias et al. (2015), modified.

The dry method, also known as natural processing, is implemented in countries such as Brazil and Ethiopia that have extended periods of sunshine (LEE et al., 2015). For this process, coffee cherries are either handpicked or machine-harvested when most of them are matured. Consequently, the levels of maturity are not consistent among the harvested coffee cherries. Following harvesting, coffee cherries are then put on patios and left to dry under the sun in layers of approximately 5-8 or 10 cm for 10–25 days, where they are constantly heaped and re-spread, until reaching a moisture of 11-12%. Along drying, natural microbial

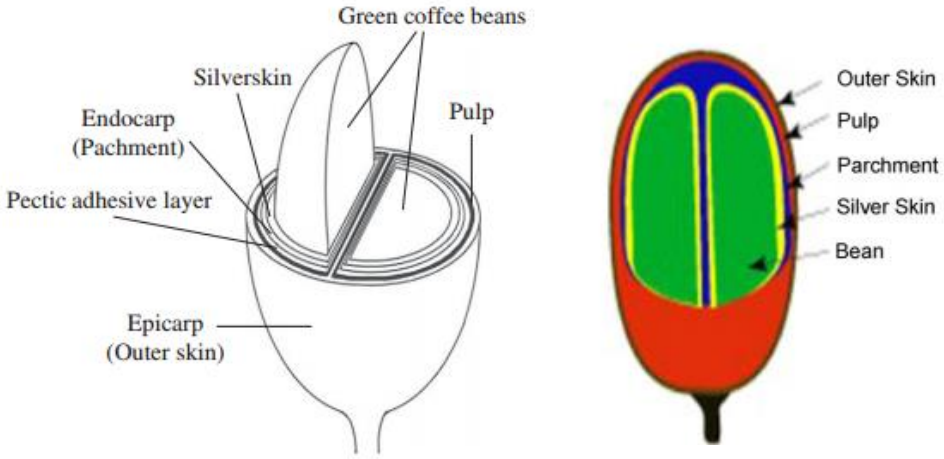
fermentation takes place and enzymatic hydrolysis leads to a breakdown of the pulp and mucilage within the coffee cherry, leaving it intact (BRANDO; BRANDO, 2015).

On the other hand, the semi-dry or pulped natural method is a variation of wet and dry processes, which started to be used in Brazil in the early 1990s. This process aims separation of ripe and unripe cherries by flotation using water. Following, ripe cherries are pulped and seeds are dried with the mucilage that was not previously removed surrounding the parchment, fermentation process occurs directly under the sun. Coffee seeds resulting from this method are called pulped natural coffees (BRANDO; BRANDO, 2015).

2.3.1 Pulp and mucilage

Before describing two important parts of coffee cherries, the pulp and mucilage, Figure 3 shows their position on a sectioned cherry. Coffee pulp is the first by-product obtained during processing and represents 29% dry-weight of the whole cherry. Coffee pulp is rich in carbohydrates, proteins and minerals (especially potassium) and it also contains appreciable amounts of tannins, polyphenols and caffeine. The organic components present in coffee pulp (dry weight) includes tannins 1.80 to 8.56%, total pectic substances 6.5%, reducing sugars 12.4%, non-reducing sugars 2.0%, caffeine 1.3%, chlorogenic acid 2.6%, and total caffeic acid 1.6% (MURTHY; NAIDU, 2012).

Figure 3 - Section of a coffee cherry.



Source: Murthy and Naidu (2012) and Narita and Inouye (2014).

The quantity of mucilage (Pectic adhesive layer) in coffee depends on the ripening stage, moisture and size of fruits. A green bean has near 1.3% of mucilage, least ripe over 8.4% and overripe between 1 to 23%. Specie Arabica has around 44% of pulp and 11% of

mucilage (QUINTERO, 2012). The mucilage is a gelatinous, translucent and sweet substance, which is richer in sugar in arabica than in robusta coffee (SAKIYAMA; GAVA, 2015); it is a source of fermentable carbohydrates for coffee fermentation, minerals, such as Ca, K and P, and amino acids (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2006). In addition, it is a layer that contains 84.2% of water, 8.9% proteins, 4.1% sugar, 0.91% pectic substances and 0.7% ash (LEE et al., 2015).

2.3.2 Beans composition

Chemical composition of green coffee from two species is shown in Table 1. Caffeine is the most known component of coffee beans (also thermostable). In raw Arabica coffee, caffeine can be found in values varying between 0.8% and 1.4% (w/w), while for the Robusta variety these values vary between 1.7% and 4.0% (w/w). However, coffee bean is constituted by several other components, including cellulose, minerals, sugars, lipids, tannin, and polyphenols. Minerals include potassium, magnesium, calcium, sodium, iron, manganese, rubidium, zinc, copper, strontium, chromium, vanadium, barium, nickel, cobalt, lead, molybdenum, titanium, and cadmium. Among the sugars, sucrose, glucose, fructose, arabinose, galactose, and mannose are present. Several amino acids such as alanine, arginine, asparagine, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine can also be found in these. Additionally, coffee beans contain vitamin of complex B, the niacin (vitamin B3 and PP) and chlorogenic acid (MUSSATTO et al., 2011).

The coffee bean is rich in polysaccharides mainly consisting of mannans or galactomannans, type II arabinogalactans, and cellulose (CAMPOS-VEGA et al., 2015). Furthermore, amino acids are considered the main components, contributing directly to the typical flavor and aroma left in roasted beans. Hence, levels of amino acids vary according to the processing method and can accumulate under stress conditions (such as osmotic pressure) (DIAS et al., 2012).

Table 1 - Chemical composition of green coffee.

Components	Arabica[a]	Robusta[a]	Constituents
Soluble carbohydrates	9 - 12.5	6 - 11.5	
Monosaccharides	0.2 - 0.5		Fructose, glucose, galactose, arabinose (traces)
Oligosaccharides	6 - 9	3 - 7	Sucrose (>90%), raffinose (0–0.9%), stachyose (0–0.13%)
Polysaccharides	3 - 4		Polymers of galactose (55–65%), mannose (10–20%), arabinose (20–35%), glucose (0–2%)
Insoluble polysaccharides	46 - 53	34 - 44	
Hemicelluloses	5 - 10	3 - 4	
Cellulose, β(1-4) mannan	41 - 43	32 - 40	
Acids and phenols			
Volatile acids	0.1		
Nonvolatile aliphatic acids	2 - 2.9	1.3 - 2.2	Citric acid, malic acid, quinic acid
Chlorogenic acid	6.7 - 9.2	7.1 - 12.1	Mono-, dicaffeoyl-, and feruloylquinic acid
Lignin	1 - 3		
Lipids	15 - 18	8 - 12	
Wax	0.2 - 0.3		
Oil	7.7 - 17.7		Main fatty acids: 16:0 and 18:2 (9,12)
N compounds	11 - 15		
Free amino acids	0.2 - 0.8		Main amino acids: Glu, Asp, Asp-NH_2
Proteins	8.5 - 12		
Caffeine	0.8 - 1.4	1.7 - 4.0	Traces of theobromine and theophylline
Trigonelline	0.6 - 1.2	0.3 - 0.9	
Minerals	3 - 5.4		

[a] Value % dry-weight basis. Source: Mussatto et al. (2011).

2.4 Fermentation of coffee

In all processing methods, the objective of fermentation is to remove the layer of mucilage from the seed to which adheres, while its degraded fruits are simultaneously dried to 11-12% of moisture. During fermentation, physicochemical changes occur in grains, such as reduction in water content, simple sugars are consumed, and aroma and flavor precursors are formed. Fermentation is a step that occurs naturally regardless of the processing method (SILVA et al., 2013).

The optimum temperature for fermentation is 30–35 ºC. The coffee masses are stirred 2–3 times or more during fermentation period, depending on the method of processing. The degradation of mucilage takes approximately between 6 to 72 h, either by drying or submerged for fermentation depending on the specie of coffee, the inherent concentration of pectinolytic enzymes, ambient temperature and elevation (BRANDO; BRANDO, 2015; MURTHY; NAIDU, 2012).

Yeast, aerobic bacteria and facultative bacteria population increase in the first hours of fermentation in open systems and without water, as the process move forward pH decreases and inhibits growth of certain organisms in the mucilage (QUINTERO, 2012).

A poor control of fermentation would be reflected in coffee aroma. Over-fermentation results in the production of black or ‘‘stinker’’ beans with poor visual and aroma characteristics. These beans are commonly associated with fruity, flora, sour and alcoholic attributes. Therefore, this shows that there is only a fine margin between the fermentation process and the quality of coffee aroma (LEE et al., 2015).

Optimization of coffee fermentations and production of special and good quality beverages can be achieved by using starter cultures. Upon selecting starters certain criteria must be used, just as, depending on their capacity to degrade mucilage, maintenance of population, metabolite production and others during fermentation drying. However, after selecting the appropriate strain, producers/farmers must carefully choose the inoculation method they will be using, since the effect can either be positive or turn into something expected.

2.4.1 Diversity and roles of microorganisms

Three important roles that microorganism execute during coffee fermentation are the production of pectinolytic enzymes to degrade the mucilage and pulp, spoilage of food, and

production of mycotoxins due to poor drying and storage. The microbiota present in coffee fruits is complex and diverse and include yeasts, filamentous fungi, and bacteria (SILVA et al., 2000).

In past studies, several species of microorganisms have been isolated from the fermentation phase of wet-processing such as aerobic bacteria - *Klebsiella ozaenae, K. oxytoca, Erwinia herbicola* and *E. dissolvens* and lactic acid bacteria such as - *Leuconostoc mesenteroides, Lactobacillus brevis* are the bacterial species that were isolated from the fermentation process. Yeast species such as *Kloeckera apis apicualata, Candida guilliermondii, Cryptococcus albidus, C. laurentii, Pichia kluyveri, P. anomala, Hanseniaspora uvarum, Saccharomyces cerevisiae, Debaryomyces hansenii* and *Torulaspora delbrueckii* have also been identified (AGATE; BHAT, 1966; AVALLONE et al., 2001; EVANGELISTA et al., 2015; MASOUD et al., 2004).

In Arabica and Robusta coffees processed by the semi-dry process, genera of *Bacillus, Flavobacterium, Serratia, Pseudomonas* and Lactic acid bacteria were found. Yeasts is the second group of microorganism in depulped fruit coffee in this process and some identified species are *S. cerevisiae, Axula spp., Candida ernobii, C. membranifaciens, Kluyveromyces spp., H. uvarum* and *Torulaspora delbrueckii* (SILVA, 2015).

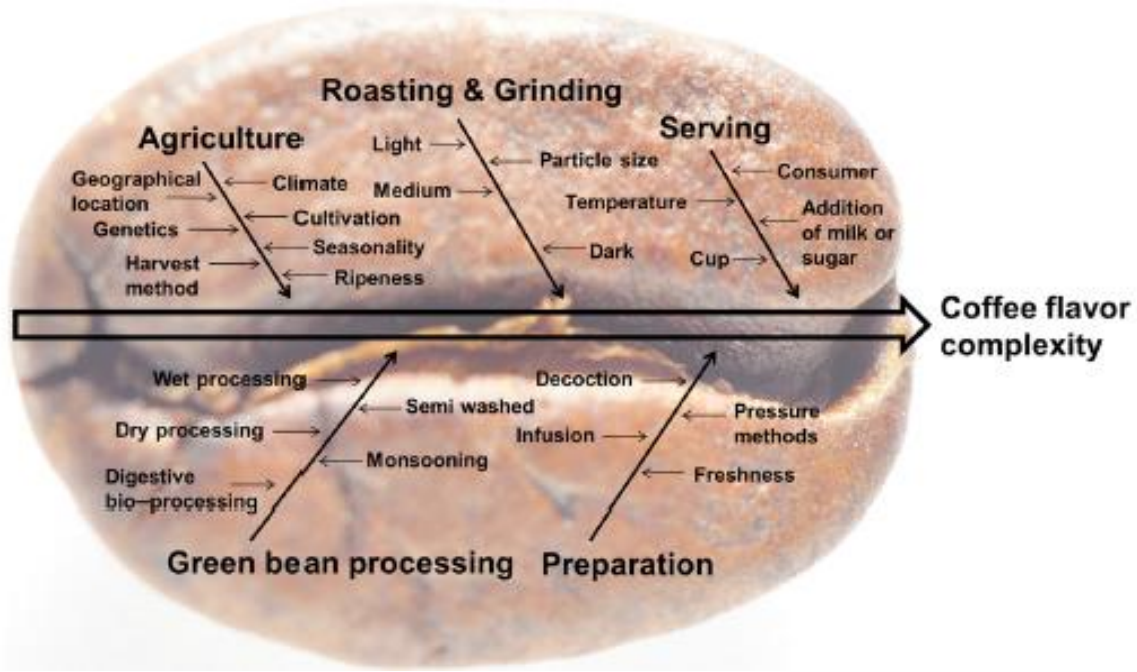
Previous studies of Silva et al. (2013) showed that indigenous and nonindigenous bacteria and yeast exhibited pectinolytic activity. Part of the potential starter cultures that were identified includes yeast cultures such as *Saccharomyces, Pichia* and *Candida*, which showed higher pectinolytic enzyme activity for efficient mucilage degradation during fermentation. Strains were also evaluated for their ability to enhance the quality of coffee fermentation in wet, dry and semi-dry processing and produced coffees with distinctive flavor. It was found that the employment of a selected culture for fermentation during coffee processing enhanced the quality of coffee aroma compared to coffees produced from fermentation involving indigenous microbiota (EVANGELISTA et al., 2013, 2014).

A culture-independent method such as quantitative real-time PCR (QPCR) is known as a fast, advantageous and sensible technique that is used for quantifying culture starters (BATISTA et al., 2015). Several recent studies in food fermentation, such as in Batista et al. (2015) with cocoa fermentations and Ribeiro et al. (2017) with coffee fermentation used this technique to monitor their yeast starters and showed promising and reliable results.

2.5 Coffee flavor and volatile compounds

Flavor of coffee is complex and it develops in various stages of processing and cup preparation as shown in Figure 4. In dry processing, a ‘hard’ coffee with a medicinal flavor is produced while wet processing yields a better quality coffee with less body, higher acidity and more aroma than the dry processing. The semi-dry (semi-washed or pulped natural) offers a coffee with intermediate body. Furthermore, roasting has the most influence on coffee flavor (Figure 5), with temperatures varying between 180 ºC to 240 ºC for 8 to 15 min. During roasting, endothermic and exothermic processes begin from heat being transferred to the bean through hot gases or contact with the metal surface of the coffee roaster, which reduces water content of the coffee beans and causes puffing and cooling to produce desirable characteristics. The impact of roasting on flavor comes from the degradation and formation or release of numerous chemical compounds through Maillard reactions, Strecker degradation, break down of amino acids, degradation of trigonelline, quinic acid, pigments, lipids and interaction between intermediate products (SUNARHARUM; WILLIAMS; SMYTH, 2014).

Figure 4 - Factors that affect coffee flavor.

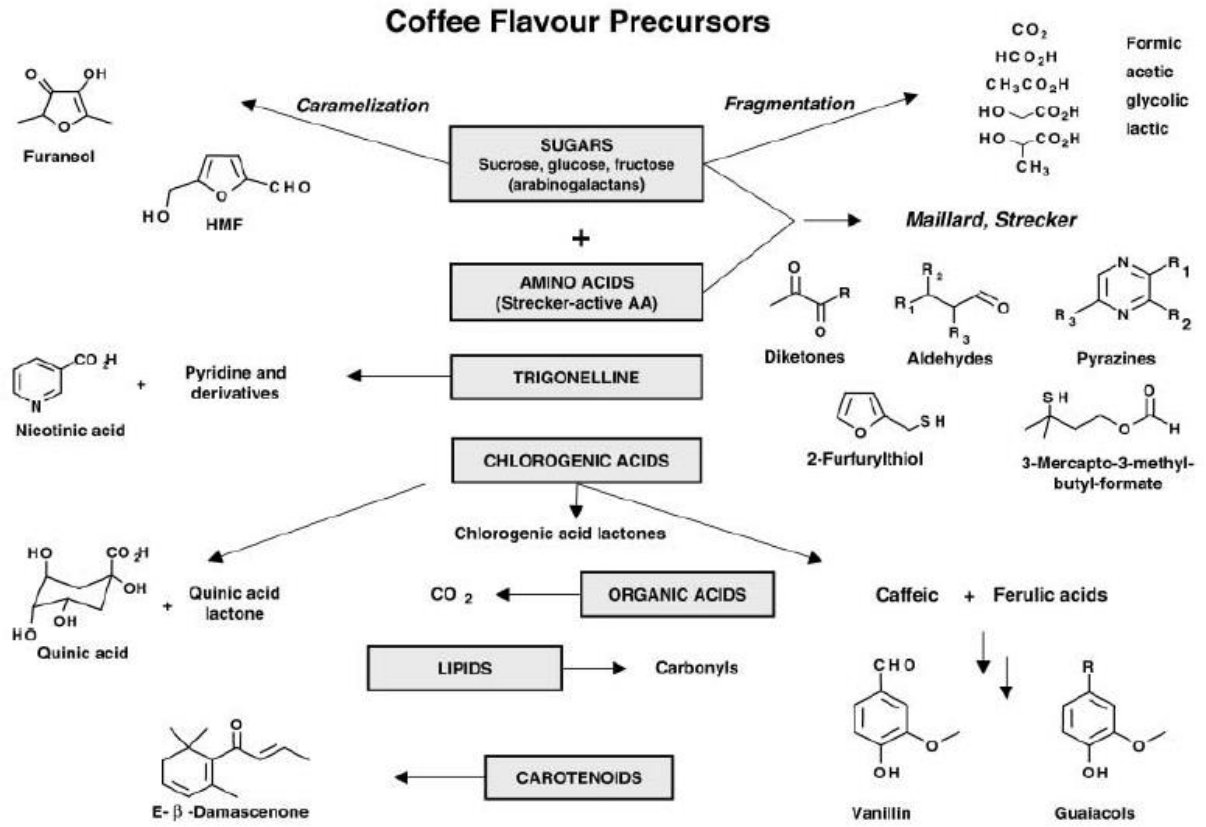


Source: Sunarharum, Williams and Smyth (2014).

Tannins are the main phenolic compounds in coffee pulp and in the seed, phenolic compounds are present predominantly as a family of esters formed between certain hydroxycinnamic acids and quinic acid, collectively known as chlorogenic acids (CGA). Other phenolic compounds, such as tannins, lignans and anthocyanins are also present in coffee seeds in smaller amounts. CGA, which are present in high concentrations in green coffee seeds (up to 14 %), have a marked influence in determining coffee quality and play an important role in the formation of coffee flavor (FARAH; DONANGELO, 2006).

A variety of other volatile compounds comprise several chemical classes including alcohols, aldehydes, ketones, carboxyclic acids, pyrazines, pyrroles, pyridines, sulfur compounds, furans, furanones, phenols, oxazoles among others. These compounds vary significantly in concentration and sensory potency which makes coffee flavor extremely complex, and explains why different coffee types may exhibit such diverse, unique and specific flavors (SUNARHARUM; WILLIAMS; SMYTH, 2014).

Figure 5 - Main classes of volatile compounds formed during roasting from non-volatile precursors in the green beans.



Source: Yeretzian et al. (2002).

Studies have shown that gas chromatography–mass spectrometry analysis (GC–MS) of volatile components in coffee together with principal component analysis (PCA) had allowed discrimination of Arabica/Robusta blends (AGRESTI et al., 2008) and analysis of compounds produced in cup (BRESSANELLO et al., 2017).

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SEGUNDA PARTE – ARTIGO

ARTIGO 1 - Different inoculation methods for semi-dry processed coffee using yeasts as started cultures

Artigo redigido conforme normas da revista International Journal of Food Microbiology

ABSTRACT

Coffee is a bitter beverage consumed worldwide due to its pleasant and stimulating properties. This study evaluates the behavior of *Saccharomyces (S.) cerevisiae* (CCMA 0543), *Candida (C.) parapsilosis* (CCMA 0544), and *Torulaspora (T.) delbrueckii* (CCMA 0684) as starter cultures for semi-dry processed coffee using two inoculation methods: (1) direct inoculation of the yeast starter culture solution by spraying the coffee beans (the direct method), and (2) inoculation in which coffee beans are fermented with a yeast starter solution in polystyrene buckets for 16 hours (the bucket method). The microbial population was evaluated by plating and real-time or quantitative polymerase chain reaction (qPCR). The microbial metabolic response of both the bucket and direct inoculation methods during fermentation was evaluated using high performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC-MS). Quantification of sugars, acids, caffeine, 5-caffeoylquinic acid (5-CGA), and trigonelline were performed in HPLC. For volatile compounds, GC-MS and a sensorial test were also carried out. Citric and succinic acids were detected throughout the fermentation period. Chlorogenic acid concentration levels were higher for the bucket method after roasting. Roasted coffee beans also had a higher caffeine concentration, with the exception of the *T. delbrueckii* (CCMA0684) assay. Acids, pyrazines and pyridines were the main volatile compounds in both green and roasted coffee beans. Coffee cupping results proved that both inoculation methods scored well in terms of coffee quality. Inoculating beans with yeast using the bucket method before sun drying encouraged microbial growth and metabolite production, generating a better quality coffee.

Keywords: coffee quality; qPCR, volatile compounds; coffee fermentation

1. Introduction

Coffee is a dark-brown, slightly bitter beverage made from ground and roast coffee beans, which is widely consumed and considered popular due to its stimulant properties and composition (Ballesteros et al., 2014; Contreras-Calderón et al., 2016). South and Central America, the Caribbean, Africa and Asia are the main coffee-producing areas (Restuccia et al., 2015). Brazil is the major global coffee producer and exporter, with 55.000 thousand bags produced in 2016 (ICO, 2017). Within Brazil, Minas Gerais, Espírito Santo and São Paulo are the main coffee producing states (CONAB, 2017).

Coffee beverages are produced mainly by the processing of two species of the *Coffea (C.)* genera: *C. canephora* (robusta coffee) and *C. arabica* (Arabica coffee). Arabica coffee is recognized to have better quality and flavor and accounts for approximately 65% of the world coffee trade and 75% of the coffee trade in Brazil. The largest numbers of Arabica coffee cultivars are found in plantations in Brazil's Mundo Novo and Catuaí (Amarelo and Vermelho) (Sakiyama et al., 2015).

Coffee fermentation occurs naturally, regardless of the process, in order to remove the mucilage from seeds and reduce water content, with beans being dried simultaneously until moisture content is reduced to between 11 and 12% (Silva et al., 2013). Most microorganisms responsible for fermentation are autochthonous (bacteria, yeast, and filamentous fungi). The population of each microbial group may vary depending on the processing method and the extent of water loss (Silva et al., 2000). During certain processing stages, the contribution of acetic acid, lactic acid, caffeine, chlorogenic acids and other compounds improves coffee flavor and gives health benefits. Coffee beans possess antioxidant and antidiabetic properties that can reduce cholesterol levels (Belguidoum et al., 2014). Measuring these compounds before and after processing is important because they are influenced by coffee variety, geographical origin, and microbiota during fermentation and roasting.

The role of yeasts is essential for coffee fermentation, preventing toxigenic filamentous fungi growth and boosting the production of pectinolytic enzymes, which help the degradation of the coffee mucilage and pulp (Ramos et al., 2010; Silva et al., 2013). Using yeasts as starter cultures during coffee fermentation can improve the quality of the end product. Silva et al. (2013) and Evangelista et al. (2014b) have reported promising results after the direct inoculation of *Saccharomyces*, *Pichia* and *Candida* yeast strains over coffee beans.

The use of molecular and biochemical techniques to evaluate starter cultures has been applied to different foods in previous research (Batista et al., 2015; Bressanello et al., 2017;

Evangelista et al., 2014b; Menezes et al., 2016). Starter culture populations have also been monitored by qPCR (Gil-Serna et al., 2009; Postollec et al., 2011; Michel et al., 2016). In combination, the methods used in GC-MS are also relevant because the process separates volatile compounds according to the stated conditions and generates data about unknown compounds from fermentation, which may derive from coffee or other samples (Batista et al., 2015; Bressanello et al., 2017; Evangelista et al., 2014b; Ziółkowskaet al., 2016).

The aim of this study was to use qPCR to evaluate the dynamic behavior of three inoculated yeasts: *S. cerevisiae* (CCMA 0543), *C. parapsilosis* (CCMA 0544), and *T. delbrueckii* (CCMA 0684). The study took place in Brazil and used direct and bucket inoculation methods to inoculate Catuaí Amarelo depulped cherries during a semi-dry process. The effect of the inoculation on the chemical composition of the bean (sugars, acids, and volatiles) was analyzed using gas and liquid chromatography. Sensorial analysis (coffee cupping) of the resulting beverage was carried out to confirm whether inoculation contributed to coffee quality.

2. Material and methods

2.1. Coffee cherries

Cherries from the *C. arabica* variant Catuai Amarelo were obtained from a producing farm near Lavras, Minas Gerais, Brazil. The cherries were processed using a semi-dry method, which consisted of depulping and washing, leaving only the mucilage and parchment (Brando and Brando, 2015).

2.2. Starter cultures

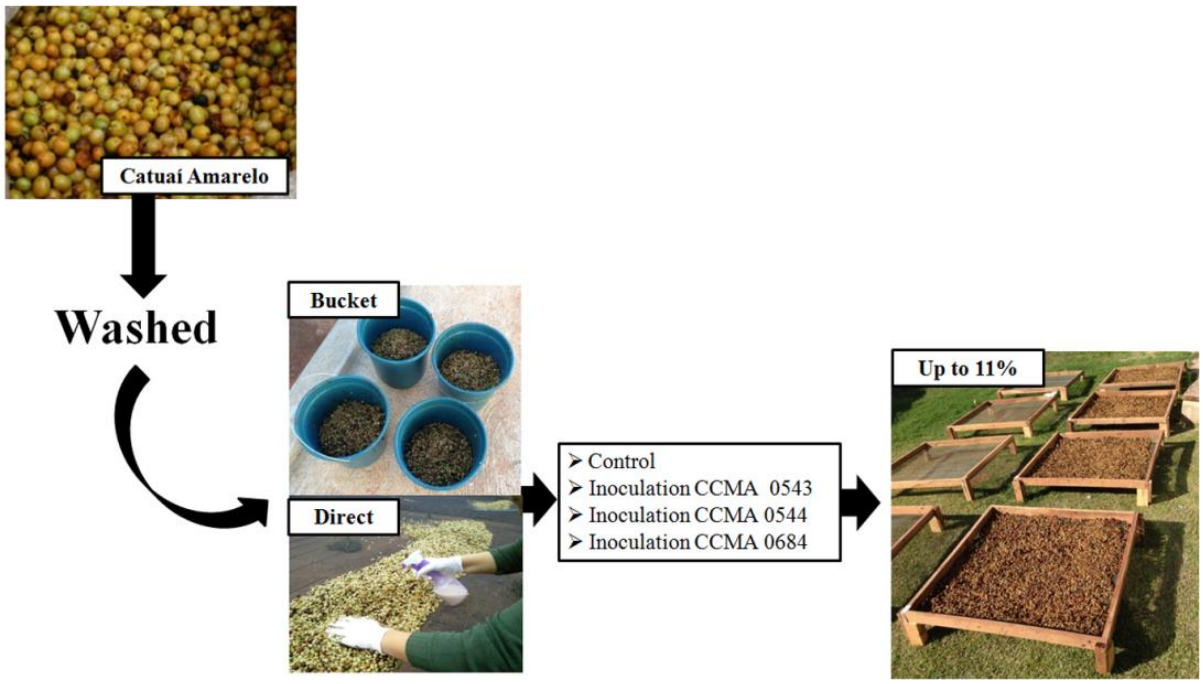
Three yeast strains—*S. cerevisiae* (CCMA 0543), *C. parapsilosis* (CCMA 0544) and *T. delbrueckii* (CCMA 0684)—from the Culture Collection of Agricultural Microbiology (CCMA) in Lavras were used as starter cultures (Evangelista et al., 2014b). Each yeast was grown in one liter of YEPG medium (20 g/L glucose, 10 g/L yeast extract, 10 g/L peptone soy, and pH 3.5) at 28ºC for 24 hours or until they reached a concentration of 10^9 cells/ml. After, cells were washed and diluted in 500 ml of water for inoculation.

2.3. Fermentation and drying

Two inoculation methods were tested: (1) direct inoculation by spraying the yeast solution on depulped cherries in wooden frames (1m x 1m) with plastic nets, and (2) inoculation of yeast solution on depulped cherries in polystyrene buckets for 16 hours, with

the fermented beans then being transferred to wooden frames for drying. A total of 8 treatments containing 10 kg of depulped cherries were carried out: three used direct inoculation; three used the bucket method; and two were used as controls (one sample was not inoculated but piled in a bucket and the other was not inoculated) (Figure 1). All treatments were fermented and sun dried until a moisture content of 10 to 11% was reached (measured using a water activity meter, provided by Pawkit). During the fermentation and drying process, samples of approximately 100 g were collected in sterile plastic bags until the appropriate moisture content was reached (after 0, 16, 64, 112, 256, and 352 hours, respectively).

Figure 1 – Flow diagram of the inoculation process and treatment.



2.4. Microbiological analysis

2.4.1. Microbial count

Initial and final samples (10 g) of fermented beans were homogenized in 90 mL saline-peptone water (0.1% (v/v) bacteriological peptone (Himedia) and 0.8% (v/v) NaCl (Merck, Whitehouse Station, NJ)) in a stomacher at normal speed for 5 minutes, serially diluted, and then plated in triplicate. YEPG (g/L glucose 20 (Merck), yeast extract 10 (Merck), peptone soy 10 (Himedia) and agar 20 (Merck), pH 3.5) with 0.01% (w/v) chloramphenicol, MRS agar containing 0.1% (w/v) nystatin, and Nutrient agar were used to

count the total yeast, lactic acid bacteria (LAB), and mesophilic bacteria, respectively. Plates were incubated at 30°C for 48 hours.

2.4.2. Quantitative polymerase chain reaction (qPCR)

Total DNA was extracted from samples at six different fermentation times (after 0, 16, 64, 112, 256, and 352 hours of drying, respectively)] using the "DNA Purification from Tissues" protocol (QIAamp DNA Mini Kit (Qiagen, Hilden, Germany)) in accordance with the manufacturer's instructions.

Specific primers for *S. cerevisiae* (Díaz et al., 2013), *C. parapsilosis* (Hays et al., 2011) and *T. delbrueckii* (Zott et al., 2010) were used (Table 1). The specificity of each primer pair was confirmed by searching in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). A qPCR analysis was used to quantify *S. cerevisiae*, *C. parapsilosis* and *T. delbrueckii,* as described by Batista et al. (2015). Three independent qPCR assays were performed for each treatment. For standard curves, all yeast species were cultivated in YEPG broth at 28ºC for 24 hours. The yeast populations were then counted using a Neubauer chamber. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and serially diluted (1:10) from 10^{10} to 10^{3} cell/mL. Each point of the calibration curve was measured in triplicate.

Table 1 – Specific primer used for qPCR analysis.

Specie	**Primers**		
	Name	Sequence	Source
S. cerevisiae	SC-5fw SC-3bw	5'AGGAGTGCGGTTCTTTGTAAAG3' 5' TGAAATGCGAGATTCCCCT3'	Díaz et al., 2013
T. delbrueckii	PRIMER 1 Tods L2 PRIMER 2 Tods R2	5' CAAAGTCATCCAAGCCAGC 3' 5'TTCTCAAACAATCATGTTTGGTAG3'	Zott et al., 2010
C. parapsilosis	SADH-F SADH-R	5' GCTGCGGCTTCAACTGATGC 3' 5' CTTGGTCACGAGCCTCC3'	Hays et al., 2011

2.5. Chemical analysis

2.5.1. Carbohydrates, organic acids, glycerol and ethanol by high performance liquid chromatography (HPLC)

The samples were evaluated after 0, 16, 112, 256, and 352 hours of fermentation. For extraction, 3 g of the sample was homogenized with 20 ml of Milli-Q water by vortexing for 10 minutes at room temperature. Then fluids were centrifuged two times at 100 x g for 10 minutes at 4ºC and were filtered through a 0.2 mm cellulose acetate filter. Later, a

chromatographic analysis was performed, according to Evangelista et al. (2014a). Calibration curves were constructed with different concentrations of standard compounds.

2.5.2. Caffeine, chlorogenic acid and trigonelline

Measurement of the non-volatile compounds (caffeine, chlorogenic acid, and trigonelline) in times 0, 112, 352 hours of fermentation and roasting was carried out by HPLC, according to Malta and Chagas (2009). Identification and quantitative analysis were performed using calibration curves of caffeine, trigonelline and 5-CGA (Sigma-Aldrich, Saint Luis, EUA).

2.5.3. Volatile compounds

Samples of green and roasted beans at both the start and end of the fermentation process were used for GC-MS analyses. Coffee samples were macerated with liquid nitrogen to extract manual headspace, according to Evangelista et al. (2014a). Volatile compounds were identified by comparing their mass spectra to those in the NIST11 Library. In addition, an alkane series (C10–C40) was used to calculate the retention index (RI) for each compound and compared with RI values found in the literature data.

2.6. Sensorial analysis—coffee cupping

Green coffee samples were prepared according to the Specialty Coffee Association of America (SCAA, 2013). The coffee was roasted in a laboratory roaster (Probatino, Leogap model, Brazil) with a capacity of 150 g and was then ground in an electric mill (Pinhalense ML-1, Brazil). A panel of three trained coffee tasters with Q-Grader Coffee Certificates evaluated the samples. The methodology applied to evaluate coffee was conducted according to SCAA standards (SCAA, 2013), which assess ten sensorial attributes: fragrance, flavor, aftertaste, acidity, body, uniformity, balance, sweetness, cleanliness, and score.

3. Results

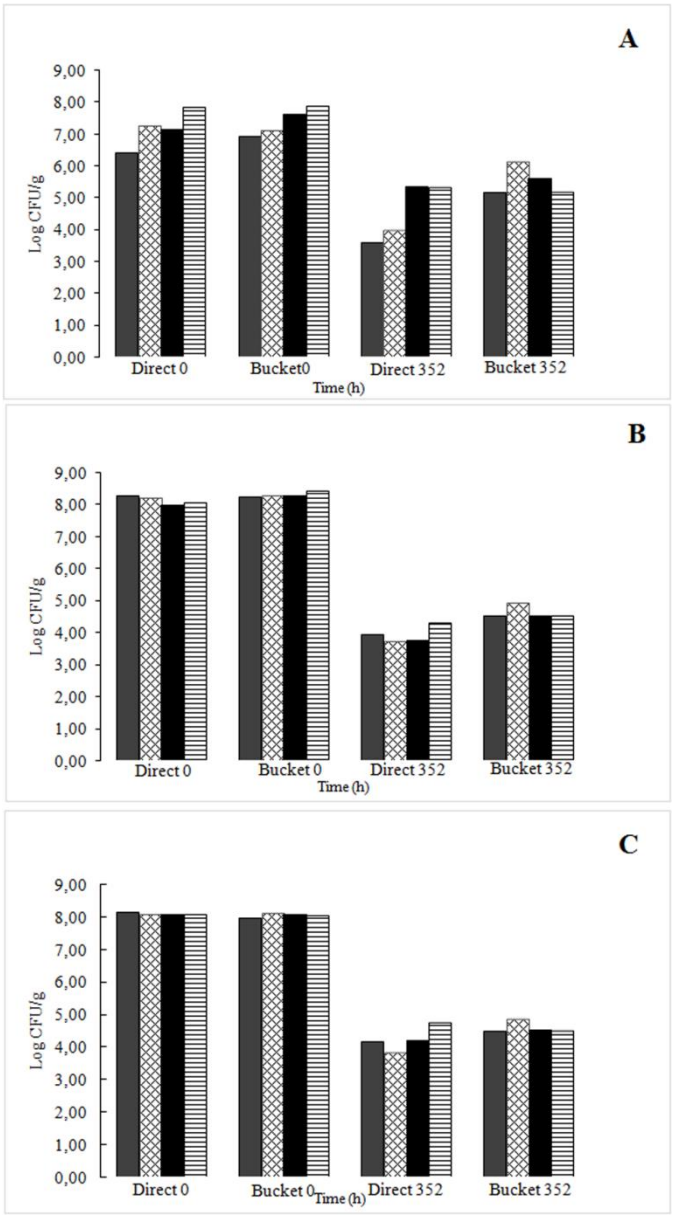
3.1. Microbiological analyses

3.1.1. Microbial count by plating

Total yeast count at the beginning of fermentation varied slightly according to the treatment (which strains were used as the starter culture) and inoculation method (direct or bucket) (Figure 2). With the direct method, the yeast count was 6.40 log CFU/g for the control, 7.25 log CFU/g for the sample inoculated with *S. cerevisiae* (CCMA 0543), 7.85 log

CFU/g for *C. parapsilosis* (CCMA 0544), and 7.15 log CFU/g for *T. delbrueckii* (CCMA 0684). With the bucket method, the yeast count was 6.90 log CFU/g for the control, 7.11 log CFU/g for the sample inoculated with *S. cerevisiae* (CCMA 0543), 7.87 log CFU/g for *C. parapsilosis* (CCMA 0544), and 7.63 log CFU/g for *T. delbrueckii* (CCMA 0684). However, at 352 hours of the fermentation process, there was a greater difference between treatments in relation to the yeast count. Treatments inoculated with the yeast *S. cerevisiae* (CMMA 0543) (6.10 log CFU/g) using the bucket method and treatments inoculated with *C. parapsilosis* (CCMA 0544) (5.30 log CFU/g) and *T. delbrueckii* (CCMA 0684) (5.34 log CFU/g) using the direct method had higher yeast counts than other treatments. The final yeast population using the direct method differed between the control (3.6 log CFU/g), *S. cerevisiae* (CCMA 0543) (4.0 log CFU/g), *C. parapsilosis* (CCMA 0544) (5.3 log CFU/g), and *T. delbrueckii* (CCMA 0684) (5.3 log CFU/g) treatments.

Figure 2 - Total population of yeast (A), lactic acid bacteria (B) and mesophilic bacteria (C) in coffee beans inoculated with the following yeasts: *S. cerevisiae* (CCMA 0543) (▨), *T. delbrueckii* (CCMA 0684) (■), *C. parapsilosis* (CCMA 0544) (▤), and Control (■).



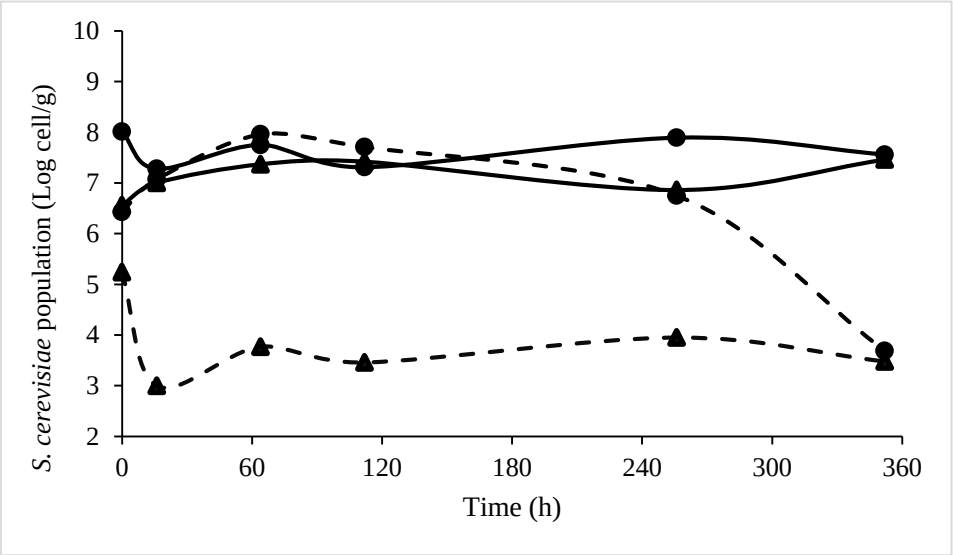
The bacteria population remained constant at the beginning of fermentation for both methods and all treatments (Figures 2B and 2C). However, at 352 hours, LAB and mesophilic populations were higher with the bucket method. The LAB population (4.88 log CFU/g) and the mesophilic bacteria count (4.87 log CFU/g) were higher with the bucket method when inoculated with *S. cerevisiae* (CCMA 0543). In addition, higher values were found at final LAB times (4.30 log CFU/g) and with mesophilic bacteria (4.77 log CFU/g) in direct treatments inoculated with *C. parapsilosis* (CCMA 0544).

3.1.2 Monitoring of starter strains by qPCR

This study used qPCR to evaluate both control coffee fermentations that were not inoculated and fermentations that were inoculated with *S. cerevisiae* (CCMA 0543), *C. parapsilosis* (CCMA 0544), and *T. delbrueckii* (CCMA 0684) (Figure 3). The specificity of primers was tested in a conventional PCR assay before running. Resulting standard curves parameters were observed, with fluctuating values for R^2 (from 0.992 to 0.998), slope (from -3.112 to -3.864), and efficiency (81% to 110%).

Yeasts used as starter cultures were quantified using inoculated coffee samples and control samples (Figure 3). Population of inoculated samples with *S. cerevisiae* (CCMA 0543) ranged from 8.0 to 7.6 log cell/g with the bucket method, and from 6.6 to 7.5 log cell/g with direct inoculation. However, in control samples, *S. cerevisiae* (CCMA 0543) behaved differently for both methods. With the bucket method, its population was higher for the first 112 hours and then declined, reaching 4.0 log cell/g at the end of processing. With the direct method, the highest *S. cerevisiae* (CCMA 0543) population was also observed at the start of fermentation (5.24 log cell/g), reaching 3.5 log cell/g at the end of processing.

Figure 3 - Dynamic behavior of *S. cerevisiae* (A), *T. delbrueckii* (B) and *C. parapsilosis* (C) during 352 hours of fermentation and drying in Catuaí semi-dry coffee beans with inoculation (full line) and control (dashed line), using direct (▲) and bucket (●) inoculation methods, measured by qPCR. (Continue)



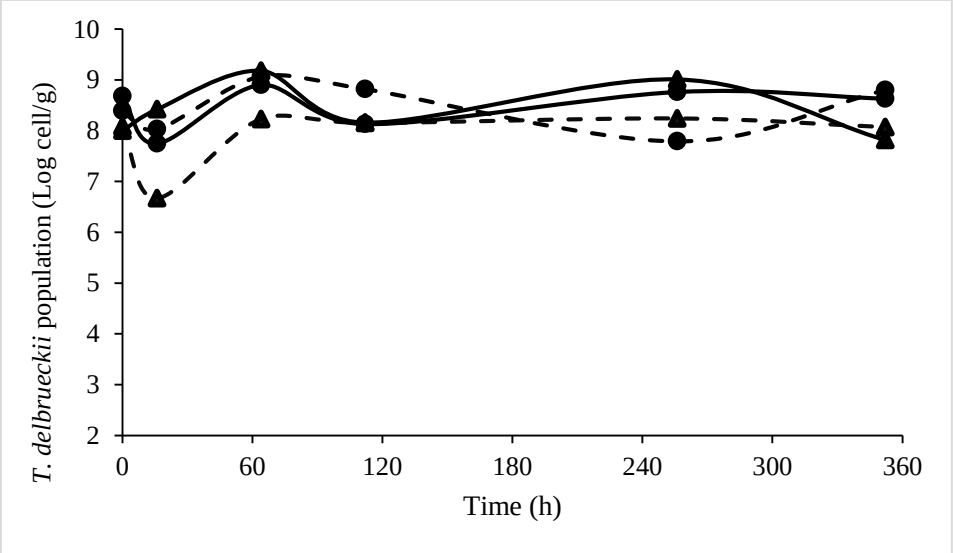
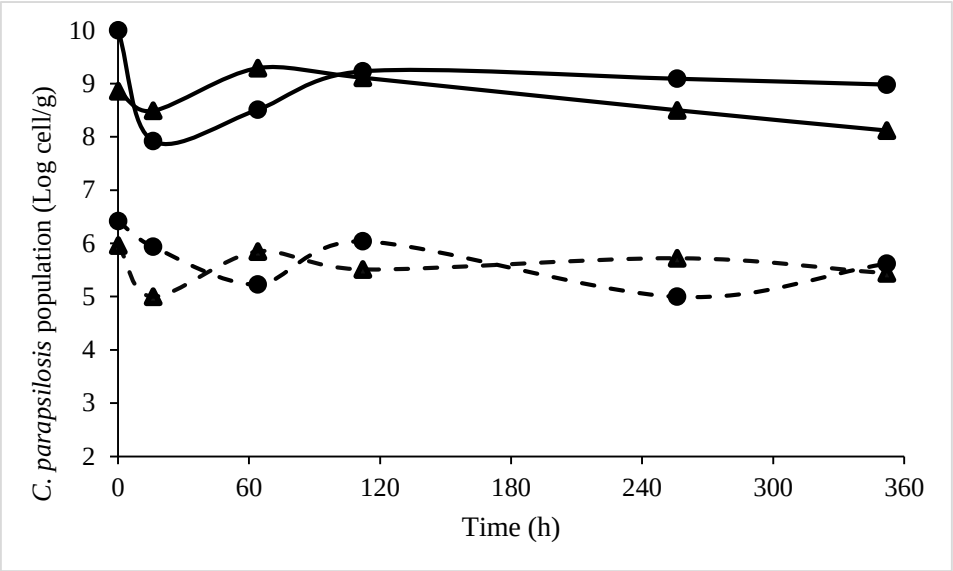


Figure 3 - Dynamic behavior of *S. cerevisiae* (A), *T. delbrueckii* (B) and *C. parapsilosis* (C) during 352 hours of fermentation and drying in Catuaí semi-dry coffee beans with inoculation (full line) and control (dashed line), using direct (▲) and bucket (●) inoculation methods, measured by qPCR. (Conclusion)



The *T. delbrueckii* (CCMA 0684) population was similar for the treatments performed in the present study, independent of inoculation method (Figure 3B). The yeast population remained higher with the bucket than with the direct method, ranging in inoculated assays from 8.7 to 8.6 log cell/g and from 8.4 to 8.8 log cell/g for the control at the end of the drying process. In relation to *C. parapsilosis* (CCMA 0544), the inoculation method contributed to the persistence of the species during coffee processing. Inoculated treatments had a larger population than the control (for both bucket and direct inoculation) (Figure 3C). The population of this yeast varied from 10 to 9 log cell/g from bucket inoculation, and from 8.9 to 8.1 log cell/g for direct inoculation. In the control, the average of the final population was 5.6 log cell/g for the bucket method and 5.4 log cell/g for the direct method.

3.2 Target metabolites

Organic compounds such as citric, malic, succinic, lactic, acetic, propionic, isobutyric, and chlorogenic acid, and glycerol and ethanol from the inside (green bean) and outside of the fermented beans were detected and quantified by HPLC (Table 2). The concentrations of citric acid were lowered during fermentation-drying. Lactic and acetic acids presented inverse behavior in relation to the inoculation method used. With the bucket method, there were higher concentrations of lactic acid (an average of 1.21 g/L on the inside and 2.25 g/L on the outside) than with acetic acid (0.20 g/L on the inside and 0.19 g/L on the outside). With the

direct method, there were higher concentrations of acetic acid (1.10 g/L on the inside and 2.07 g/L on the outside) than with lactic acid (0.13 g/L on the inside and 0.22 g/L on the outside).

Although lactic acid was produced throughout 352 hours of fermentation with both inoculation methods, when *C. parapsilosis* (CCMA 0544) and *T. delbrueckii* (CCMA 0684) were inoculated, there was a decrease in lactic acid production with direct inoculation (data not shown). Lactic and malic acid were produced, but only on the outside of the coffee bean after 112 hours of fermentation (from 0.07 to 0.13 g/Kg with the bucket method and from 0.26 to 0.30 g/Kg with the direct method). Malic concentrations were reduced for the same period on the inside of the grain.

At the start of the fermentation process, no acetic acid was detected on the outside of the grain with either inoculation method. However, at the end of the fermentation, higher concentrations were observed with the direct method (from 0.82 to 1.14 g/Kg on the inside and from 0.15 to 2.12g/Kg on the outside) when compared with the bucket method (from 0.20 to 1.61 g/Kg on the inside and from 0.10 to 0.30 g/Kg on the outside) (Table 2). Propionic acid was presented in both inoculation methods. Isobutyric acid had the highest values on the inside of the bean (ranging from 1.35 to 4.73 g/Kg with the bucket method and from 2.24 to 6.04 g/Kg with the direct method). Neither isovaleric nor butyric acid was detected during fermentation in any of the treatments studied. Chlorogenic acid increased in all fermentation samples, but decreased after roasting (18.39 g/Kg in grains inoculated using the bucket method and 14.91 g/Kg in grains inoculated using the direct method).

The inoculation methods affected ethanol production, with higher concentrations when the bucket inoculation method was used. On the outside of the grain, the concentration ranged from 0.88 to 0.16 g/Kg, and from 1.04 to 0.17 g/Kg on the inside. With the direct inoculation method, the ethanol concentration was similar both on the inside and the outside of the coffee grains (Table 2). Where bucket inoculation was used, coffee beans had a higher glycerol concentration (ranging from 0.17 to 0.88 g/Kg on the inside and from 0.36 to 2.25 g/Kg on the outside). With the direct method of inoculation, the concentration ranged from 0.12 to 0.59 g/Kg on the inside and from 0.19 to 0.96 g/Kg on the outside.

Table 2 - Average values of organic compounds for all yeast treatments and control from inside and outside the bean.

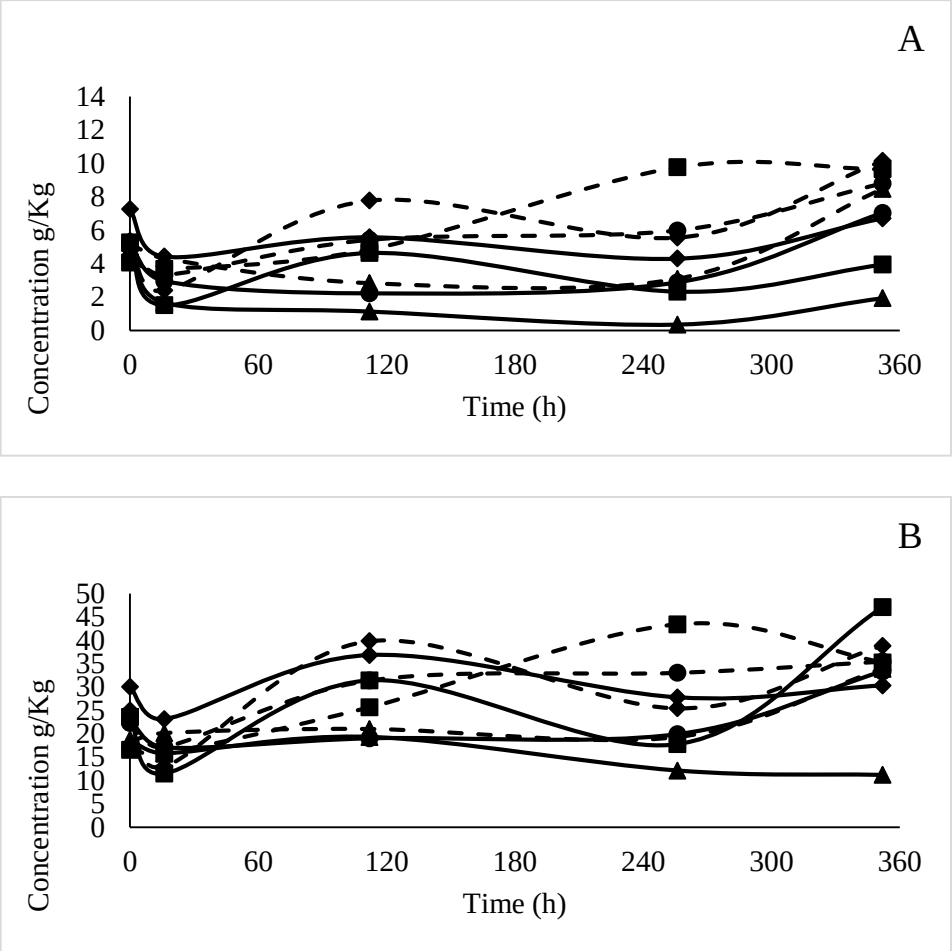
Sample	Ferm. time (h)	Organic compounds in beans (g/Kg)																				Chlorogenic Acids
		Citric		Malic		Succinic		Lactic		Acetic		Propionic		Isobutyric		Isovaleric		Glycerol		Ethanol		
		In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	
Box																						
	0	2.15	0.40	0.28	0.09	1.41	0.39	0.31	0.51	1.37	ND	0.27	0.04	2.96	0.10	ND	ND	0.28	0.62	1.04	0.88	9.42
	16	3.39	0.25	0.42	0.10	0.80	0.25	0.06	0.16	0.44	0.30	0.03	0.01	4.36	0.03	ND	ND	0.17	0.36	0.66	0.24	
	112	3.07	0.06	0.63	0.08	0.63	0.22	0.83	1.81	1.61	0.15	0.14	0.07	4.73	0.04	ND	ND	0.40	1.63	0.15	0.26	20.69
	256	2.34	0.06	0.45	0.07	0.22	0.20	0.38	1.50	0.41	0.10	0.06	0.05	3.19	0.04	ND	ND	0.81	1.15	0.21	0.16	
	352	1.96	0.07	0.32	0.13	0.07	0.30	1.21	2.25	0.20	0.19	0.20	0.07	1.35	0.16	ND	ND	0.88	2.25	0.17	0.16	21.83
	T																					18.39
Direct																						
	0	2.16	0.42	0.27	0.08	1.46	0.39	0.42	0.52	1.14	ND	0.18	0.04	2.62	0.15	ND	ND	0.34	0.86	0.81	0.86	8.76
	16	3.06	0.20	0.53	0.11	0.58	0.97	0.02	0.02	0.82	0.15	0.07	ND	3.25	0.06	ND	ND	0.12	0.19	1.08	ND	
	112	3.74	0.31	0.63	0.26	0.97	0.94	0.16	0.15	0.85	1.25	0.12	0.08	6.04	0.06	ND	ND	0.59	0.81	0.46	0.09	23.39
	256	2.34	0.16	0.47	0.26	0.54	0.69	0.07	0.12	1.00	2.12	0.20	0.03	3.04	0.11	ND	ND	0.30	0.91	0.31	0.08	
	352	1.77	0.23	0.33	0.30	0.15	0.50	0.13	0.22	1.10	2.07	0.30	0.01	2.24	0.08	ND	ND	0.27	0.96	0.20	ND	21.95
	T																					14.91

T= Roasted beans; ND= Not detected

3.3 Carbohydrates, trigonelline and caffeine

Sucrose, fructose, glucose, trigonelline and caffeine concentrations were analyzed by HPLC (Figure 4). All treatments showed sucrose consumption after 112 hours of fermentation (with the bucket method, sucrose concentration ranged from 6.7 to 18.2 g/Kg for the control, from 4.5 to 18.6 g/Kg when inoculated with *S. cerevisiae* (CCMA 0543), from 5.2 to 17.8 g/Kg when inoculated with *C. parapsilosis* (CCMA 0544), and from 7 to 14.5 g/Kg when inoculated with *T. delbrueckii* (CCMA 0684). With the direct method, the concentration ranged from 0.9 to 23.4 g/Kg for the control, from 3.8 to 19.9 g/Kg when inoculated with *S. cerevisiae* (CCMA 0543), from 4.5 to 22.1 g/Kg when inoculated with *C. parapsilosis* (CCMA 0544), and from 5.5 to 21.2 g/Kg when inoculated with *T. delbrueckii* (CCMA 0684)).

Figure 4 - Carbohydrates detected in coffee beans during fermentation and drying: glucose (A) and fructose (B), exposed to treatments with inoculation—by *S. cerevisiae* (CCMA 0543) (▲), *T. delbrueckii* (CCMA 0684) (■), and *C. parapsilosis* (CCMA 0544) (●)—and without inoculation—the control (♦)—using the bucket (full line) and the direct inoculation methods (dashed line).



In contrast to fructose, glucose concentrations were lower for all fermentation times as shown in Figure 4: from 7.2 to 4.3 g/Kg with the bucket method and from 10.1 to 2.3 g/Kg with the direct method for the control; from 4.8 to 0.3 g/Kg for the bucket method and from 8.5 to 2.8 g/Kg for the direct method for samples inoculated with *S. cerevisiae* (CCMA 0543); from 7.0 to 2.2 g/Kg for the bucket method and from 8.7 to 3.3 g/Kg with the direct method for samples inoculated with *C. parapsilosis* (CCMA 0544); and from 5.2 to 1.5 g/Kg with the bucket method and from 9.7 to 3.6 g/Kg with the direct method for samples inoculated with *T. delbrueckii* (CCMA 0684).

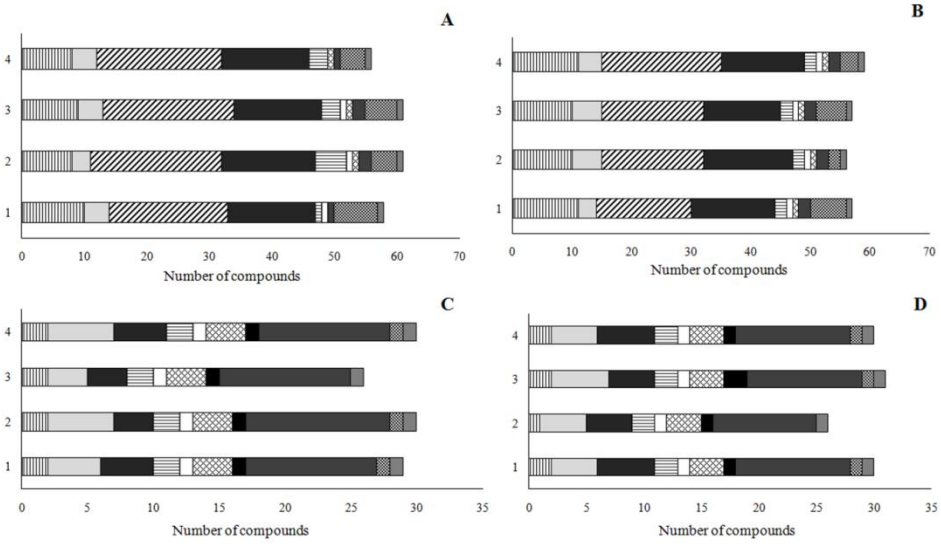
Trigonelline concentrations in roasted beans were higher when the bucket inoculation method was used for all treatments studied, with *C. parapsilosis* (CCMA 0544) having the highest concentration (11.68 g/Kg). In green coffee, trigonelline concentrations were higher

only in the control treatment. In all other treatments via bucket inoculation, concentrations were lower than with direct inoculation. Caffeine concentration in roasted beans was also higher when the bucket method was used with *S. cerevisiae* (CCMA 0543) (24.76 g/Kg), *C. parapsilosis* (CCMA 0544) (21.94 g/Kg) and the control (18.79 g/Kg) assays. This was not the case for *T. delbrueckii* (CCMA 0684) (15.40 g/Kg). In green beans, caffeine was more stable for all yeasts when direct inoculation was used, apart from small variations in concentration levels.

3.4 Volatiles compounds

A total of 112 compounds were detected using the headspace-MS method and their IDs are displayed in the supplementary material. The compound groups detected in green and roasted coffee beans include aldehydes, ketones, alcohols, acids, esters, lactones, furans, pyrroles, pyrazines, aromatics, and others (Figure 5). Some of the detected compounds include important sensory values involved in flavor and aroma, including fruit-like, sweet, caramel, nutty, and floral tastes and smells, among others.

Figure 5 – Profile of volatile compounds identified by HS-SPME GC-MS during coffee fermentation, with bucket inoculation of green (A) and roasted beans (C), and with direct inoculation of green (B) and roasted beans (D). Exposed treatments: (1) control (without inoculation); (2) inoculation with *S. cerevisiae* (CCMA 0543); (3) inoculation with *C. parapsilosis* (CCMA 0544); and (4) inoculation with *T. delbrueckii* (CCMA 0684). Aldehydes ( ), Ketones ( ), Alcohols ( ), Acids ( ), Esters ( ), Lactones ( ), Furans ( ), Pyrroles ( ), Pyrazines and pyridines ( ), Aromatics ( ), and others ( ).



In the green beans, alcohols and acids were the groups with more volatile compounds, regardless of the inoculation method (Figure 5). As can be seen in Figures 5A and 5B, the bucket and direct inoculation methods resulted in a small difference between the compound groups identified. However, the treatments used with bucket inoculation (Figure 5A) had more compounds than with direct inoculation (Figure 5B). Vanillin was only found in the direct treatment inoculated with *S. cerevisiae* and control, resulting in a vanilla-like flavor (Supplemental material).

After roasting, there was a greater difference between the bucket inoculation method (Figure 5C) and the direct method (Figure 5D). Treatment with *S. cerevisiae* (CCMA 0543) and *T. delbrueckii* (CCMA 0684) using the bucket method showed higher numbers of the identified compounds. With direct inoculation, treatment with *C. parapsilosis* (CCMA 0544) showed a greater number of volatile compounds.

3.5 Sensorial analyses

With regard to sensorial analysis, coffee from all the treatments achieved scores above 80, which indicates good quality. The sensorial results showed that there were no differences between treatments and inoculation methods. Coffee beverages produced using direct inoculation methods achieved scores of 81.5, 80.8, 81.0, and 81.3 for the control treatment, and treatment with *T. delbrueckii* (CCMA 0684), *C. parapsilosis* (CCMA 0544) and *S. cerevisiae* (CCMA 0543), respectively. Coffee beverages produced using the bucket method achieved scores of 81.4, 81.0, 81.3, and 81.4 for the control treatment, and treatment with *T. delbrueckii* (CCMA 0684), *C. parapsilosis* (CCMA 0544) and *S. cerevisiae* (CCMA 0543), respectively.

4 Discussion

First, this study measured plate counts of LAB, mesophilic bacteria and yeast (Fig. 2). Our main objective was to evaluate whether the bucket inoculation method would contribute to the persistence of the starter cultures during the coffee fermentation process. In general, the bucket method maintained a higher population of yeast, lactic acid and mesophilic bacteria at the end of fermentation (Figures 2A, 2B and 2C). Probably due to the favorable environmental conditions this method provided, such as availability of oxygen, temperature, and pH which lead to their survival and growth. Factors such as moisture and the temperature of the coffee beans can affect the degree of colonization and the colonization species (Silva et al., 2008), this may offer an explanation for a yeast and bacteria population above 6 log

CFU/g and for the similarity between treatments at the beginning of fermentation for both the bucket and direct methods. It can be assumed that inoculation with the yeast *C. parapsilosis* (CCMA 0544) using the direct method favors the growth of yeast and bacteria, resulting in slightly higher counts than with other treatments. In a previous study using the same process (semi-dry), including the direct inoculation method but with different coffee varieties of coffee, Evangelista et al. (2014b) observed that *C. parapsilosis* stood out from the other treatments with a higher population (approximately 7.5 log CFU/g) after 30 days of fermentation and drying. In our study, *C. parapsilosis* (CCMA 0544) prevailed in all treatments when using the bucket inoculation method and it can therefore be assumed that this method favors the growth of the inoculant and other coffee microbiota.

In this study, three potential yeasts—*S. cerevisiae (*CCMA 0543), *C. parapsilosis* (CMMA 0544) and *T. delbrueckii (*CCMA 0684)—previously selected and tested based on their pectinolytic activity and organic acid production by Silva et al. (2013), were monitored using two inoculation methods (the bucket and the direct method), which had not previously been evaluated. The study used qPCR to evaluate the Catuai Amarelo variety (Figure 3). Our results demonstrate that the starter cultures gave positive results for Catuai Amarelo. Evangelista et al. (2014b) tested the same cultures using the direct method but with another variety of coffee. In that study, the direct inoculation method had positive results when using starter cultures.

The bucket method showed a higher population at 352 hours with samples inoculated with *S. cerevisiae* than with non-inoculated coffee beans, indicating that this method helps the multiplication of cells and probably of specie *S. cerevisiae*, which is considered to be an epiphytic strain of coffee (Evangelista et al., 2014a; Masoud et al., 2004; Silva et al., 2000; Silva et al., 2013). Although the concentration of cells at the beginning of fermentation was supposed to be the same for all the yeast treatments whatever method was used, cell concentration was higher with the bucket method. This proves that some cell concentration is lost when spraying by air flow. The different inoculation methods did not affect the *T. delbrueckii* population. The bucket method favored the growth of the non-saccharomyces yeast *Candida parapsilosis.*

As the concentration of acids may vary depending on the process used, the variety of coffee, the inoculation method and other factors, their presence may act as good quality indicators of the final product (Silva et al., 2008; Sunarharum et al., 2014; Vilela et al., 2010). Each yeast behaved differently with varying metabolite concentration levels. Some desirable dominant acids, such as citric and succinic acid, were detected throughout fermentation

(Table 2). In contrast to the study by Evangelista et al. (2014b), which tested the same inoculants, citric and succinic acid concentrations on the outside i8-of the bean were higher at the beginning of direct fermentation. There were lower acid concentrations at the end of fermentation, although a more stable population was observed with the bucket method. The bucket inoculation method maintained microbial population levels during the fermentation process (Figures 2 and 3, respectively) and generated a good quality final beverage.

Chlorogenic acids are a major family of phenolic compounds and are responsible for coffee pigmentation and astringency (Duarte et al., 2010). The bucket method showed a constant increase in their levels before roasting (Table 2). This can be explained in accordance with Fagan et al. (2011) who have argued that chlorogenic acid is transported from the inside to the outside surface of the fruits to protect against attack from microorganisms when fruits are mature. After roasting, lower concentrations of these compounds were detected (with the bucket method, the concentration was 18.39 g/Kg and with the direct method 14.91 g/Kg). With its antioxidant properties, this result has potential for human health (Farah et al., 2006). As for trigonelline, after roasting, beans inoculated using the bucket method maintained higher concentrations (data described only), leading to desirable aroma compounds.

Carbohydrates, glucose, fructose and sucrose were found in the mucilage and may be degraded by enzymatic reactions, with a positive impact on coffee aroma (Lee et al., 2015). All sucrose was degraded as expected (data described only). Lower glucose concentrations were observed for both inoculation methods when compared with fructose (Figure 4A). The level of maturation and the processing method used can influence sugar concentration (Knopp et al., 2006; Quintero, 2012). A possible explanation for lower glucose concentration is the microorganism's presence as it is a primary energy source in metabolic processes. As with glucose, fructose was also consumed (Figure 4B). However, at the end of fermentation, both sugars increased for most of the treatments. There no clear explanation for this but it was probably due to enzymatic reactions inside the coffee bean (Figure 5), which may be responsible for fresh and floral notes (Toledo et al., 2016). In roasted beans, pyrazines and pyridines were the most abundant group, in accordance with Sunarharum et al. (2014), who argue that pyrazines are part of the top two main classes of compounds. Arising after roasting, they add significantly to coffee flavor by exhibiting nutty, earthy, roasted, and green aromas. Both main groups (acids and pyrazines) can be correlated with attributes perceived by the certified tasters in this study, including yellow fruits, caramel and almonds. This study identified important volatile markers of raw and roasted coffee such as, 2, 3-butanediol, 2, 5-dimethylpyrazine, and 2, 3-dimethylpyrazine (complementary material).

Conclusions

The bucket inoculation method favored microorganisms and metabolic response. The starter yeasts—*S. cerevisiae* (CCMA 0543), *C. parapsilosis* (CCMA 0544), and *T. delbrueckii* (CCMA 0684)—worked well with the Catuaí Amarelo coffee variety because they changed the behavior of the microbiota and the chemical composition during the process. In addition, all the coffees produced in this study resulted in scores over 80, which indicates good quality.

Acknowledgments

The authors thank the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico of Brasil (CNPQ), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We also thank Dra. Suzana Evangelista Reis and Resfriado farm, from Lavras in the state of Minas Gerais, for the collected samples.

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Supplemental material

Volatile compounds found in treatments inoculated with yeasts and without inoculation (continue)

Group	Compound	Sample of treatment	Sensory attributes* (Odor or flavor)
Aldehydes	Hexanal	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	Fruity
	2-Butenal, 3-methyl-	1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	Almond
	2-Octenal, (E)-	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	
	2-Nonenal, (E)-	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	Fatty, cardboard-like
	Benzaldehyde	3, 5, 7, 8, 9, 11, 14, 15, 16	Almond, sweet
	2-Pentenal, (E)-	1, 2, 3, 4, 9, 11, 12	
	Pentadecanal-	1, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 16	
	2-Heptenal, (Z)-	1	Apple-like
	Heptanal	5, 6, 7, 15, 16	Fruity-like
	Nonanal	5, 6, 7, 8, 13, 14, 16	
	Decanal	7, 8	
	2,4-Nonadienal, (E,E)-	9, 10, 12	Oily, Green
	2-Hexenal	15	
	2,6-Nonadienal, (E,Z)-	13, 14, 15, 16	
	2-Furancarboxaldehyde	17, 18, 19, 20, 21, 22, 23, 24	Sweet, bread-like
	5-Hydroxymethylfurfural	17, 18, 19, 20, 21, 23, 24	
	Vanillin	9, 10	Vanilla-like
Ketones	2-Heptanone	1, 2, 3, 4, 9, 10, 11, 12, 13, 14, 16	
	5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	
	2-Butanone, 3-hydroxy-	3	
	2-Pentadecanone, 6,10,14-trimethyl-	1, 2, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 23	
	3-Octen-2-one, (E)-	5, 7, 8, 15	Earthy
	2,3-Octanedione	10, 12, 14, 15, 16	Cheese
	2-Undecanone, 6,10-dimethyl-	10	Citronella
	Acetoin	17, 18, 19, 20, 21, 22, 23, 24	

Supplemental material

Volatile compounds found in treatments inoculated with yeasts and without inoculation (continue)

Group	Compound	Sample of treatment	Sensory attributes* (Odor or flavor)
	2-Propanone, 1-hydroxy-	17, 18, 19, 20, 21, 22, 23, 24	Sweet, caramel
	2-Butanone, 1-hydroxy-	17, 18, 19, 20, 21, 22, 23, 24	
	Ethanone, 1-(1H-pyrrol-2-yl)-	17, 18, 20, 21, 22, 23, 24	
Alcohols	(S)-(+)-2-Pentanol	3	
	1-Butanol, 3-methyl-	1, 2, 3, 4, 5, 6, 7, 8, 12, 16	Fruity
	1-Pentanol	2, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16	
	2-Buten-1-ol, 3-methyl-	2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 16	
	2-Heptanol	1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 16	Nutty, sweet
	1-Hexanol	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	Fruity
	1-Hexanol, 2-ethyl-	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	Fermented-yeast
	Linalyl alcohol	2, 3, 4, 7	Floral
	1-Octanol	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16	Coconut
	2,3-Butanediol, [R-(R*,R*)]-	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	
	2-Octen-1-ol, (E)-	1, 3, 4, 5, 6, 7, 8, 13, 14, 15, 16	Fruity
	Benzyl alcohol	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	
	Phenylethyl Alcohol	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	Floral
	1-Pentanol, 4-methyl	2, 3	
	1-Penten-3-ol	1, 2, 3, 4	
	3-Buten-1-ol, 3-methyl-	1, 3	
	3-Hexen-1-ol, (Z)-	2, 3, 4, 9, 11, 12	
	1-Heptanol	1, 2, 3, 4, 5, 6, 7, 13, 14, 15, 16	Dairy, lactonic
	1-Propanol, 2-methyl-	3, 10, 11, 12	
	5-Hepten-2-ol, 6-methyl-	1, 4, 9, 10, 11, 12	
	1,6-Octadien-3-ol, 3,7-dimethyl-	1, 2, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16	Floral
	2-Hexen-1-ol, (E)-	1, 2, 4, 9, 10, 11, 12	
	Cyclohexanol, 5-methyl-2-(1-methylethyl)	5, 7	Mint-like

Supplemental material

Volatile compounds found in treatments inoculated with yeasts and without inoculation (continue)

Group	Compound	Sample of treatment	Sensory attributes* (Odor or flavor)
	1-Nonanol	5, 6, 7, 8, 14, 15, 16	
	3-Octanol, 2-methyl-	6	
	1-Butanol	6, 8, 16	
	1-Octen-3-ol	13, 14, 15, 16	
Acids	Butanoic acid, 3-methyl-	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 17, 18, 19, 20, 21, 22, 23, 24	
	Pentanoic acid	1, 2, 3, 4, 5, 6, 8, 13, 14, 15, 16	Fruity, sweaty
	2-Butenoic acid, 3-methyl-	1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24	
	Hexanoic acid	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16	
	Hexanoic acid, 2-ethyl-	1, 2, 3, 4, 5, 6, 14, 20, 21, 22, 23, 24	Herbal
	Octanoic acid	1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 16	
	Nonanoic acid	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	
	Tetradecanoic acid	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14, 15, 16	
	Hexadecanoic acid	1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24	
	Hexadecanoic acid, ethyl ester	3, 4, 9, 10, 11, 12, 13	
	Pentadecanoic acid	1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 21, 24	
	Hexadecanoic acid, methyl ester	1, 2, 3, 4, 9, 13, 14, 16	
	Hexanoic acid, ethyl ester	2	
	Hexanoic acid, methyl ester	1	Pineapple-apricot
	Dodecanoic acid	2, 9, 10, 11, 12, 13, 15, 16	
	Benzoic acid	1, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	
	Decanoic acid	1, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16	
	Butanoic acid	7	

Supplemental material

Volatile compounds found in treatments inoculated with yeasts and without inoculation (continue)

Group	Compound	Sample of treatment	Sensory attributes* (Odor or flavor)
Esters	1-Butanol, 3-methyl-, acetate	3, 6, 9, 10, 12, 15	Fruity, earthy
	Methyl salicylate	1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16	
	Butanedioic acid, diethyl ester	2, 4	
	Ethyl 9-hexadecenoate	2	
	Furfuryl formate	17, 18, 19, 20, 21, 22, 23, 24	Floral
	2-Furanmethanol, acetate	17, 18, 19, 20, 21, 22, 23, 24	Floral
	Benzeneacetic acid, ethyl ester	2, 3, 4	
Lactones	Butyrolactone	5, 6, 7, 17, 18, 19, 20, 21, 22, 23, 24	
	2(3H)-Furanone, dihydro-5-pentyl-	10, 11, 12, 13, 14, 15, 16	Fruity
Furans	Furan, 2-pentyl-	2, 3, 4, 9, 11, 12, 13, 14, 15, 16	Earthy
	Ethanone, 1-(2-furanyl)-	17, 18, 19, 20, 21, 22, 23, 24	
	2-Furancarboxaldehyde, 5-methyl-	17, 18, 19, 20, 21, 22, 23, 24	Sweet, caramel
	2-Furanmethanol	17, 18, 19, 20, 21, 22, 23, 24	
Pyrroles	Pyrrole	17, 18, 19, 20, 21, 22, 23, 24	
	Indole	23	
Pyrazines and pyridines	Pyrazine, 2-methoxy-3-(2-methylpropyl)-	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	
	Pyrazine, methyl-	17, 18, 19, 20, 21, 22, 23, 24	Sweet. Nutty
	Pyrazine, 2,5-dimethyl-	17, 18, 19, 20, 21, 22, 23, 24	
	Pyrazine, 2,6-dimethyl-	17, 18, 19, 20, 21, 22, 23, 24	Sweet
	Pyrazine, 2,3-dimethyl-	17, 18, 19, 20, 21, 22, 23, 24	Nutty
	Pyrazine, 2-ethyl-6-methyl-	17, 18, 19, 20, 21, 22, 23, 24	
	Pyrazine, 2-ethyl-3-methyl-	17, 18, 19, 20, 21, 22, 23, 24	Nutty
	Pyrazine, trimethyl-	17, 18, 19, 20, 21, 22, 23, 24	
	Acetylpyrazine	17, 18, 19, 20, 21, 22, 23, 24	
	Pyrazine, ethenyl-	18, 19	
	Pyrazine	17, 18, 20, 21, 23, 24	

Supplemental material

Volatile compounds found in treatments inoculated with yeasts and without inoculation (conclusion)

Group	Compound	Sample of treatment	Sensory attributes* (Odor or flavor)
	Pyridine, 2,6-dimethyl-	6, 7, 13, 14, 15, 16	
	Pyridine	17, 18, 19, 20, 21, 22, 23, 24	
Aromatics	Mesitylene	3, 4, 5, 6, 7, 11	
	o-Xylene	1, 2, 3, 5, 7, 9, 11	
	Styrene	1, 2, 4, 5, 6, 9, 10, 11, 12, 13, 14, 15, 16	
	p-Xylene	1, 5, 9	
	Benzene, 1-ethyl-4-methyl-	1, 4, 7, 8, 11, 13, 15, 16	Earthy
	Benzene, 1,2,4-trimethyl-	1, 9, 10, 11, 13, 14, 15, 16	
	p-Cymene	5, 6, 7, 8, 13	
	Benzene, 1,2-dimethyl-	7	
	Maltol	17, 18, 20, 21, 23, 24	Fruity, caramel
Others	2-Buten-1-ol, 3-methyl-, acetate	1, 2, 3, 4, 9, 10, 11, 12	Banana-like
	2-Butanone, 1-(acetyloxy)	17, 18, 19, 20, 21, 22, 23, 24	Coffee

Green beans without yeast inoculation (control): Bucket inoculation 1= initial time (0 h) and 5= final time (352 h) of fermentation; Direct inoculation 9= initial time (0 h) and 13= final time (352 h) of fermentation

Green beans with yeast *Saccharomyces cerevisiae* (CCMA 0543): Bucket inoculation 2 =initial time (0 h) and 6 =final time (352 h) of fermentation; Direct inoculation 10 =initial time (0 h) and 14 =final time (352 h) of fermentation

Green beans with yeast *Candida parapsilosis* (CCMA 0544): Bucket inoculation 3 =initial time (0 h) and 7 =final time (352 h) of fermentation; Direct inoculation 11 =initial time (0 h) and 15 =final time (352 h) of fermentation

Green beans with yeast *Torulaspora delbrueckii* (CCMA 0684): Bucket inoculation 4 =initial time (0 h) and 8 =final time (352 h) of fermentation; Direct inoculation 12 =initial time (0 h) and 16 =final time (352 h) of fermentation

RB = Roasted beans: Bucket inoculation 17 =control, 18 =*Saccharomyces cerevisiae* (CCMA 0543), 19 =*Candida parapsilosis* (CCMA 0544) and 20 = *Torulaspora delbrueckii* (CCMA 0684)

RD =Roasted beans: Direct inoculation 21 = control, 22 =*Saccharomyces cerevisiae* (CCMA 0543), 23 = *Candida parapsilosis* (CCMA 0544) and 24 =*Torulaspora delbrueckii* (CCMA 0684)

* Sensory attributes are taken from: Flament, I. and Bessière-Thomas, Y.(2001) and Czerny and Grosch (2000).

FINAL CONSIDERATIONS

Information regarding culture starters and optimization of coffee fermentations with different kind of varieties tested with all coffee process and other strains is still lacking for literature review since the topic is recent and is still been under research. Based on the review, final coffee beverages are affected by a diversity of factors such as environmental conditions, inoculation strains, quality of grains, concentration of starters, natural microbiota and more. As a result, the biochemical compounds are always changing and coffee fermentations are considered complex.

In this work, the objectives stated were reached, such as evaluating the effect the inoculation method has on Catuai Amarelo coffee using previously selected strains as starters. The results obtained showed that bucket fermentation is a promising method for producers or farmers to do when inoculating, since generated special coffees with different attributes. In addition, this kind of inoculation (bucket) becomes an advantage for farmers, since materials are available and present low cost.

Due to coffee fermentations, special coffees with a variety of sensorial differences can be obtained. Therefore, the steps to make fermentation successfully must be taught in farmers because it can raise the value of their crop and income. In future studies, it would be significant to correlate the selected strains used for inoculation with the metabolites they actually produce.