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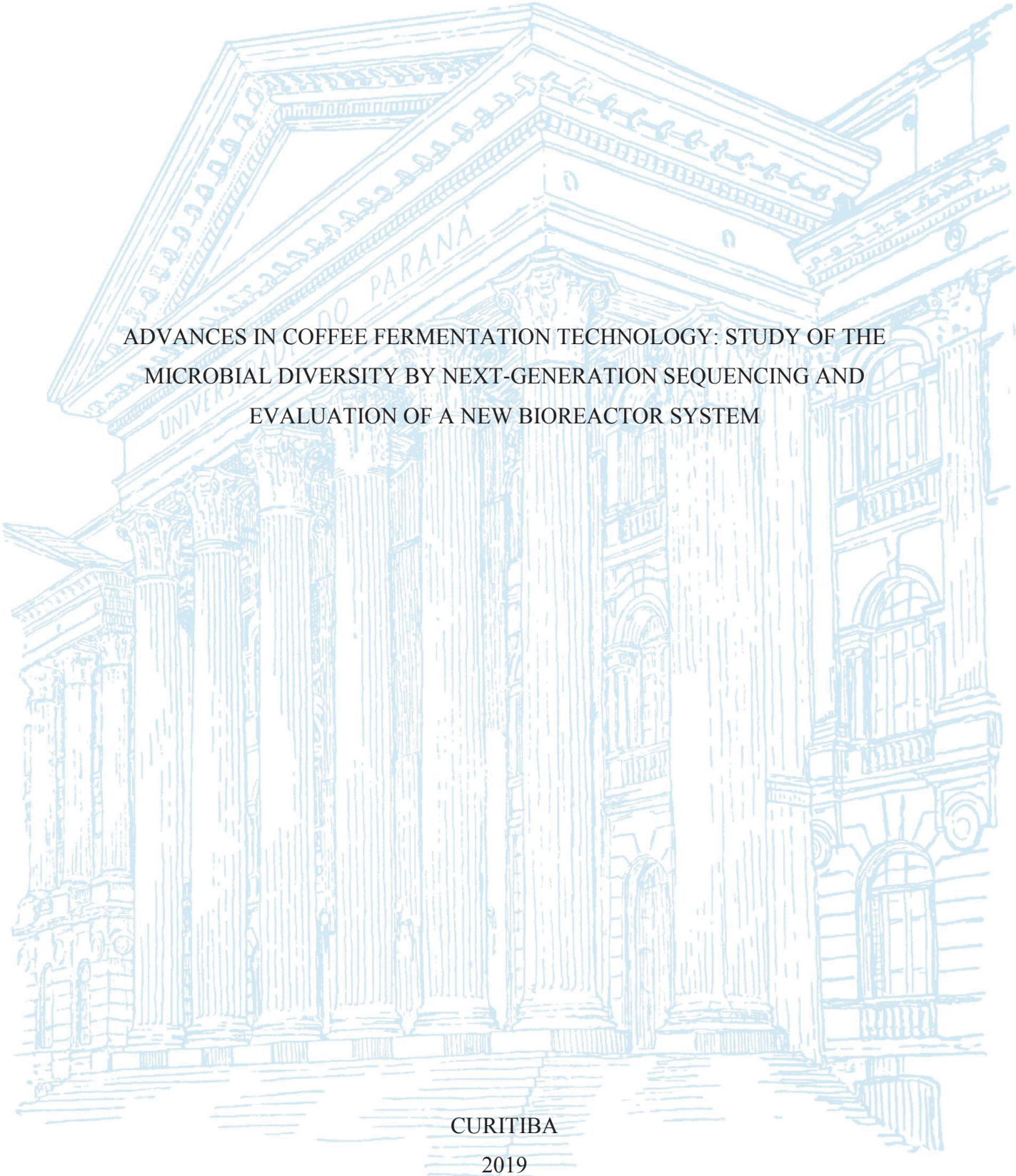

ADVANCES IN COFFEE FERMENTATION TECHNOLOGY: STUDY OF THE MICROBIAL DIVERSITY BY NEXT-GENERATION SEQUENCING AND EVALUATION OF A NEW BIOREACTOR SYSTEM

CURITIBA

2019

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Tese apresentada como requisito parcial à obtenção do grau de Doutor em Engenharia de Bioprocessos e Biotecnologia, no Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia da Universidade Federal do Paraná.

Orientador: Prof. Dr. Gilberto Vinícius de Melo Pereira
Co-Orientador: Prof. Dr. Carlos Ricardo Soccol

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **DÃO PEDRO DE CARVALHO NETO** intitulada: **ADVANCES IN COFFEE FERMENTATION TECHNOLOGY: STUDY OF THE MICROBIAL DIVERSITY BY NEXT-GENERATION SEQUENCING AND EVALUATION OF A NEW BIOREACTOR SYSTEM**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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CURITIBA, 14 de Março de 2019.

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RESUMO

A produção e exportação do café é uma grande força motriz para a economia dos principais países produtores, tais como Brasil, Honduras e Colômbia, chegando a contribuir em mais de 3% para o Produto Interno Bruto (PIB). No entanto, não há o reflexo desse impacto econômico nas linhas produtivas de café, uma vez que a etapa de processamento pós-colheita ocorre de maneira rudimentar. Por essa razão, o presente trabalho visou: i) o estudo da diversidade, composição e dinâmica de leveduras e bactérias durante o processo fermentativo dos grãos de café; ii) e o estudo do comportamento de fermentações (i.e. espontânea e inoculada) conduzidas em biorreatores de tanque agitado em condições controladas de temperatura, agitação e aeração. Em fermentações espontâneas e em condições de campo na região de Minas Gerais, o sequenciamento do gene ITS-rRNA revelou que a população de leveduras foi representada, principalmente, por *Saccharomyces* sp., seguido de *Torulaspora delbrueckii, Pichia kluyveri, Hanseniaspora uvarum, H. vineae,* e *Meyerozyma caribicca*. Utilizando a técnica de Sequenciamento de Nova Geração na plataforma Illumina, foi possível observar a presença de mais de 80 gêneros bacterianos, muitos dos quais foram descritos pela primeira vez em uma fermentação de grãos de café. Dentre estes estão inclusos *Fructobacillus, Pseudonocardia, Pedobacter, Sphingomonas* e *Hymenobacter*. A análise temporal demonstrou uma forte dominância de bactérias láticas, representando mais de 97% do total de sequências analisadas ao final da fermentação. As fermentações de café conduzidas em bioreatores em condições controladas permitiram um crescimento eficiente das culturas iniciadoras *Lactobacillus plantarum* LPBR01 (pico de 10,7 log CFU/mL em 10 h) e *Pichia fermentans* YC5.2 (8,85 log CFU/mL), resultando em uma rápida acidificação do meio (pH <4,0 em 6 h), elevadas produções de ácido lático (8,602 g/L) e acetato de etila (pico de 97,93 μmol/L em 8 h). A difusão desses compostos para o interior do grão resultou em bebidas com notas sensoriais superiores a 91 pontos na escala Specialty Coffee Association of America (SCAA), a qual foi significativamente superior às bebidas produzidas pelo método convencional. Este novo modelo de fermentação pode ser utilizado para conduzir fermentações de café controladas e fornecer grãos homogêneos e de elevada qualidade para a indústria.

Palavras-chave: Café, microorganismos, processamento via úmida, biorreator de tanque agitado.

ABSTRACT

The production and export of coffee is a major driving force for the economy of the main producing countries, such as Brazil, Honduras and Colombia, which contributes with over 3% of the Gross Domestic Product (GDP). However, there is no reflection of this economic impact on coffee production sites, since the post-harvest processing stage occurs rudimentary. For this reason, the present work aimed at: i) the study of the diversity, composition and dynamics of yeasts and bacteria during the fermentative process of coffee beans; ii) and the behavior study of fermentations (i.e., spontaneous and inoculated) conducted in stirred tank bioreactors under controlled conditions of temperature, agitation and aeration. In spontaneous fermentations conduced at on-farm conditions at Minas Gerais region, the sequencing of the ITS-rRNA gene revealed that the yeast population was represented mainly by *Saccharomyces* sp., followed by *Torulaspora delbrueckii, Pichia kluyveri, Hanseniaspora uvarum, H. vineae*, and *Meyerozyma caribicca*. The New Generation Sequencing technique on the Illumina platform revealed the presence of over 80 bacterial genera, many of which were described for the first time in coffee beans fermentation. These include *Fructobacillus, Pseudonocardia, Pedobacter, Sphingomonas* and *Hymenobacter*. The temporal analysis demonstrated a strong dominance of lactic bacteria, representing more than 97% of the total read sequences at the end of the fermentation. The coffee fermentations conducted in bioreactors under controlled conditions allowed efficient growth of the *Lactobacillus plantarum* LPBR01 (peak of 10.7 log CFU/mL in 10 h) and *Pichia fermentans* YC5.2 (8.85 log CFU/mL) starter cultures. This domination resulted in a rapid acidification of the coffee-pulp bean mass (pH <4.0 in 6 h) and high production of lactic acid (8.602 g / L) and ethyl acetate (peak of 97.93 μmol/L in 8 h). The diffusion of these compounds into the beans resulted in beverages scores above 91 points on the Specialty Coffee Association of America (SCAA) scale, which was significantly higher than those produced by the conventional method. This new fermentation model can be used to conduct controlled coffee fermentations and provide homogenous and high quality grains for the industry.

Keywords: Coffee, microorganisms, wet processing, stirred-tank bioreactor.

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SUMMARY

1. INTRODUCTION

The coffee (*Coffea arabica* L.) belongs to the Rubicaceae family, which comprises more than 600 genera and 10,000 species of tropical trees and shrubs (BREMER; ERIKSSON, 2009). The coffee originates from Ethiopian highlands, where it is still possible to observe the growth of wild coffee plants. Until the fifteenth century, coffee production was restricted to Arabia, more precisely to Yemen, where the coffee beans were of great economic importance to producers and traders. In 1615 the first sacks of coffee reached the non-Islamic world, and coffee plantations soon gained the European colonies in Central and South America (GRIGG, 2002; PEREIRA et al., 2017).

Exceeding a global production of 9.5 million tons and a global industry valued at more than US$ 200 billion, coffee is in a prominent position in the world economic scenario. With a production of 3.6 million tons, Brazil is the largest producer and exporter of coffee, followed by Vietnam, Colombia, Indonesia and Honduras (ICO, 2018). Although this cultivar has a significant impact on the economy of producing countries, the fermentation stage is carried out in a traditional way, via spontaneous methods. This fact contrasts with fermented products produced in developed countries, where fully controlled processes are performed, such as wines, beers and dairy products (AYAD et al., 2003; BRÁNYIK et al., 2005; SINGH; SOOCH, 2009; UGLIANO; GENEVESE; MOIO, 2003).

Coffee beans fermentation stage is performed by indigenous bacteria and yeasts present in the surface of coffee cherries. Spontaneous fermentations have several disadvantages, such as the lack of predictability and control over the final quality of the beverage produced, resulting in the commercial depreciation of the final product. In this sense, the search for starter cultures that can promote a homogeneous degradation of the mesocarp, reduction of fermentation time, and production of flavoring compounds has become the target of several recent studies (SILVA et al., 2013; PEREIRA et al., 2014, 2015, 2016; LEE et al., 2016a, 2017a). However, the rudimentary conditions and the lack of control (i.e., temperature, agitation, aeration control or anaerobic system) in which the fermentations are performed hinder the reproducibility and standardization necessary for the development of commercial lines of starter cultures.

In order to contribute to a better understanding of microbial diversity and the improvement of coffee bean fermentation technology in Brazil, the present study aimed to carry out studies using dependent- and independent-cultivation methodologies to

evaluate the dynamics of the bacterial population and to propose a new fermentation model.

2. OBJECTIVES

2.1. Main objective

The present study aims to characterize the diversity, composition and dynamics of the microbiota in spontaneous coffee fermentations conducted in the region of Minas Gerais and to perform kinetic, metabolic and sensorial studies of inoculated and spontaneous fermentations in stirred tank reactors.

2.2. Secondary objectives

- Characterize the different microbial groups present in the region of Minas Gerais and estabilish a relationship between microbiome *versus* quality;
- Standardize and apply Next Generation Sequencing tools through the Illumina Platform in coffee fermentations;
- Establish the relationship between the dominant microorganisms observed in the fermentation processes and the difference between the profile of aromatic compounds in the coffee beans obtained;
- Determine the kinetic parameters of fermentations conducted in stirred-tank bioreactors;
- Characterize the coffee fruits' profile of organic volatile compounds, sugars and organic acids (GC-MS and HPLC) and establish a correlation with the dominant microbiota in the fermentation process;
- Evaluate the effect of the implementation of starter cultures on the natural microflora of coffee processing through the Illumina MiSeq Next Generation Sequencing Platform.
- Produce coffee beans with high content of aromatic compounds;
- Produce high quality coffee beverages.

CHAPTER I (LITERATURE REVIEW) – EXPLORING THE IMPACTS ON THE AROMA FORMATION OF COFFEE BEANS – A REVIEW

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ABSTRACT

The aim of this review is to describe the volatile aroma compounds of green coffee beans and evaluate sources of variation in the formation and development of coffee aroma through postharvest processing. The findings of this survey showed that the volatile constituents of green coffee beans (e.g., alcohols, aldehydes, and alkanes) have no significant influence on the final coffee aroma composition, as only a few such compounds remain in the beans after roasting. On the other hand, microbial-derived, odor-active compounds produced during removal of the fruit mucilage layer, including esters, higher alcohols, aldehydes, and ketones, can be detected in the final coffee product. Many postharvest processing including drying and storage processes could influence the levels of coffee aroma compositions, which remain to be elucidated. Better understanding of the effect of these processes on coffee aroma composition would assist coffee producers in the optimal selection of postharvest parameters that favor the consistent production of flavorful coffee beans.

Keywords: Green coffee beans; volatile aroma compounds; coffee aroma; postharvest processing

1.1. INTRODUCTION

The popularity of coffee products is related to their unique sensory and pleasant flavor. A critical contributor to coffee beverage quality is the series of postharvest practices performed to obtain dried beans suitable for roasting (HUCH; FRANZ 2015). These practices involve a number of relatively complex steps, including fruit harvesting, depulping, drying, and storage. Following on-farm postharvest processing, coffee beans can be transported to industrial plants, where semi-manufactured or finished products are obtained for commercialization (PEREIRA et al., 2017).

More than 90 years of scientific studies exploring the complex volatile aroma composition of coffee have yielded over 1,000 volatile compounds (LEE; SHIBAMOTO, 2002; CZERNY; MAYER; GROSCH, 1999; GROSCH, 1998; SANZ et

al., 2002). These volatiles, including sulfur compounds, pyrazines, pyridines, oxazoles, pyrroles, furans, aldehydes, higher alcohols, ketones, esters, and phenols, are mostly generated by thermal reactions that occur during roasting and brewing (BUFFO; CARDELLI-FREIRE, 2004; BRÖHAN et al., 2009; RODRIGUEZ; DURAN; REYES, 2010). However, the chemical composition of green coffee beans, as well as chemical changes that occur through postharvest processing, can have a direct impact on the quality and value of the final product (WINTGENS, 2004; BHUMIRATANA; ADHIKARI; CHAMBERS, 2011; SUNARHARUM; WILLIAMS; SMYTH, 2014).

The influence of genotype, cultivation method and postharvest treatment (harvesting, depulping, drying, and storage) on the final coffee quality is still under study (AVALLONE et al., 2002; BYTOF et al., 2005; KNYSAK, 2017; LEE et al., 2015; LEE; SHIBAMOTO, 2002; PEREIRA et al., 2016; RENDÓN; SALVA; BRAGAGNOLO, 2014; SELMAR; BYTOF; KNOPP, 2008; SELMAR et al., 2006). The aim of this review is to examine all the factors related to coffee volatile composition and variation through postharvest processing. Additionally, we discuss strategies that can be exploited to improve the flavor of coffee with a special focus on the microbial-derived metabolites generated during removal of the fruit mucilage layer.

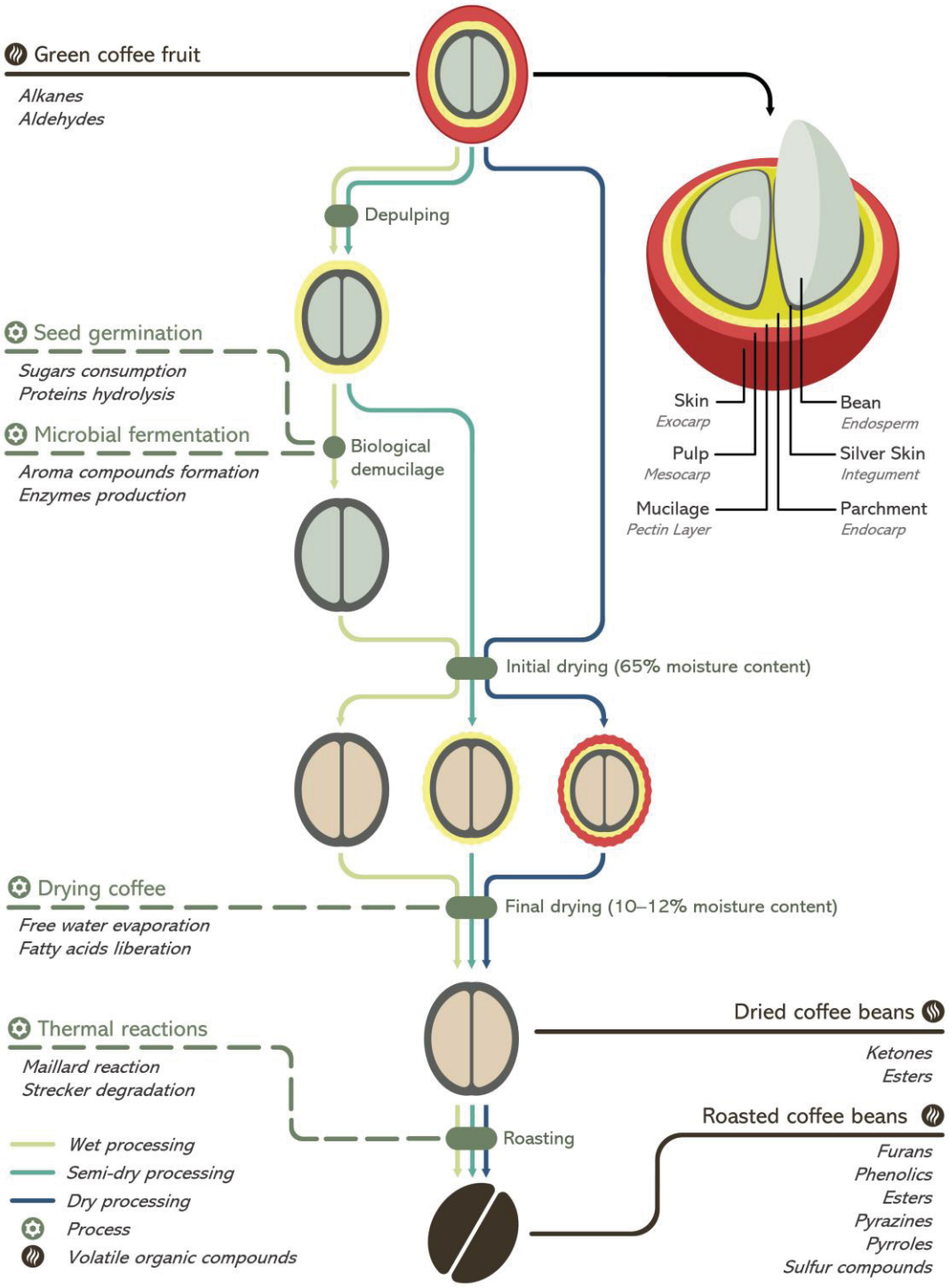
1.2. COFFEE PRODUCTION AND POSTHARVESTING PROCESSING

Coffee is derived from the tree of the genus *Coffea*, including more than 103 species of tropical trees and shrubs (FERRÃO et al., 2015). The coffee tree is commercially cultivated throughout the geographic region between latitudes 30° N and 30° S, known as the “coffee belt.” Brazil is one of the leading coffee producers, supplying about a third of total world production, followed by Vietnam, Indonesia, Colombia, India, Peru, Honduras, Ethiopia, Guatemala, Mexico, and 60 other countries (PEREIRA et al., 2017). The total worldwide production of coffee beans exceeded 9 million tons in the 2015–2016 crop, with a turnover close to US$21 billion (ICO, 2017). It is one of the most traded and consumed agricultural products worldwide, at times surpassed only by oil (LEE et al., 2015).

The fruit of the coffee tree consists of an orange-red to red skin on ripening (exocarp), a fleshy yellow-white pulp and mucilage (mesocarp), and a plain yellow parchment (endocarp) and silver skin (integument) surrounding the seeds (endosperm) (FIGURE 1.1). The exocarp gives the fruit external resistance. It is a monocellular layer

protected by a waxy substance; when unripe, the fruits are green and turn to red-violet, deep red, yellow, or orange (depending on the genotype) when ripe. The mesocarp is a fleshy, fibrous, and sweet pulp, which is rich in carbohydrates (glucose, fructose, and pectin), proteins, fat, lipid minerals and considerable amounts of tannins, poliphenols, and caffeine (JANISSEN; HUYNH, 2018; MURTHY; NAIDU, 2012). The endocarp, the so-called parchment layer, is a thin, yellowish, crumbly, paper-like polysaccharide composed principally of α-cellulose, hemicellulose, lignin, and ashes (ESQUIVEL; JIMÉNEZ, 2012). The silver skin is predominantly composed of polysaccharides, especially cellulose and hemicelluloses, in addition to monosaccharides, proteins, polyphenols, and other minor compounds (FARAH; DOS SANTOS, 2014). This layer is high in total dietary fibers and phenolic compounds with significant antioxidant activity (JANISSEN; HUYNH, 2018). The silver skin covers two hemispheres of elliptical seeds which, in turn, contain the endosperm and embryos (FARAH; DOS SANTOS, 2014; ESQUIVEL; JIMÉNEZ, 2012).

FIGURE 1.1 – COFFEE FRUIT STRUCTURE AND SCHEMATIC PRESENTATION OF POSTHARVEST PROCESSING METHODS CORRELATED WITH MAJOR BIOCHEMICAL CHANGES THAT IMPACT ON COFFEE VOLATILE FORMATION. THIS FIGURE IS ADAPTED FROM BYTOF et al. (2005), LEE et al. (2015), SELMAR et al. (2006), AND PEREIRA et al. (2017).



Fruit harvesting is the first step in postharvest coffee processing. The heterogeneous development of coffee fruits leads to a simultaneous presence of different maturation stages in the same coffee tree—that is, green (immature), cherry (ripe), and raisin (overripe) (PEZZOPANE et al., 2003). When mature, the coffee fruits

present lower concentrations of phenolic compounds, which implies a reduction of astringency; moreover, coffee cherries show a higher content of volatile compounds (aldehydes, ketones, and higher alcohols) in comparison to immature fruits (WINTGENS, 2004). Thus, coffee harvesting should be initiated when the plant reaches a homogeneous stage of maturation with a minimum prevalence of immature fruits (BEE et al., 2005).

Harvesting of coffee is predominantly performed by handpicking or by stripping the fruits onto sheets placed beneath the tree; however, the use of mechanical harvesters based on the vibration of tree branches (e.g., self-propelled machines, portable and mechanical stripping machines) has greatly increased all around the world (BEE et al., 2005). The choice of method employed will interfere directly in the quality of the fruit used for further steps of on-farm processing. Handpicking allows the exclusive selection of fruits in their ideal stage of maturation (i.e., coffee cherries). Obtaining only ripe coffee cherries through selective handpicking is, however, expensive and laborious. In this sense, many producers choose between stripping or mechanical harvesting of coffee fruits, followed by removal of immature beans through sorting (HUCH; FRANZ, 2015).

After harvesting, coffee processing should begin as quickly as possible to prevent fruit spoilage by unfavorable fermentation or mold formation (BEE et al., 2005; ILLY, 2002). The outer layers of the coffee fruit (i.e., the skin and pulp) are easily removed, while the mucilage, parchment, and silver skin are firmly attached to the beans (DE BRUYN et al., 2017). FIGURE 1.1 illustrates the three different methods commonly used to eliminate these layers. In the dry processing, seeds are exposed to the sun or air dryers until the moisture content is approximately 10%–12%. After drying, the fruits are cleaned and dehulled, and then the dried skin and pulp are removed. Wet processing, in contrast, involves a relatively complex series of steps, including mechanical removal of the coffee skin and pulp, microbial degradation (fermentation) of the mucilage layer and, finally, water removal by sun-drying (FIGURE 1.1). This process reduces the time (from 3–5 weeks to 8–10 days) and area required for drying the beans in relation to dry processing (BEE et al., 2005). Finally, semi-dry processing presents stages of both dry and wet methods, where the coffee fruits are mechanically depulped and then submitted to sun-drying (PEREIRA et al., 2017; BEE et al., 2005).

The beans resulting from any processing method must be dried to a final water content of 10–12%. This process can be performed by sun-drying or using various mechanical dryers (i.e., static dryers, column dryers, round dryers, or forced air dryers).

The choice of the drying technique used is linked to economic factors and/or to the type of processing employed. For example, in dry processing, the preservation of the exocarp and mesocarp extends the time required to reach the desirable humidity levels because the moisture of the harvested fruits is approximately 70%. For this reason, the use of mechanical dryers is not recommended because it would represent a high and unnecessary cost (KLEINWÄCHTER; BYTOF; SELMAR, 2015). On the other hand, the use of mechanical dryers for wet-processed coffees has gained greater visibility because of the reduction in both drying time and risk of microbial contamination.

1.3. GREEN COFFEE BEAN COMPOSITION AND FLAVOR PRECURSOR FORMATION

The term "green coffee beans" in this study refers to the raw, unprocessed seeds of *Coffea* fruits (FIGURE 1.1). The chemical composition of green coffee beans is very complex, including more than 1,000 substances with different chemical and physical properties (BAGCHI; MORUYAMA; SWAROOP, 2016). The main aroma precursors are insoluble carbohydrates (cellulose and hemicellulose), soluble carbohydrates (i.e., arabinose, fructose, galactose, glucose, sucrose, raffinose, and stachyose), lipids, chlorogenic acids, and nitrogen (N)-containing compounds (FADAI et al., 2017; POISSON et al., 2017). Low-molecular-weight carbohydrates, such as sucrose, glucose, and fructose, contribute to the formation of acids and other volatile compounds during roasting (CLARKE; VITZHUM, 2008). In addition, polysaccharides are important constituents for the retention of volatiles and, consequently, flavor formation (BUFFO; CARDELLI-FREIRE, 2004). The lipid fraction of coffee is mainly composed of triacylglycerols, sterols, coffeadiol, arabiol, and tocopherols. These compounds are located mostly in the endosperm of green coffee beans, and only a small amount, the coffee wax, is located in the outer layer of the bean (SPEER; KÖLLING-SPEER, 2006; ESQUIVEL; JIMENEZ, 2012).

Nitrogen-containing compounds, such as alkaloids (e.g., caffeine and trigonelline) and proteins, as well as nonvolatile, aliphatic acids (citric, malic, and quinic acids) and volatile acids (such as acetic, butanoic, decanoic, formic, hexanoic, isovaleric, and propanoic acids), are also found in high concentrations in green coffee beans. These compounds break down during the roasting process and generate

important flavor-active metabolites such as pyridines and pyrroles (SUNARHARUM et al., 2014; POISSON et al., 2017).

Other minor compounds present in green coffee beans include phenolics, thiols, and minerals. Thiols are crucial compounds that are responsible for the distinctive “coffee” and “roasty” smell notes, influencing the sensory perception of coffee (DULSAT-SERRA; QUINTANILLA-CASAS; VICHI, 2016). The most important phenolic compounds in coffee are caffeoylquinic acid-CQA, feruloylquinic acid-FQA, and dicaffeoylquinic acid-diCQA. These components are known to have antioxidant activity and various beneficial properties in human health (OGAWA, 2014). The mineral composition of green coffee beans is related to soil constitution as well as to other environmental factors, such as altitude, humidity, temperature, and shading during the formation of the coffee fruits. In general, potassium appears in high amounts followed by phosphorus, magnesium, and calcium (CARVALHO NETO et al., 2017).

1.4. AROMA FORMATION THROUGH POSTHARVEST PROCESSING

1.4.1. Green coffee beans volatile constituents.

Green coffee beans are characterized by an unpleasant taste, and development of the characteristic beverage flavor is achieved through thermal reactions during roasting and brewing. In fact, green coffee beans have only a basic composition of chemical volatiles when compared to roasted beans. Whereas more than 1,000 volatile compounds are generally detected in roasted coffee, on average, only 200 are found in green beans. In addition, heterocyclic compounds, important components in providing the distinctive flavor of roasted beans, are generally not found in green coffee beans (LEE; SHIBAMOTO, 2002). Lee and Shibamoto (2002) reported that of the more than 350 heterocyclic compounds identified in roasted coffee (e.g., pyrroles, furans, pyrazines, thiazoles, oxazoles, thiopheones, and imidazoles), only 2-methoxy-3-(2-methylpropyl)-pyrazine was found in green coffee bean samples. Thus, these and other odorous molecules are mostly formed during the roasting process from nonvolatile precursors present in green coffee beans, such as polysaccharides, lipids, proteins, and free amino acids (LEE; SHIBAMOTO, 2002).

TABLE 1.1 summarizes the volatile compounds reported in green, dried, and roasted coffee beans. The volatile compounds of green coffee beans comprise

hydrocarbons, higher alcohols, aldehydes, ketones, acids, esters, lactones, sulfur compounds, furans, and phenols. Among these, aldehydes and alkanes are the most abundant chemical classes found (FIGURE 1.1). Of the 21 volatile compounds reported by Poyraz et al. (2016) in Turkish green coffee bean samples, isoamyl alcohol (10.4%), hexanal (10.4%) and hexacosane (8.2%) were the most predominant. In Hawaiian green coffee samples, Lee and Shibamoto (2002) identified 3-methyl butanoic acid (32.8%), phenyl ethyl alcohol (17.3%), hexanol (7.2%), 4-hydroxy-3-methylacetophenone (3.7%), and 3-methyl butanol (3.6%) as the major constituents, and aldehydes (hexanal and benzaldehyde) and alkanes (tetradecane and cyclotetrasiloxane, octamethyl-) were detected in high concentration in Thai green coffee beans by Somporn et al. (2011).

TABLE 1.1 – COFFEE VOLATILE COMPOSITION (GREEN, DRIED, AND ROASTED BEANS) THROUGH POSTHARVEST PROCESSING

Major odorous compounds*	Aroma perception	Processing stage detection**	References***
Acids (9)			
Acetic acid[Ar;Ro]	Pungent, sour, vinegar-like	G,F,R	c,d,i,j,k,l,m,n,o,v
Butyric acid[Ar]	Acidic, sour, cheesy	R	e,f
Isobutyric acid[Ar]	Acidic, sour, cheesy	G,R	e,f,h
Hexanoic acid[Ar]	Fatty, cheese-like	F,R	e,f,i
Nonanoic acid[Ar]	Waxy, cheese-like	G,F,R	c,h,i,j
Decanoic acid[Ar]	Rancid, sour, fatty	R	e,f
Benzoic acid[Ar]	Faint, balsamic	F,R	i,j,k,l
Isovaleric acid[Ar]	Dairy, acidic, sour	G,R	b,c
Valeric acid[Ar]	Acidic, sweaty, rancid	F	k
Aldehydes (11)			
Acetaldehyde[Ar]	Fruity	F,R	d,e,f,h,m,n,o,s
Benzaldehyde[Ar;Ro]	Fruity, cherry	G,F,R	a,c,j,l,t,u
Hexanal[Ar]	Green, fatty, grassy	G,F,R	a,c,e,h,i,j,k,m,o,t
Octanal[Ar]	Citrus, orange with a green peely nuance	F,R	e,f,h,k
Nonanal[Ro]	Citrus, with a fresh green lemon peel-like nuance	G,F	e,h,u
2-Methylbutanal[Ar]	Green, malty	G,R	c,p
Butyraldehyde[Ar]	Pungent, cocoa	F,R	e,f,h
3-Methylbutanal[Ar]	Malty, cocoa	G,R	c,p
Vanillin*,**	Sweet (vanilla)	F,R	i,k,l
2-Phenyl-2-butenal[Ar]	Musty, floral, cocoa	R	l
2-Phenylacetaldehyde[Ar]	Sweet, flora, honey-like, cocoa-like	F	i,k
Esters (17)			
Ethyl acetate[Ar]	Fruity, grape	F,R	e,f,m,n
Isoamyl acetate[Ar]	Banana	F,R	d,e,f,m,n
Ethyl hexanoate[Ar]	Sweet, fruity, pineapple	F	n

Hexyl acetate[Ar]	Fruity, with apple and pear notes	F	d,n
Ethyl butyrate[Ar]	Sweet, fruity, tutti-frutti	F,R	d,e,f,n
Isobutyl acetate[Ar]	Fruity, with an apple and banana nuances	F	e,f,n
Ethyl isobutyrate[Ar]	Sweet, ethereal and fruity	F	d
Ethyl octanoate[Ar]	Pineapple and fruity with a creamy, dairy nuance	F,R	d,e,f
Propyl acetate[Ar]	Sweet and fruity	F,R	c,d,e,f,h
Propyl butyrate[Ar]	Fruity, sweet, bubble gum and tutti-frutti-like	R	e,f
Phenyl acetate[Ar]	Floral, rosy with balsamic dark chocolate notes	R	c,e,f,h
Diethyl malonate[Ar]	Sweet, apple, pineapple	R	e,f
Ethyl octanoate[Ar]	Pineapple and fruity with a creamy, dairy nuance	F,R	d,e,f
Methyl acetate [Ro]	Fruity, winey	G,R	c,u
Methyl salicylate[Ar]	Wintergreen, mint-like	F,R	i,j,k,l
Methyl palmitate[Ar]	Waxy,orris	F,R	k,l
Ethyl palmitate[Ar]	Waxy, fruity, creamy, balsamic	F,R	i,j,k,l
Furanone (7)			
3-Hydroxy-4,5-dimethyl-2(5H)-furanone[Ar;Ro]	Fenugreek, curry	G,R	p,r
4-Hydroxy-2,5-dimethyl-3(2H)-furanone[Ar;Ro]	Caramel, fruity	G,R	l,p,q
2,5-Dimethyl-4-hydroxy-3(2H)-furanone[Ar;Ro]	Caramel-like	R	q
4-Methyl-5-ethyl-3-hydroxy-2(5H)-furanone[Ar;Ro]	Buttery, seasoning-like	R	q
2(5H)-furanone[Ar]	Buttery	F,R	k,l
5-methyl-2(5H)-furanone[Ar]	-	F,R	k,l
Dihydro-2-methyl-3(2H)-furanone[Ar;Ro]	-	R	l,s,v
Furans (7)			
Furfural[Ar;Ro]	Almond-like, woody, bready	F,R	e,f,i,j,k,l,r,s
5-methyl-Furfural[Ar;Ro]	Sweet, caramellic, coffee-like	G,F,R	a,c,e,f,h,r,s
Furfuryl alcohol[Ar]	Sweet, caramellic, brown	G,F,R	c,e,f,h,i,j,k,l
Furfuryl acetate[Ar;Ro]	Sweet, fruity, banana	F,R	e,f,j,l,v
Furfuryl propanoate[Ar;Ro]	-	R	j,l,v
Furfuryl formate[Ar;Ro]	Etheral	R	j,l,v

Furfuryl propionate[Ar]	Sweet, fruity, green	R	e,f,j,l
Higher alcohols (15)			
Isoamyl alcohol[Ar]	Fruity, banana	G,F	a,b,c,d,e,f
Isobutyl alcohol[Ar]	Etheral, wine	G	c,e
1-Octen-3-ol[Ar;Ro]	Green, oily, vegetative	G	a,b,c,g,u
Benzyl alcohol[Ar]	Floral, fruity with chemical nuances	G,F,R	a,b,c,g,k,l,t
2-Phenylethyl alcohol[Ar;Ro]	Floral, rose	G,F,R	b,e,f,,h,i,j,k,l,t,u
1-Pentanol[Ar]	Sweet, balsam	G	a,c,g
1-Hexanol[Ar]	Fruity and alcoholic	G	a,b,c,t
2-Heptanol[Ar]	Fresh, lemon grass, herbal	G,F	a,g,e
n-Butanol[Ar]	Fusel, oily	G,F,R	e,f,t
3-methyl-1-Pentanol[Ar]	Fruity, wine and cocoa	F	e,f
Trans-3-hexen-1-ol[Ar]	Green	R	i,j
2,3-Butanediol[Ar]	Fruity, creamy, buttery	F	i,j,t
1-Decanol[Ar]	Floral, orange	F	d
1,2-Propanediol[Ar]	Sweet	R	e,f
1-Propanol[Ar]	Floral with a sweet, coconut nuance	F,R	e,f
Hydrocarbons (6)			
Pentane[Ar]	-	G	t
Toluene[Ar;Ro]	Sweet	G,F,R	c,k,t,u
Ethylbenzene[Ar]	-	G,F	k,t
Styrene[Ar]	Sweet, balsamic, floral	G	k,t
Tetradecane[Ar;Ro]	Waxy	G,F	k,o,u
Pentadecane[Ar;Ro]	Waxy	G,F	k,u
Ketones (13)			
Acetoin[Ar]	Buttery, creamy, dairy-like	G,F,R	c,h,i,j,k,l
2-Butanone [Ro]	Etheral, fruity, camphoreous	R	v
2,3-Butanedione[Ar;Ro]	Buttery, creamy, caramellic	G,F,R	d,e,f,h,i,j,k,l,m,s,t,v

2-methyl-3-Pentanone[Ar]	-	F,R	k,l
2-Pentanone [Ro]	Sweet, fruity	R	v
2,3-Pentanedione[Ar]	Buttery	R	c,l,p
1-hydroxy-2-Propanone[Ar]	Pungent, caramellic, ethereal	F,R	j,k,l
1-hydroxy-2-Butanone[Ar]	Sweet with malt and butterscotch nuance	F,R	j
1-(acetyloxy)-2-Propanone[Ar]	-	F,R	k,l,m
1-(acetyloxy)-2-Butapanone[Ar]	-	F,R	k,l,m
2-Nonanone[Ar]	Fruity, coconut-like	G,F,R	c,e,f,h
3-hydroxy-2-Butanone[Ar]	Sweet, buttery, creamy	G,R	c
2-Heptanone[Ar]	Fruity, spice, herbal	F	d,n
Lactones (4)			
γ-Butyrolactone[Ar;Ro]	Creamy, oily, fatty, caramellic	G,F,R	a,i,j,k,l,s,t,u
β-Butyrolactone[Ar]	Creamy, oily with fatty nuances	R	c
γ-Hexanolactone[Ar]	Sweet, creamy, vanilla	F	k
γ-Nonanolactone[Ar]	Sweet, coconut	F	k
Phenols (7)			
Guaiacol[Ar;Ro]	Phenolic, spicy	G,F,R	c,e,f,h,i,j,k,l,r
4-vinyl guaiacol[Ar;Ro]	Spicy	G,R	a,c,i,j,k,l,q,r
4-ethyl guaicol[Ar;Ro]	Spicy	F,R	j,q,r,s
4-vinyl phenol[Ar]	Phenolic, sweet, chemical-, medicine-like	F,R	i,j,k,l
Phenol[Ar;Ro]	Rubbery, plastic-like	G,F,R	i,j,k,l,s,t,u,v
3-methyl phenol[Ar]	Woody, leather-like	F,R	i,j,k,l
4-methyl phenol[Ar]	-	F,R	i,j,k,l
Pyrazines (14)			
Pyrazine[Ar;Ro]	Pungent, sweet, hazelnut-like	F,R	j,k,l,r,s
2-Methyl pyrazine[Ar;Ro]	Nutty, cocoa-like, roasty	G,F,R	i,j,k,l,r,s,u,v
2,5-Dimethyl pyrazine[Ar;Ro]	Nutty, roasty, earthy, cocoa-like	F,R	i,j,k,l,r,v
2,6-Dimethyl pyrazine[Ar;Ro]	Nutty, roasty, cocoa-, coffee-like	G,F,R	i,j,k,l,r,u,v

2-Diethyl pyrazine[Ar;Ro]	Nutty, woody, roasty, cocoa-like	F,R	i,j,k,l,v
2,3-Dimethyl pyrazine[Ar;Ro]	Nutty, coffee-like, caramellic, cocoa-like	F,R	i,j,k,l,r,s,v
2-ethyl-6-methylpyrazine[Ar;Ro]	Roasted, hazelnut-like	F,R	j,k,l,r,v
2-ethyl-5-methylpyrazine[Ar;Ro]	-	R	j,l,r,v
2-ethyl-3,5-dimethylpyrazine[Ar]	-	R	a,p,k,r
2-methoxy-3-isobutylpyrazine[Ar]	Musty, nutty, coumarin-like	F,R	i,j,p,q
2-ethenyl-3,5-dimethylpyrazine[Ar]	Earthy,musty	R	p
2,3-diethyl-5-methylpyrazine[Ar]	Toasted, nutty, cocoa	R	j,p,q
2,3-Diethylpyrazine[Ar;Ro]	-	R	j,l,r
2,3,5-Trimethylpyrazine[Ar;Ro]	Nutty, earthy, cocoa	R	j,l,q
Pyridines (6)			
Pyridine[Ar;Ro]	Sour, fishy	G,R	a,c,o,r,s,u,v
3-Ethylpyridine[Ar;Ro]	Caramellic, roasted, hazelnut	R	r
2-Methoxypyridine[Ar]	Green, fermented, tea	F	k
2-Acetylpyridine[Ar]	Corn, nutty	F,R	k,l
2-Amino-5-methylpyridine[Ar]	-	R	L
3-Pyridinol[Ar]	-	R	j,l
Pyrroles (10)			
1-Methyl pyrrole[Ar;Ro]	Woody, herbal	G,R	c,j,l,r,v
2-Acetyl pyrrole[Ar]	Musty, nutty, coumarin-like	F,R	i,j,k,l
2-Formyl pyrrole[Ar]	Musty, beefy, coffee-like	F,R	i,k,
3-Methyl pyrrole[Ar]	-	F,R	j,k
2-Methyl pyrrole[Ar]	-	F	K
1-Furfuryl pyrrole[Ar]	Vegetal, cereal, bready	F,R	j,k,l
2-Formyl-1-methyl pyrrole[Ar]	-	R	r,l
Indole[Ar;Ro]	-	F,R	k,l,v
1-H-pyrrole [Ro]	Sweet, warm, nutty	R	v
1-H-pyrrole carboxyaldehyde[Ar;Ro]	-	R	j,r,v

Sulfur compounds (10)			
Methanethiol[Ar]	Sulfur, garlic	R	p,s
2-Furanmethanethiol [Ro]	Roasted coffee	R	v
3-Methyl-2-buten-1-thiol [Ro]	Amine-like	R	q
2-Methyl-3-furanthiol[Ar;Ro]	Meaty	R	q
2-Furfurylthiol[Ar;Ro]	Roasty (coffee)	R	l,q,r,s
Dimethylsulfide[Ar;Ro]	Vegetable, tomato	G	t,v
Benzothiazole[Ar]	Cooked, coffee, nutty	F	k
Furfuryl methyl sulfide[Ar]	Pungent, garlic	R	j,l
3-ethyl-2-formylthiophene[Ar]	-	R	j,l
2-Formylthiophene[Ar]	-	R	j
Terpenes (8)			
Linalool[Ar;Ro]	Citrus, orange, lemon	F,R	a,e,f,j,l
Limonene[Ar;Ro]	Sweet, citrus, orange	G,F,R	k,l,s,u
α-Terpeniol[Ar]	Citrus, woody, lemon	G,R	c,h
β-Citronelol[Ar]	Citronella, rose, leafy	R	e,f
β-Myrcene[Ar]	Woody, citrus, mango	F,R	j,k
β –Damascenone[Ar;Ro]	Honey-like, fruity	G,F,R	k,q
Geraniol[Ar]	Floral, sweet, rosey	R	e
Menthol[Ar]	Cooling, mentholic	F,R	f

*AR= compound detected in Arabica coffee; Ro= compound detected in Robusta coffee; Ar;Ro= compound detected in both Arabica and Robusta coffees

** G= green coffee beans; F= fermented, dried coffee beans; R= roasted coffee beans.

***a= Poyraz et al., 2016; b= Lee and Shibamoto, 2002; c= Gonzalez-Rios et al., 2007a; d= Pereira et al. 2016; e= Evangelista et al. 2014a; f= Evangelista et al. 2014b; g= Holscher and Steinhart, 1995; h= Evangelista et al. 2015; i= Lee et al., 2016a; j= Lee et al., 2016b; k= Lee et al., 2017a; l= Lee et al. 2017b; m= Pereira et al. 2014; n= Pereira et al. 2015; o= Somporn et al., 2011; p= Poisson et al., 2017; q= Blank et al., 1991; r= Caporaso et al., 2018b; s= Dryahina, Smith and Španěl, 2018; t= Bertrand et al., 2012; u= Dong et al., 2015; v= Akiyama et al., 2005.

The survey shown in TABLE 1.1 shows that many raw coffee volatiles are lost during the roasting process, especially those of nonthermal origin, such as hexanal, isoamyl alcohol, 1-hexanol, 1-pentanol, 2-heptanol, 1-octen-3ol, benzyl alcohol, and benzaldehyde (GONZALEZ-RIOS et al., 2007a; POYRAZ et al., 2016). On the other hand, some compounds are reported to be common in both green and roasted samples, including γ-butyrolactone, linalool, guaiacol, pyridine, furfural, 5-methylfurfural, 1-methylpyrrole, and β-damascenone (GONZALES-RIOS et al., 2007a; 2007b; HOLSCHER; STEINHART, 1995; POYRAZ et al., 2016), which are responsible for mainly spicy or green, vegetable-like flavors of the final coffee beverage (HOLSCHER; STEINHART, 1995; POYRAZ et al., 2016). Furthermore, some raw coffee volatiles (e.g., geosmin, 2,4,6-trichloroanisol-phenol, and 4-heptenal) are externally caused by insect attacks or immaturity of coffee beans and generally associated with sensory defects (OESTREICH-JANZEN, 2010).

The species *C. arabica* L. (Arabica coffee) and *C. canephora* Pierre (Robusta coffee) economically dominate the world coffee trade, accounting for about 99% of world bean production. Presently, Arabica coffee dominates the volume of world production (about 75% of total production) because of its superior bean quality (BELITZ; GROSCH; SCHIEBERLE, 2009). In this sense, there is a price difference between the above-mentioned types of coffee on the global market. The average price of Arabica on the US market is 170 cents per pound, while the price of Robusta is 100 cents per pound (INDEXMUNDI, 2017a; 2017b). The high caffeine concentration in Robusta beans in relation to Arabica coffee is the main chemical difference between them. However, volatile chemical constituents can also serve as markers in the differentiation of these beverages; for instance, 2-methylisoborneol has been detected in high concentrations in Robusta coffees and may be responsible for their typical earthy flavor (HOLSCHER; STEINHART, 1995). Further studies on this topic are necessary in order to obtain volatile markers for differentiation of these two coffee species (KNYSAK, 2017).

1.4.2. Impact of processing methods.

Coffee fruit can be processed according to the three above-mentioned postharvest processing methods. The chosen method will have a direct influence on the quality of the final coffee beverage (GONZALEZ-RIOS et al., 2007a; JÖET et al.,

2010; PEREIRA et al., 2017; SELMAR et al., 2006). In general, wet-processed coffees are known to present higher acidity and more aroma than dry-processed coffees (MAZZAFERA; PURCINO, 2004). This fact can be attributed to the different metabolic activities of sugar and free amino acids inside the seeds as a result of the chosen conditions (FIGURE 1.1). These compounds are important aroma precursors in the formation of many volatiles during roasting, such as furans, diketones, pyrazines, pyrrolines, lactones, and phenolic acids (BYTOF et al., 2005; KNOPP; SELMAR; BYTOF, 2006; SELMAR et al., 2006). In a study conducted by Knopp et al. (2006), glucose and fructose content was significantly lowered through wet processing. This outcome may be associated with sugar metabolism (i.e., alcoholic or lactic fermentation due the anoxic conditions of wet processing) and inter-conversions that are expected during the seed-germination process (JÖET et al., 2010). In addition, the superior concentration of free amino acids (i.e., aspartate, glutamate, and alanine) in wet-processed coffee is associated with the hydrolysis of proteins in order to generate raw materials for the germination process (BYTOF et al., 2005; SELMAR; BYTOF; KNOPP, 2002). On the other hand, the accumulation of γ-aminobutyric acid in dry-processed coffees could be associated with a response to drought stress during long exposure while drying (FIGURE 1.1). These alterations in the pool of free amino acids and low-weight sugars can explain the differences between the aroma profiles of wet- and dry-processed coffee beans.

1.4.3. Mucilage removal

After harvesting and pulping, the coffee beans are submitted to underwater tank fermentation (wet process) or placed on a terrace (semi-dry process) for mucilage breakdown and removal. The sugars present in the mucilage will allow microorganisms' growth, especially yeasts (e.g., *Pichia guilliermondii, P. anomala Kluvyeromyces marxianus*, and *Saccharomyces cerevisae*) and lactic acid bacteria (e.g., *Leuconostoc mesenteroides, Lactobacillus plantarum* and *Lb. brevis*) (EVANGELISTA, 2014b; LEONG et al., 2014; PEREIRA et al., 2017; VILELA, 2010). The microbial growth generates a range of end-metabolites, which can diffuse into the seeds and have an impact on the final coffee quality (EVANGELISTA et al., 2014a; PEREIRA et al., 2015; SILVA et al., 2013). In this respect, yeasts have a pivotal influence through the generation of different aroma-influencing molecules via central carbon and nitrogen

metabolism. In addition, various studies have shown that coffee-associated yeast strains of *Candida parapsilosis, Debaryomyces hansenii*, *Kluyveromyces marxianus, Pichia guilliermondii, P. fermentans, P. kluyveri*, and *Saccharomyces cerevisiae* are capable of promoting pectin breakdown (the major carbohydrate polymer present in coffee mucilage) through the production of different hydrolytic enzymes, such as pectin methyl esterase, pectin lyase, and polygalacturonase (MASOUD; JESPERSEN, 2006; PEREIRA et al., 2014; SILVA et al., 2008; Silva et al., 2013). The hydrolysis of pectin releases simple sugars (i.e., glucose, rhamnose, L-arabinose and D-galacturonate) as an additional carbon source for yeast metabolism and aroma formation (GERMANE et al., 2015; KIM et al., 2016).

Ethanol, acetaldehyde, and acetic acid are the primary metabolites produced by yeast during coffee mucilage fermentation (FIGURE 1.2). Coffee yeasts appear to have low aldehyde dehydrogenase activity because no acetic acid is produced by selected single cultures (TABLE 1.2). Along with ethanol, fermenting yeast cultures produce many low-molecular-weight flavor compounds during the mucilage removal process, including esters, higher alcohols, aldehydes, ketone, and terpenoids (FIGURE 1.2). Among these compounds, esters (acetate and ethyl esters) are quantitatively the most abundant group of volatiles formed. They are generated by a condensation reaction between fatty acids and an alcohol molecule (SAERENS et al., 2010). In addition, the direct connection to higher alcohols and their amino acid precursors makes ester production highly dependent on a nitrogen source (DZIALO et al., 2017). Esters are widely known to contribute to floral and fruity sensory notes in alcoholic beverages (PROCOPIO; QIAN; BECKER, 2011). The exploration of yeast-derived esters to assist coffee quality is, however, a relatively recent and recurrent approach. In this context, different studies are currently dedicated to the selection of ester-producing coffee yeasts (e.g., *Pichia fermentans, P. guilliermondii, Candida parapsilosis, Saccharomyces cerevisiae, Torulaspora delbrueckii*, and *Yarrowia lipolytica*) with the potential to increase the contents of these compounds in the beans (BRESSANI et al., 2018; EVANGELISTA et al., 2014a; LEE et al., 2017a; PEREIRA et al., 2014, 2015; SILVA et al., 2013). The production of ethyl acetate, isoamyl acetate, propyl acetate, ethyl hexanoate, and n-butyl acetate has been shown to contribute to the development of exotic sensory notes in coffee beverages, such as “Sicilian lemon,” “apricot,” “caramel,” “nutty” and “banana raisin” (EVANGELISTA et al., 2014a; EVANGELISTA et al., 2014b; PEREIRA et al., 2015). In addition, the metabolism of

lactic acid bacteria has also recently been used for ester formation (e.g., ethyl acetate, ethyl isobutyrate, and hexyl acetate) and the production of flavorful coffee beans (TABLE 1.2).

TABLE 1.2 – VOLATILE ORGANIC COMPOUNDS (VOC) PRODUCED BY COFFEE-ASSOCIATED MICROORGANISMS AND DETECTION AFTER THE ROASTING PROCESS

Microorganism	VOC production	Roasting detection	References
Pichia fermentans YC5.2	***Aldehyde*** (Acetaldehyde; Hexanal) ***Acetate esters*** (Hexyl acetate; Isobutyl acetate and Isoamyl acetate) ***Ethyl ester*** (Ethyl acetate) ***Higher alcohol*** (2-methyl-1-Butanol; 3-methy-1-Butanol; 1-Pentanol; 2-Hexanol; 1-Octanol; 1-Decanol) ***Diketones*** (2,3-Butanedione)	Acetaldehyde, Hexyl acetate, Isobutyl acetate, Ethyl acetate and Isoamyl acetate	PEREIRA et al., 2014; 2015
Saccharomyces cerevisae YC9.15	***Aldehyde*** (Acetaldehyde; Hexanal) ***Ethyl ester*** (Diethyl succinate; Ethyl acetate) ***Higher alcohol*** (1-Octanol; 2-methyl-1-Butanol) ***Ketone*** (2-Hexanone)	NE	PEREIRA et al., 2014
P. guilliermondii YC1.2	***Aldehyde*** (Acetaldehyde) ***Ethyl ester*** (Ethyl acetate)	NE	PEREIRA et al., 2014
P. kluyveri YH7.16	***Acetate ester*** (Isoamyl acetate) ***Ethyl ester*** (Ethyl acetate)	NE	PEREIRA et al., 2014
Lactobacillus plantarum LPBR01	***Ethyl ester*** (Ethyl propionate; Ethyl acetate; Ethyl isobutirate; Ethyl hexanoate; Ethyl octanoate) ***Acetate ester*** (Propyl acetate; Isoamyl acetate; n-Butyl acetate) ***Higher alcohol*** (1-Octanol)	NE	PEREIRA et al., 2016
L. plantarum LPBL01	***Ethyl ester*** (Ethyl propionate)	NE	PEREIRA et al., 2016

L, paracasei spp. *paracasei* LPBL07	***Ethyl ester*** (Ethyl acetate)	NE	PEREIRA et al., 2016
S. cerevisae UFLACN 727	***Aldehyde*** (Acetaldehyde; Decanal) ***Acetate ester*** (Propyl acetate; Phenylethyl acetate) ***Ethyl ester*** (Ethyl lactate; Ethyl acetate) ***Ketone*** (Acetoin) ***Higher alcohol*** (3-methyl-1-Butanol; 2-methyl-1-Butanol; 1,3-Butanediol; 2-Phenylethanol) ***Furan*** (Furfural; Furfuryl alcohol) ***Terpene*** (Linalool; Geraniol; α-Terpeniol; Citronellol)	Acetaldehyde, 3-Methyl butanol, Furfuryl alcohol, Furfural and Linalool	SILVA et al., 2013
S. cerevisiae UFLACN 724	***Aldehyde*** (Acetaldehyde; Butyraldehyde; Decanal) ***Ethyl ester*** (Mono ethyl succinate; Ethyl lactate) ***Ketone*** (Acetoin) ***Higher alcohol*** (2-Phenylethanol; 1,2-Propanediol) ***Furan*** (Furfural; Furfuryl alcohol) ***Terpene*** (b-citronelol) ***Phenol*** (Guaiacol)	Acetaldehyde, Furfural, 2-Phenylethanol, 1,2-Propanediol, Furfuryl alcohol and Guaiacol	SILVA et al., 2013
P. guilliermondii UFLACN 731	***Aldehyde*** (Acetaldehyde; Decanal) ***Ethyl ester*** (Ethyl lactate; Diethyl malate) ***Higher alcohol*** (2-methyl-1-Butanol; 1,2-Propanediol; 1,3-Butanediol; 2-Phenylethanol) ***Ketone*** (Acetoin; 2-Nonanone) ***Furan*** (Furfural; Furfuryl alcohol) ***Terpene*** (Linalool)	Acetaldehyde, Furfural, Linalool, Furfuryl alcohol, 1,3-Butanediol and 2-Phenylethanol	SILVA et al., 2013
Candida parapsilosis UFLACN 448	***Aldehyde*** (Acetaldehyde; Furfural; Decanal) ***Ketone*** (Acetoin)	Acetaldehyde, 1-Pentanol, Furfural, Furfuryl alcohol and 2-Phenylethanol	SILVA et al., 2013

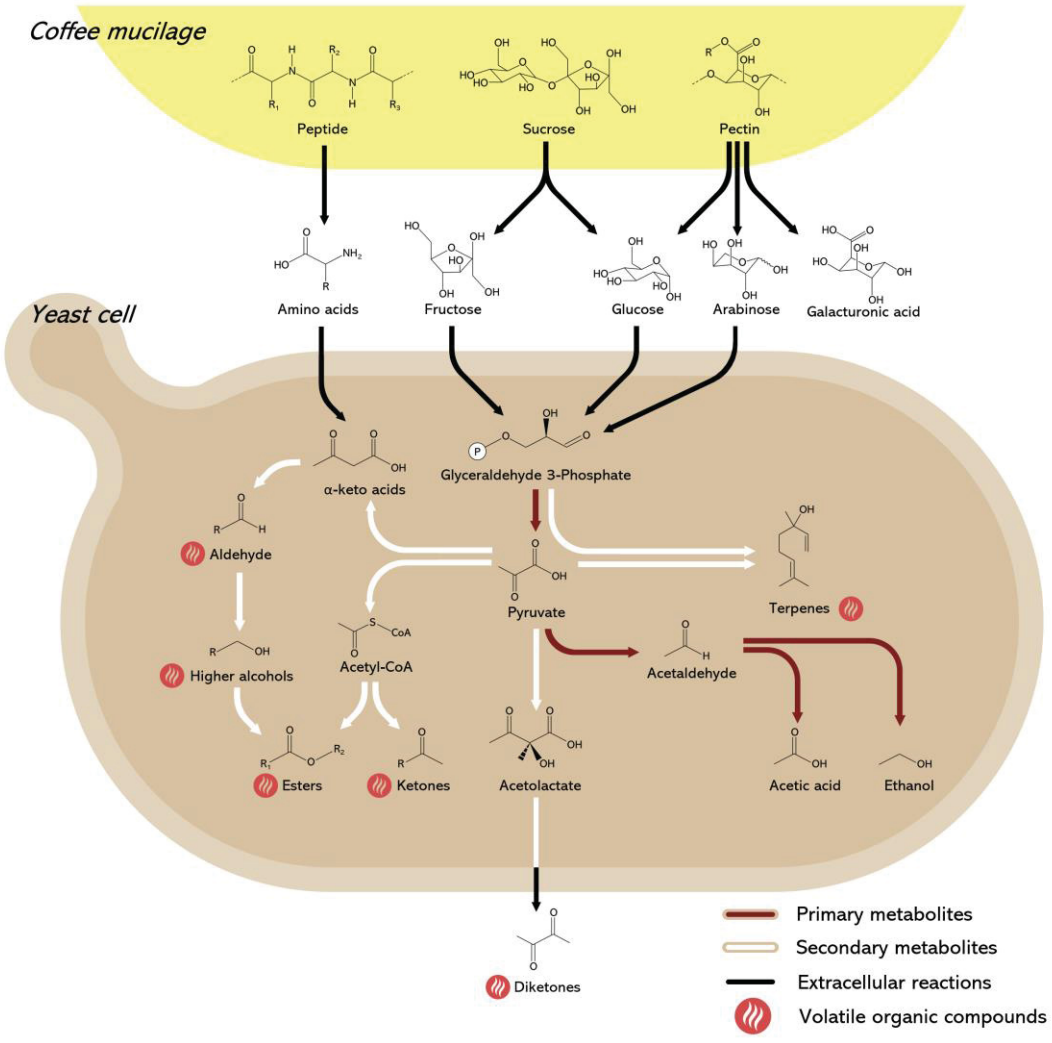
	Higher alcohol (2-methyl Butanol; 1-Pentanol; 2-Heptanol; Furfuryl alcohol; 2-Phenylethanol) ***Terpene*** (Linalool; b-Citronellol; Menthol)		
Saccharomyces cerevisiae CMA 0200	***Aldehyde*** (Octanal; Nonanal; Decanal; Tetradecanal; Pentadecanal; 2-Heptenal; 2-Octenal; 2-methyl-2-Butenal; 2-Nonenal; 2-Undecenal; 3-methyl-2-Butenal; 2,4-Heptadienal; 2,4-Nonadienal; Benzaldehyde; 3-methyl-Benzaldehyde; 2-Furaldehyde) ***Ketone*** (2-Heptanone; 2-Octanone; 2-Nonanone; 6-Methyl-3,5-heptadiene-2-one) ***Higher alcohol*** (1-Hexanol; 1-Heptanol; 2-Heptanol; 1-Octanol; 1-Nonanol; 2-ethyl-1-Hexanol; 3-methyl-2-buten-1-ol; 1-Octen-3-ol; Benzylalcohol; 2-Phenylethanol)	2-Phenylethanol and 2-methyl-2-Butenal	RIBEIRO et al. 2017
Saccharomyces cerevisiae CMA 0543	***Aldehyde*** (Octanal; Nonanal; Decanal; Tetradecanal; Pentadecanal; (E)-2-Heptenal; 2-Octenal; 2-Nonenal; 2-Undecenal; 2-methyl-2-Butenal; 3-methyl-2-Butenal; 2,4-Heptadienal; 2,4-Nonadienal; Benzaldehyde; 3-methyl-Benzaldehyde; 2-Furaldehyde) ***Ketone*** (2-Heptanone; 2-Octanone; 2-Nonanone; 6-methyl-3,5-Heptadiene-2-one) ***Higher alcohol*** (1-Hexanol; 1-Heptanol; 2-Heptanol; 1-Octanol; 1-Nonanol; 2-ethyl-1-Hexanol; 3-methyl-2-Buten-1-ol; 1-Octen-3-ol; Benzylalcohol; 2-Phenylethanol)	2-Phenylethanol and 2-methyl-2-Butenal	RIBEIRO et al. 2017
P. kluyveri PK-KR1	***Acetate ester*** (Isoamyl acetate)	NE	SAERENS; SWIEGERS, 2016

	Higher alcohol (isoamyl alcohol)		
Yarrowia lipolytica NCYC 2904	***Aldehyde*** (3-Methylbutanal; Hexanal; Octanal; Nonanal; trans-2-Octenal; Benzaldehyde; 2-Phenylacetaldehyde; Dodecanal; trans,trans-2,4-Decadienal; Vanillin) ***Hexyl ester*** (Hexyl 2-methyl butyrate; Hexyl 3-methyl butanoate) ***Ethyl ester*** (Ethyl linoleate) ***Methyl ester*** (Methyl salicylate; Methyl palmitate) ***Ketone*** (3-Octen-2one; 1-(acetyloxy)-2-Propanone; 2-methyl-3-Pentanone; 1-(acetoloxy)-2-Butanone; 3,5-Octadien-2-one; 6,10,14-trimethyl-2-Pentadecanone; γ-Butyrolactone) ***Higher alcohol*** (1-Phenylmethanol; 2-Phenylethanol)	2-Phenylethanol; Benzaldehyde; Vanillin; Methyl salicylate; Methyl palmitate; 2-Methyl-3-pentanone; γ-Butyrolactone	LEE et al., 2017a; 2017b
Rhizopus oligosporus	***Aldehyde*** (Heptenal) ***Methyl ester*** (Methyl salicylate) ***Ethyl ester*** (Ethyl palmitate; Ethyl 3-hydroxybutanoate) ***Ketone*** (γ-Butyrolactone) ***Higher alcohol*** (2,3-Butanediol; 2-Phenylethyl alcohol; 1-Octen-3-ol) ***Phenol*** (p-Vinylguaiacol)	Ethyl palmitate	LEE et al. 2016a; 2016b

NE.: Not evaluated

Yeast commonly synthesizes a great variety of higher alcohols during coffee fermentation, such as n-butanol, isobutanol, 1-propanol, 2-phenylethanol, isoamyl alcohol, 2,3-butanediol, and 2-methyl-1-butanol (TABLE 1.1). These compounds are derived from amino acid catabolism via the Ehrlich pathway (FIGURE 1.2). The coffee mucilage is rich in amino acids that are assimilated by the Ehrlich pathway, including leucine, valine, phenylalanine, threonine, and isoleucine (ELÍAS, 1979; PEREIRA et al., 2014). The direct relationship between microbial-derived higher alcohols and coffee quality is not yet known. Higher alcohols are known for their higher sensory threshold, which differs by several orders of magnitude from that of their corresponding acetate esters (DZIALO et al., 2017). Thus, it is possible to speculate that an intense diffusion process during fermentation is required to impact overall coffee quality.

FIGURE 1.2 – PROPOSED SCHEMATIC REPRESENTATION OF POTENTIAL AROMA COMPOUNDS THAT CAN BE GENERATED IN YEAST FERMENTATION OF COFFEE MUCILAGE FROM EXISTING PRECURSORS.



A range of ketone compounds are formed during the coffee bean fermentation process (TABLE 1.1). Some of these compounds are associated with yeast metabolism, especially diacetyl (2,3-butanedione) (FIGURE 1.2). The presence of 2,3-butanedione in roasted beans is linked to the generation of a buttery-like aroma in coffee beverages (EVANGELISTA et al., 2015). Through yeast metabolism, this compound is formed extracellularly by chemically driven decarboxylation of α-acetolactate (HIRST; RICHTER, 2016). Thus, since the conversion of α-acetolactate into diacetyl is a nonenzymatic process, fermentations conducted at high temperatures may increase the conversion rate of excess α-acetolactate into flavor-impacting 2,3-butanedione (KOBAYASHI; KUSAKA; SATO, 2005).

The terpenes formed during the coffee mucilage removal process (β-citronelol, linalool, geraniol, α-terpeniol, citronellol, and β-citronelol) originate from glycoside precursors through yeast β-glucosidase enzymes (HERNÁNDEZ et al., 2003; MENDES-FERREIRA et al., 2009). In addition, some yeast species found in coffee fermentation (e.g., *Sacharomyces cerevisiae, Torulaspora delbrueckii*, and *Hanseniaspora uvarum*) can produce terpene derivatives through the mevalonic acid pathway (CARRAU et al., 2005; GRUCHATTKA et al., 2013). Silva et al. (2013) showed that linalool produced by coffee-associated *Saccharomyces cerevisiae* and *Pichia guilliermondii* yeasts can be detected in beans after the roasting process (TABLE 2). The aroma of linalool has been described as fresh, citrusy, and woody in various food products (GÓRSKA et al., 2017). For coffee, however, more studies are necessary to evaluate the direct impact of this compound on the final product quality.

The aldehydes generated during the coffee mucilage removal process are important precursors in the formation of aromatic compounds such as higher alcohols and esters (FIGURE 1.2). These compounds are catabolized by the interconversion of alcohols and the corresponding aldehydes or ketones via alcohol dehydrogenase activity (HIRST; RICHTER, 2016). In addition, some aroma-active aldehydes (*e.g.* 2-methyl-2-butenal and acetaldehyde) can be released from yeast cells during the apoptosis event and diffuse into the beans, affecting the fruity and floral aromas of the coffee beverage (BRESSANI et al., 2018; PEREIRA et al., 2014, 2015; RIBEIRO et al. 2017).

TABLE 1.2 outlines suitable starter cultures used to conduct a controlled coffee mucilage removal process. It shows the diverse range of volatile compounds produced by the different microbial strains selected. Thus, the selection criteria of fermenting microorganisms should be driven by the targeted flavor with a focus on the relevant

metabolic activities. Preference should be given to strains that deliver an overall pleasant aroma to the end product with no off-flavors or unwanted acids such as acetic, succinic, or butyric (PEREIRA et al., 2016). The combination of starter cultures possessing different aroma-producing profiles can be considered for flavor enhancement. In addition, the application of stressor factors, such as high or low temperatures, can be exploited to modulate the concentration of individual or groups of compounds for flavor improvement (PEREIRA et al., 2014).

1.4.4. Drying and storage processes

During the drying process, coffee beans remain viable with intense metabolic activities (BYTOF et al., 2005; KNOPP et al., 2006). These include reactions of interconversion of low-molecular-weight sugars (i.e., glucose, fructose, and mannose) and hydrolysis of proteins, resulting in the accumulation of a wide variety of free amino acids (JÖET et al., 2010; KNOPP et al., 2006; SELMAR et al., 2002). So far, no research has investigated changes in specific volatile compounds through the drying process, and some studies have focused only on the major chemical compounds (i.e., sugar and proteins). More detailed chemical studies could assist in the understanding of volatile losses caused by evaporation and/or oxidation reactions during drying.

After the drying process, the freshly processed coffee beans are stored for a period of up to 3 years. The storage process must be maintained under ideal conditions of humidity (e.g., 11% humidity), in a low temperature, and in an inert atmosphere in order to preserve bean quality. During the storage process, coffee beans can remain viable for up to 6 months. However, if the beans are stored within the parchment layer (FIGURE 1.1), living seeds can be found for up to one year. After this period, the coffee beans die, starting the senescence reactions (SPEER; KÖLLING-SPEER, 2006; RIBEIRO et al., 2011; TOCI et al., 2013). In general, a decline in cup quality is linked to the loss of bean viability. This is mainly correlated with the chemical reactions occurring during the senescence process, such as chlorogenic acid oxidation, which leads to the development of a bluish-green color in the beans.

Works dedicated to biochemical alterations that occur during storage have reported mainly on the major nonvolatile constituents, including sugars, lipids, and carbohydrates. Selmar et al. (2008) found that the relatively high content of glucose present in on-farm processed coffee beans decreased markedly through the storage time.

On the other hand, Bucheli et al. (1998) found an increase in glucose under storage conditions; these authors also detected a significant decrease in sucrose, which is hydrolyzed to produce glucose and fructose. Thus, the carbohydrate fraction present in freshly processed coffee beans has a direct influence on the germination process during storage. For example, the glucose and fructose concentration is markedly higher in dry-processed beans than in wet-processed beans, while that of beans originating from a semi-dry process is between those of wet- and dry-processed beans (RENDÓN et al., 2014).

During storage, undesired changes within the lipid fraction occur due to oxidation processes (BORÉM; MARQUES; ALVES, 2008; SPEER; KÖLLING-SPEER, 2006). Lipid oxidation is favored during the first three months because of a high rate of respiration and the accumulation of reactive oxygen species. After this period, the respiration is interrupted and the products of lipid oxidation react with proteins, forming polymers (RENDÓN et al., 2014; SPEER; KÖLLING-SPEER, 2006).

While many storage-associated reactions are already known, the specific causes of the progressive weakening of cup quality are still unclear. Carbohydrates, lipids, and proteins are all important precursors in the development of chemical aromas during the roasting process. However, at present, there are no studies demonstrating the changes of specific volatile molecules during storage. Coffees prepared by beans stored for only 6 months are attributed raspy, woody, or stale notes, while after one year stored, flat aromas and old and woody notes are detected (RENDÓN et al., 2014; SELMAR et al., 2008). These data demonstrate a possible direct influence of volatile constituents formed and/or generated during the storage process.

1.5. ROASTING PROCESS

The color, taste, and aroma of coffee is related to chemical reactions that occur during roasting. In general, this process takes between 3 and 20 minutes and can be classified into three different stages: (i) the initial stage of water removal, where the temperature is raised to 180 °C and the moisture of the beans changes from 10–12.5% to 2.5% moisture; (ii) the second stage at 200–300 °C, during which physical-chemical transformation occurs, leading to flavor development; and (iii) the phase of cooling via cold air or water jets (FADAI et al., 2017).

A wide variety of volatile compounds are present in roasted coffee beans, such as alcohols, aldehydes, amines, carboxylic acids, dicarbonyls, enoles, esters, furans, furanones, hydrocarbons, imidazoles, indoles, ketones, lactones, oxazoles, phenols, pyrazines, pyridines, pyrroles, quinoxalines, sulfur compounds, terpenes, and thiazoles (TABLE 1) (BUFFO; CARDELLI-FREIRE, 2004; POISSON et al., 2017; SUNARHARUM et al., 2014). However, only some of these compounds have a major impact on human perception, such as pyrazines, furans, esters, ketones, phenols, and sulfur compounds (FIGURE 1.1). These compounds can undergo dramatic changes depending on the thermal profile applied during the roasting process (CAPORASO et al., 2018a). Several chemical events take place in the coffee seeds during the formation of such molecules, such as cleavage, cyclization, dehydration, enolization, epimerization, fragmentation, hydrolysis, isomerization, lactonization, and recombination (AGUIAR; ESTEVINHO; SANTOS, 2016; POISSON et al., 2017). The predominant chemical processes are the Strecker degradation and the Maillard and pyrolysis reactions (FADAI et al., 2017; FLAMENT; BESSIÈRE-THOMAS, 2002). The Maillard reaction is a chemical reaction between reducing sugars and amino acids, leading to the formation of a range of important volatile compounds such as pyridines, pyrazines, dicarbonyls, diacetyl, oxazoles, thiazoles, pyrroles and imidazoles, enolones (furaneol, maltol, cyclotene), and formic and acetic acids (LEE et al., 2015; POISSON et al., 2017). The Strecker degradation consists of a chemical reaction in which α-amino acids are converted into aldehydes and sulfur compounds (e.g., 3-2-methylbutanal, methional, 3-mercapto-3-methylbutyl formate, 3-methyl-2-butene-1-thiol, 2-furfurylthiol, methanethiol, and phenylacetaldehyde), contributing to the complex aroma composition of coffee (POISSON et al., 2017). In the pyrolysis reaction, structural carbohydrates of the intercellular coffee bean matrix are degraded, increasing internal porosity and CO_2 production (FADAI et al., 2017). Other minor reactions, including the degradation of specific individual amino acids (i.e., proline and hydroxy amino acids), aliphatic acids (particularly quinic acid) and lipids, have important impacts on the formation of volatiles during roasting. For more information concerning these reactions, readers are directed to the reviews published by Buffo and Cardelli-Freire (2004), Martins et al. (2001), and Toledo et al. (2016).

The concentration of volatile compounds in roasted coffee strongly depends on the genetic differences in the plant (CAPORASO et al., 2018a). Arabica is known to contain high concentrations of 2,3-butanedione, 2,3-pentanedione, furfural, 1-

(acetyloxy)-2-propanone, 2-acetylfuran, ethyl propanoate, furaneol, 2,3-butanediol, acetoin, and 1-hydroxy-2-butanone, whereas Robusta is generally associated with pyrazine compounds such as 2-methyl-pyrazine, 2,6-dimethylpyrazine, 2,5-dimethylpyrazine, ethylpyrazine, 2-ethyl-6-methylpyrazine, and 2-ethyl-5-methylpyrazine (BLANK; SEN; GROSCH, 1991; CAPORASO et al., 2018a). Other factors that influence roasted volatile constitution include green coffee bean composition, geographic origin, postharvest processing of beans, environmental factors in pre-harvest processing, presence of defective beans, and ripening stage (FREITAS; MOSCA 1999; JÖET et al., 2010; TOLEDO et al., 2016).

1.6. CONCLUSION

The findings presented in this review show that the main odor-active compounds in coffee beverages, such as furans, pyrazines, and pyrroles, are not found in raw green coffee beans. Thus, green coffee bean quality is determined by major, nonvolatile constituents present in the raw material, such as sugar, amino acids, and lipids. These aroma precursors will further undergo modifications in the postharvest processing steps due to the seed germination process and lipid oxidation.

Among the different steps in postharvest coffee processing, microbial mucilage removal has a major influence on the volatile composition of processed beans. Various studies have shown that microbial-derived metabolites can diffuse into seeds and remain after the roasting process, including esters, higher alcohols, aldehydes, ketone, and terpenoids. Among these, flavor-active esters show great potential to influence the quality of the final coffee beverage. So far, no research has investigated the changes of specific volatile compounds through drying and storage. With respect to this issue, the volatile kinetics that take place during these steps should be studied further.

CHAPTER II (RESEARCH RESULTS) – YEAST DIVERSITY AND PHYSICOCHEMICAL CHARACTERISTICS ASSOCIATED WITH COFFEE BEAN FERMENTATION FROM THE BRAZILIAN CERRADO MINEIRO REGION

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ABSTRACT

The aim of this study was to evaluate yeast diversity and physicochemical characteristics of spontaneous coffee beans fermentation conducted in the coffee-producing region at Cerrado Mineiro, Brazil. During 48 h of fermentation, yeast population increased from 6.60 to 7.89 log CFU/mL with concomitant pulp sugar consumption and organic acids production (mainly lactic (3.35 g/L) and acetic acids (1.27 g/L)). According to ITS-rRNA gene sequencing, yeast population was mainly represented by *Saccharomyces* sp., followed by *Torulaspora delbrueckii*, *Pichia kluyveri, Hanseniaspora uvarum, H. vineae,* and *Meyerozyma caribicca*. SPME-GC-MS analysis revealed a total of 25 volatile organic compounds with predominance of hydrocarbons (9 compounds) and higher alcohols (6 compounds). The resulting fermented, roasted coffee beans were analyzed by diverse chemical analysis methods, including Fourier Transform Infrared (FTIR) spectroscopy and mineral and thermogravimetric analysis. The thermal decomposition of the coffee beans occurred in four stages between 90 and 390 ºC, with significant mass loss (68%) after the second stage at 190 ºC. FTIR spectroscopy confirmed the presence of the main organic functions associated with the coffee aroma, such as aromatic acids, ketones, aldehydes and aliphatic esters. The results presented in this study enrich our knowledge concerning yeast diversity and physicochemical characteristics associated with coffee beans fermentation, and can be used to promote a controlled on-farm processing.

Keywords: Wet processing, coffee, yeasts, physicochemical characteristics

2.1. INTRODUCTION

Coffea is a genus of flowering plants whose seeds are used to make coffee beverage. Although there are many steps in the manufacturing of coffee beverage, microbial fermentation plays an important role with great impacts on product quality and value (EVANGELISTA et al., 2014a, 2015; PEREIRA et al., 2014, 2015, 2016). Coffee fermentation consists of an on-farm process, occurred in the so-called wet processing, during which microorganisms grow in the pulp material that surrounds the seeds of the coffee fruit (PEREIRA et al., 2017). In this processing method, the pulp (the exocarp and a part of the mesocarp) is removed mechanically. Subsequently, the beans are submitted to 24–48 h of underwater tank fermentation to allow microbial degradation of the remaining mesocarp layer (called mucilage) adhering to the parchment. In addition, the microbiota responsible for the fermentation may also contribute to the beverage's sensory characteristics and other qualities due to the excretion of metabolites produced during this process (PEREIRA et al., 2017). The main chemical changes that occur during coffee fermentation are pectin degradation and microbial production of organic acids, ethanol, esters and other metabolites from the carbohydrates (PEREIRA et al., 2014; SIVERTZ, 1963; WOOTTON, 1963).

Yeasts are among the most frequently isolated microorganisms from fermenting coffee beans. They are considered to be important to the fermentation process and coffee flavor development. Consequently, yeast is the microbial group most widely studied in coffee fermentations, which metabolic function has been elucidated in recent studies (EVANGELISTA et al., 2014a; PEREIRA et al., 2014). The most frequently occurring yeast species during coffee processing are *Pichia kluyveri, Pichia anomala, Hanseniaspora uvarum, Saccharomyces cerevisiae, Debaryomyces hansenii and Torulaspora delbrueckii* (MASOUD et al., 2004; SILVA et al., 2008; VILELA et al., 2010).

Recent studies published by our research group have reported the yeast and bacteria diversity associated with coffee beans fermentation in Brazil (PEREIRA et al., 2014, 2015, 2016, 2017). *Pichia fermentans* (YC5.2), *Saccharomyces* sp. (YC9.15) and *Lactobacillus plantarum* (LPBR01) were studied as having a potential for use as starter cultures for coffee wet fermentation (PEREIRA et al., 2015, 2016). However, studies still are needed to improve the knowledge of the microbiota present in coffee processing due to the variation in climate and altitude (EVANGELISTA et al., 2015). Cerrado

Mineiro is one of the largest coffee-producing regions in Brazil, located at Alto Paranaíba, Mineiro Triangle and Norwest Minas Gerais. This region presents a uniform edaphoclimatic pattern with an average temperature of 23 ºC and flat relief situated at 800-1300 m, which enables the production of high-quality coffees (ABOPCAFE, 2017). To the best of our knowledge, there is no study on the yeast diversity and physicochemical characteristics associated with coffee bean fermentation performed in this coffee-producing region. In this regard, the aim of this study was to study the yeast diversity and physicochemical characteristics associated to coffee beans fermentation during on-farm wet processing in the Cerrado Mineiro region.

2.2. MATERIAL AND METHODS

2.2.1. Spontaneous coffee beans fermentation and sampling

Coffee cherries (*Coffea arabica*) were manually harvested at the mature stage from the Fazenda Shalon (Patrocínio, Minas Gerais State, Brazil). FIGURE 2.1 shows the experimental setup and analytical procedures of each step conducted in this study. The fruits were mechanically depulped and approximately 75 kg of depulped beans were then conveyed in a clear water stream to tanks and left to ferment for 48 h in accordance with local wet processing method (PEREIRA et al., 2015, 2016). Every 12 h, liquid fraction samples were withdrawn from the middle depth of the tank fermentation and transferred to the laboratory in ice boxes for microbiological and chemical analyses.

FIGURE 2.1. EXPERIMENTAL SETUP OF THE CASE STUDY OF COFFEE-PROCESSING EXPERIMENTS CARRIED OUT AT THE CERRADO MINEIRO REGION

2.2.2. Yeast isolation

Ten milliliters of each sample were added to 90 mL sterile saline-peptone water, followed by serial dilutions. Yeasts were enumerated by surface inoculation on YEPG

agar containing 100 mg/L chloramphenicol (Sigma, São Paulo, Brazil) to inhibit bacterial growth. Plating was performed with 100 µL of each dilution and cultures were incubated at 30 ºC for 5 days (PEREIRA et al., 2014). According to the macroscopic observations (texture, surface, margin, elevation, and color), colonies of different types on YEPG medium were counted separately, and representatives isolated from different fermentation times were purified by repetitive streaking. The purified isolates were stored at –80 °C in YEPG broth containing 20% (v/v) glycerol (Difco, Franklin Lakes, NJ).

2.2.3. Identification of yeast isolates

Yeast cultures were grown under appropriate conditions, collected from agar plates with a sterile pipette tip and resuspended in 50 µL of ultra-pure water. The suspension was heated for 15 min at 95 °C, and 1 µL of this suspension was used as a DNA template in PCR experiments. The 5.8S ITS rRNA gene region of yeast isolates was amplified using the primers ITS1 and ITS4. The 55 µL volume reaction consisted of 5.5 µL of 10x PCR buffer (Invitrogen, Carlsbad, CA), 2 µL of $MgCl_2$ (50mM), 1.21 µL of dNTP Mix (10mM), 4 µL of the combined forward and reverse primers (ITS5 and ITS4), 0.4 µL of 5U/µL Platinum® Taq DNA polymerase (Invitrogen, Waltham, MA, USA). The 5.8S ITS rRNA gene region was sequenced using an ABI3730 XL automatic DNA sequencer. The sequences obtained were compared with sequences available in the GenBank database through a basic local alignment search tool (BLAST).

2.2.4. High performance liquid chromatography (HPLC) analysis of fermenting coffee beans samples

The concentration of the reducing sugars (glucose and fructose) and organic acids (acetic, citric, succinic, lactic, propionic and butyric acids) of fermenting coffee-pulp bean mass was monitored during the course of fermentation. Samples (2 µL) of each time were centrifuged at 6000 g and filtered through 0.22-µm pore size filter (Sartorius Stedim, Goettingen, Germany). The samples were analyzed through a HPLC apparatus (Aglient Technologies 1260 Infinity Series; Aglient Technologies, Santa Clara, CA, USA) equipped with a Hi-Plex H column (300 x 7.7 mm; Aglient

Technologies, Santa Clara, CA, USA) connected to a refractive index (RI) detector (Aglient Technologies, Santa Clara, CA, USA). The column was eluted with a mobile phase containing 5mM H_2SO_4, at 60 ºC and a flow rate of 0.6 mL/min.

2.2.5. Physicochemical characterization of fermented and roasted coffee beans

2.2.5.1. Volatile organic compounds determination by Gas Chromatography coupled to mass spectrometry (GC-MS)

The resulting parchment coffee was dried in a laboratory oven at 35–40 °C until a water content of 12% was achieved. The extraction of volatile compounds from the fermented, dried coffee bean samples (FIGURE 2.1) were performed using a headspace (HS) vial coupled to a Solid Phase Micro Extraction (SPME) fiber (Carboxen® (CAR)/Polydimethylsiloxane (PDMS) df75µm partially crosslinked, Supelco, St. Louis, MI, USA). For each determination, 1 g of sample was stored in a 20 mL HS vial. The flask was heated at 70 °C for 10 min without shaking, followed by 15 min of fiber exposure in COMBI-PAL system for balancing the volume within the vial. The compounds adsorbed by the fiber were desorbed into the gas chromatograph injection system gas phase (CGMS TQ Series 8040 and 2010 Plus GC-MS Shimadzu, Tokyo, Japan) to 250 °C. The compounds were separated on a column 95% PDMS/5% PHENYL (30 m x 0.25 mm, 0.25 mm film thickness, Shimadzu, Tokyo, Japan). The GC was equipped with an HP 5972 mass selective detector (Hewlett Packard Enterprise, Palo Alto, CA, USA). Helium was used as carrier gas at a rate of 1.0 mL/min. Mass spectra were obtained by electron impact at 70 eV. The compounds were identified by comparison to the mass spectra from the library database (Nist'98 and Wiley7n).

2.2.5.2. Metal analysis

Metal analysis was performed of fermented and roasted coffee beans. The fermented, dried coffee samples were roasted in a semi-industrial roaster (Probatino, Leogap model, Brazil) at 140 °C for 30 min. For sample preparation, fermented and roasted coffee samples were transferred to a 250 mL volumetric flask and acidified with 5 mL of concentrated P.A. HNO_3 and H_2O_2 30%. Subsequently, the system was allowed to heating for 40 min at 80 ºC. The extract was filtered with a 0.45 µm pore size filter

and the volume was completed to 100 mL. Reference solutions were prepared using deionized water with resistivity of 18.2 MΩ/cm through a Milli-Q water purification system linked to a water distillatory Fisatom (Model 534, Brazil). Glassware used in this procedure was subjected to a decontamination treatment with HNO_3 10% (w/v) for 24 hours prior to use.

For metal content determination, an Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES, Varian, Model ES 720, Palo Alto, CA, USA) was used simultaneously with axial arrangement and solid-state detector. The torch was aligned horizontally and vertically with a Mn^{2+} standard solution concentration of 5.0 mg/L. The optical system of the ICP OES was calibrated with multi-element stock solution of scanned patterns. Spectral lines were selected considering the absence of interferences and appropriate sensitivity for determining elements in high and low concentrations. The operation conditions were as follows: power of 1.10 kW, plasma gas flow of 15 L/min, auxiliary gas flow of 1.5 L/min, nebulizer pressure of 180 kPa, triplicate time read of 3 s, stabilization time of 15 s, sample delay of 30 s, pump speed of 15 rpm and sample washing time of 3 s.

2.2.5.3. Fourier transform infrared (FTIR) spectroscopy

Functional groups in samples of grounded coffee beans (fermented and roasted) were determined by FTIR on a VERTEX 70 (Bruker, Billerica, MA, USA) containing a DRIFT accessory with 64 scans and a 4 cm^{-1} resolution at the 4000 to 400 cm^{-1} wave length region. The samples were crushed, pulverized and oven dried. Before determination, about 20 mg of the samples were mixed and homogenized with 100 mg of Potassium bromide (KBr), and the reads were recorded.

2.2.5.4. Thermal stability

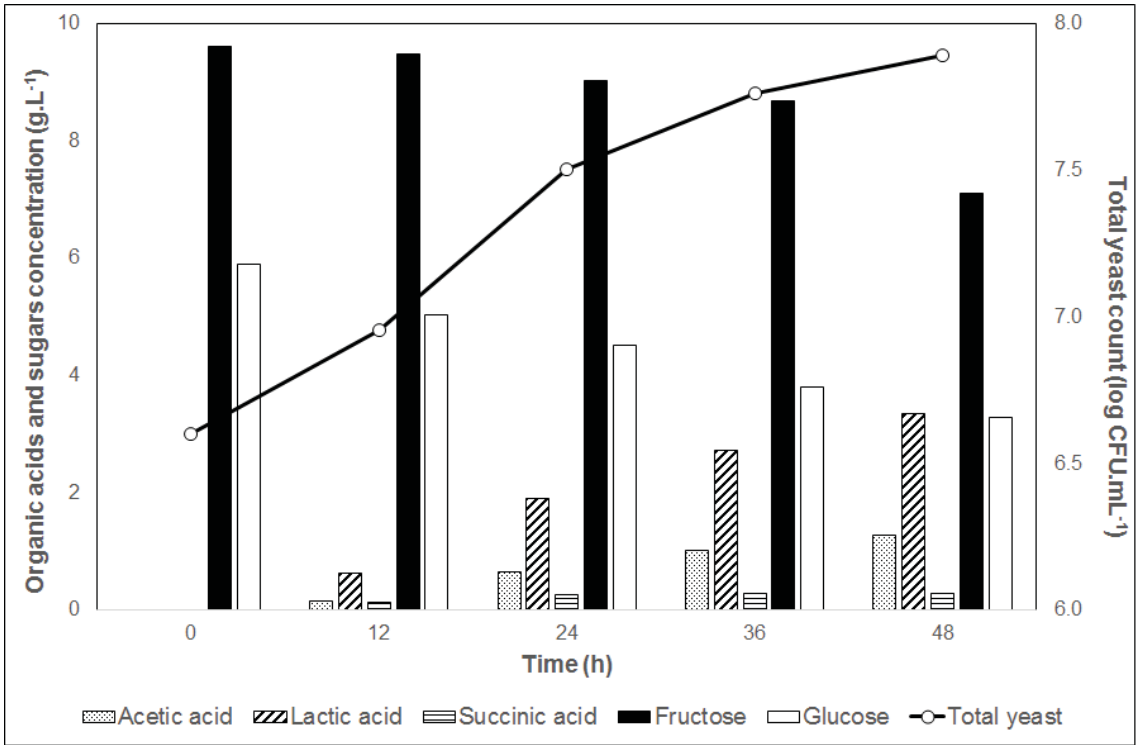
Thermal stability of fermented, dried coffee beans was evaluated by Thermogravimetry (TG) analysis. Analyzes were performed under an O_2(g) atmosphere at 20 °C/min rates to a maximum temperature of 800 °C in a Setsys Evolution TG/DTA/DSC (SETARAM, Hillsborough, NJ, USA) system.

2.3. RESULTS AND DISCUSSION

2.3.1. Microbiological and chemical characterization of coffee beans fermentation

Descriptions of the load of yeast in coffee fermentation have been provided by some studies (AVALLONE et al., 2001; MASOUD et al., 2004; SILVA et al., 2010; VELMOUROUGANE, 2013). This has been reported to range between 2 to 7 log CFU/mL, depending on the study. Factors affecting the initial yeast load include the quality and integrity of the coffee beans and the hygiene of fermentation tank, utensils and water used at the commencement of the fermentation process (PEREIRA et al., 2017). In this study, the average number of yeast, which was 6.60 log CFU/mL early in the fermentation process, increased to 7.89 CFU/mL by the end of 48 h of fermentation (FIGURE 2.2). This growth is favored by the ability of yeast cells to metabolize coffee pulp sugars as well to adapt and to cope with the hostile environment and stress conditions prevailing in coffee fermentation matrix (PEREIRA et al., 2014).

FIGURE 2.2 – TOTAL YEAST COUNT, ORGANIC ACIDS PRODUCTION AND PULP-SUGAR CONSUMPTION DURING THE ON-FARM WET PROCESSING



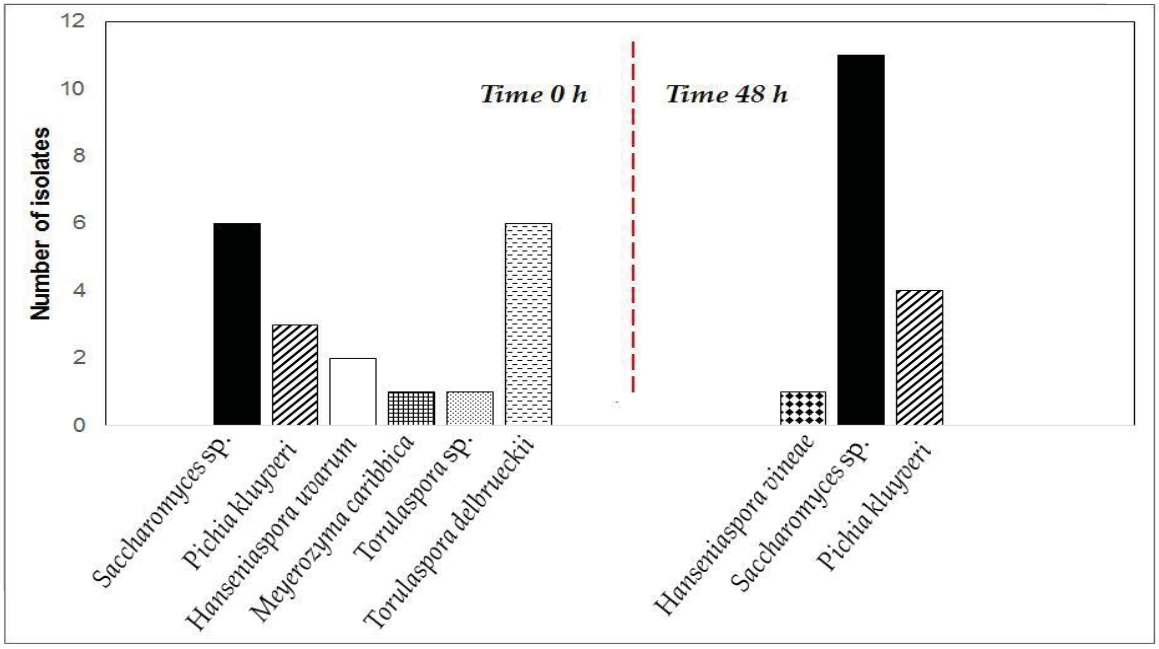
The yeast growth was accompanied by a regular consumption of the pulp sugars (glucose and fructose) and their conversion into organic acids. Lactic acid was the major

metabolite produced reaching a concentration of 3.28 g/L at 48 h, followed by acetic and succinic acids (1.27 and 0.30 g/L, respectively). The low production of acetic acid (<1.5 g/L) and the absence of butyric and propionic acids minimize the formation of off-flavors in the final beverage (LOPEZ et al., 1989; SILVA et al., 2013). On the other hand, lactic acid production can assist in the coffee-pulp acidification process without interfering in the product final quality (PEREIRA et al., 2017). Such organic acids production during coffee fermentation is mainly associated with lactic acid bacteria metabolism (PEREIRA et al., 2016). However, yeasts of the genera *Saccharomyces*, *Pichia* and *Hanseniaspora* may also have produced a fraction of the concentration of organic acids found in the coffee pulp beans mass in this study (BLOMBERG; ALDER, 1992; CIANI et al., 2006; SAUER et al., 2008).

2.3.2. Yeast identification

A total of 35 yeasts were isolated at the beginning and end of the fermentation process and identified by ITS-rRNA gene sequencing (FIGURE 2.3). The most frequently detected species were *Saccharomyces* sp. (17 isolates), *Torulaspora delbrueckii* (6 isolates) and *Pichia kluyveri* (7 isolates). However, *Saccharomyces* sp. was found to be dominant at the end of the fermentation process proving its easy adaptation to the coffee fermentation environment. In addition, the ability to metabolize pulp coffee pectin showed by some *Saccharomyces* species might be considered an advantage over other non-pectinolytic yeasts (PEREIRA et al., 2014).

FIGURE 2.3 – DISTRIBUTION AND FREQUENCY OF ISOLATES DURING ON-FARM COFEE WET PROCESSING



Two isolates of *Hanseniaspora uvarum* and one isolate of each *Meyerozyma caribbica, Torulaspora* sp. and *Hanseniaspora vineae* were identified at the beginning of the fermentation process. These yeast species have been previously found in coffee processing environments (MASOUD et al., 2004; PEREIRA et al., 2014; VILELA et al., 2010), except for *H. vineae* which was isolated for the first time. *H. vineae* is mainly associated with grapes and has been demonstrated to increase fruity aromas of wine by producing a high amount of acetate esters, such as 2-phenylethyl acetate and ethyl acetate (LLEIXÀ et al., 2016; MEDINA et al., 2013; VIANA et al., 2013). For coffee fermentation, these flavor-active esters could attribute distinct fruity sensory notes to the coffee bean through their diffusion during the fermentation process, enriching the flavor of the final beverage (PEREIRA et al., 2016). Thus, this yeast species should be included in research programs for the selection and development of functional starter cultures.

2.3.3. Volatile organic compounds determination of fermented coffee beans by gas chromatography coupled to mass spectrometry (GC-MS)

Yeast fermentation of pulp sugars produces a vast array of volatile metabolites that are well known for their aromatic and flavorant properties (PEREIRA et al., 2015;

SWIEGERS; PETRORIUS, 2005). These volatiles can diffuse into the coffee beans which may influence in its chemical composition (PEREIRA et al., 2015). In this study, a total of 25 volatile organic compounds were identified in the fermented coffee beans by SPME-GC-MS analysis, with a predominance of hydrocarbons (9 compounds) and higher alcohols (6 compounds) (TABLE 2.1). Although most of the compounds identified originate from the bean itself, some are known to be related to bacterial (i.e. nonanal, citric acid and heptanal) and yeast (i.e., hexane, heptane and tiophenes) metabolism (DAMIANI et al., 1996). Despite the diffusion mechanism has not yet been elucidated, it is often referenced in the literature that these volatile organic compounds diffuse into the beans (OWUSU; PETERSEN; HEIMDAL, 2012; PEREIRA et al., 2014, 2016). Further research to understand how these volatiles are conserved during roasting operation and reach the final product is required.

TABLE 2.1 – VOLATILE COMPOUNDS IDENTIFIED IN FERMENTED COFFEE SAMPLES BY GC-MS

Organic Functional Groups	**Volatile organic compounds**
Aldehydes (2)	Nonanal Heptanal
Alcohol (6)	1,3-Cyclohexanediol, 5-(1,1-dimethylethyl) 2-Propyl-1-pentanol 1-Octynol, 4-ethyl 1-Decanol, 2-ethyl-
Carboxylic acid (4)	Benzyloxy tridecanoic acid Dodecanoic acid, 3-hydroxy- Methacrylic acid Acetic acid
Ester (1)	Heptyl valerate
Hydrocarbons (9)	Heptane, 2,2,3,5-tetramethyl- Pentane, 2,2,3,4-tetramethyl- Heptane, 2,2,6,6-tetramethyl- Hexane, 2,2,5-trimethyl- Eicosane, 3-methyl- Dodecane, 2,6,11-trimethyl- Heptane, 5-ethyl-2,2,3-trimethyl- Hexadecane 9-Octadecene, 1,1-dimethoxy-
Sulfur Compounds (1)	3-Methyl-4-(phenylthio)-2-prop-2-enyl-2,5-dihydrothiophene, 1,1-dioxide
Ketone (1)	p-Benzoquinone
Pyrazine (1)	2-Isobutyl-3-methoxypyrazine

2.3.4. Physicochemical characterization of coffee beans

The mineral composition of fermented, dried and roasted samples is shown in Table 2.2. Potassium displayed the higher amount among the minerals analyzed in fermented and roasted coffee samples (12,453.10 and 13,117.50 mg/kg, respectively), followed by phosphorus (1,932.76 and 2,110.71 mg/kg, respectively), magnesium (1,554.31 and 1,772.65 mg/kg, respectively) and calcium (1,360.19 and 1,192.32 mg/kg, respectively). These results are in agreement with those found by Martín et al. (1998), except for the high aluminum content present in our samples. Although the *Coffea arabica* is not reported as an aluminum accumulator, the levels of such metal present in the analyzed samples may be indicative of a soil with a high availability of this metal to the plants (FRANKAVÁ et al., 2009).

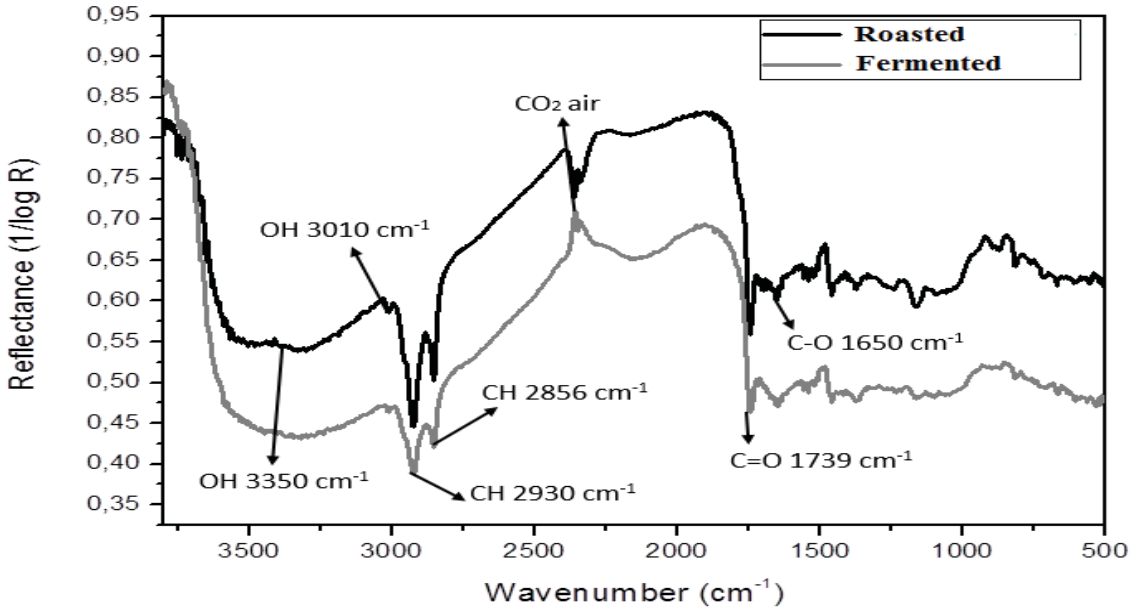
TABLE 2.2 – CONTENT OF METALS ON FERMENTED AND ROASTED WET PROCESSED COFFEE CHERRIES

Metals (mg/kg)	**Trait**	
	Fermented Coffee	**Roasted Coffee**
Al	234.71 ± 29.22[a]	362.56 ± 106.12[b]
Ba	3.40 ± 0.49[c]	2.61 ± 0.09[c]
B	7.28 ± 0.30[c]	6.26 ± 0.21[c]
Cd	ND[c]	ND[c]
Ca	1360.19 ± 20.53[d]	1192.32 ± 0.88[e]
Co	ND[c]	ND[c]
Cu	16.51 ± 0.38[c]	17.57 ± 0.25[c]
Fe	33.75 ± 1.32[c]	33.12 ± .44[c]
P	1932.76 ± 43.56[f]	2110.71 ± 28.40[g]
Li	ND[c]	ND[c]
Mg	1554.31 ± 17.47[h]	1772.65 ± 24.87[i]
Mn	16.32 ± 0.01[c]	17.43 ± 0.07[c]
Mo	ND[c]	ND[c]
Ni	ND[c]	ND[c]
K	12453.10 ± 8.35[j]	13117.50 ± 16.41[k]
Se	ND[c]	ND[c]
Na	350.565 ± 17.83[b]	380.99 ± 16.40[b]
V	0.86 ± 0.05[c]	0.47 ± 0.04[c]
Zn	10.15 ± 0.12[c]	9.78 ± 0.30[c]

*Means of triplicate in each row bearing the same letters are not significantly different ($p > 0.05$) from one another using Duncan's Test (mean ± standard variation). ND: not detectable.
Al = Aluminum; Ba = Barium; B = Boron; Cd = Cadmium; Ca = Calcium; Co = Cobalt; Cu = Copper; Fe = Iron; P = Phosphorus; Li = Lithium; Mg = Magnesium; Mn = Manganese; Mo = Molybdenum; Ni = Nickel; K = Potassium; Se = Selenium; Na = Sodium; V = Vanadium; Z = Zinc.

Over the last two decades, vibrational spectroscopy methods have proven to be a reliable and fast technique for the identification and quantification of several primary and secondary metabolites generated during fermented processes or to estimate the quality of the food itself (GIOVENZANA; BEGHI; GUIDETTI, 2014; KRÄHMER, 2015; LYMAN et al., 2003; PARADKAR; IRUDAYARAJ, 2002; SINIJA; MISHRA, 2009). In this study, FTIR spectroscopy analysis showed a quite similar spectrum for both fermented and roasted coffee beans (FIGURE 2.4). It was possible to verify the presence of the main organic functions associated with the coffee aroma, such as aromatic acids (1700-1680 cm^{-1}), ketones (1725-1705 cm^{-1}), aldehydes (1739-1724 cm^{-1}) and aliphatic esters (1755-1740 cm^{-1}). Those results corroborates with the wide variety of volatile organic compounds identified by the SPME-GC-MS technique (TABLE 2.1).

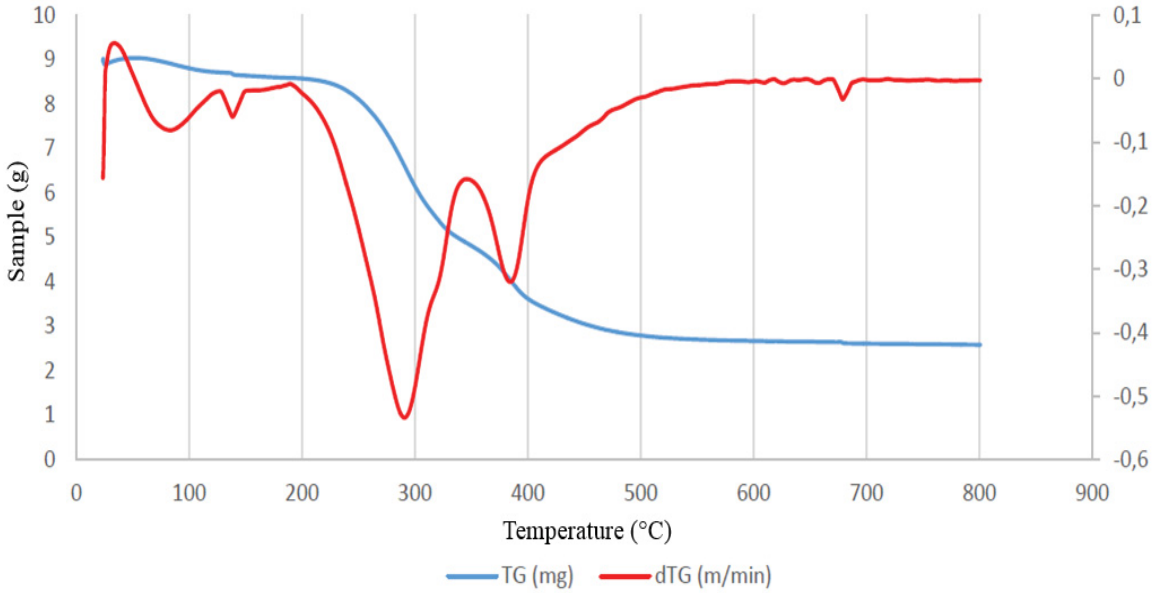
FIGURE 2.4 – FUNCTIONAL GROUPS PRESENT IN FERMENTED AND ROASTED SAMPLES OF COFFEE BEANS DETERMINED BY FOURIER TRANSFORM OF INFRARED (FTIR) SPECTROSCOPY ON RANGE OF 400 – 4000 WAVENUMBER. THE MAIN BANDS IDENTIFIED AND ORGANIC FUNCTIONS ASSOCIATED WERE: O-H (3350 AND 3010 CM^{-1}; ALCOHOLS); C-H (2930 AND 2856 CM^{-1}; ALKANES); C=O (1739 CM^{-1}; CARBOXYLIC ACIDS AND ESTERS); C-O (1650 CM^{-1}; ALCOHOLS OR PHENOLS).



Thermogravimetric analysis, especially thermogravimetry/differential thermogravimetry (TG/dTG), are already being utilized to measure the physical and chemical proprieties of coffee samples as a function of temperature or time. For the coffee samples analyzed in this study, the thermal decomposition occorred in four stages between 90 and 390 ºC (FIGURE 2.5). A significant mass loss (68%) can be

observed after the second stage at 190 ºC. At temperatures above 420 ° C only ashes remain in the final matter.

FIGURE 2.5 – THERMOSTABILITY OF FERMENTED COFFEE CHERRIES SUBMITTED TO AN OXYGEN ATMOSPHERE HEATED UP TO 800ºC. DTG: PROFILES OF MAIN VOLATILE PRODUCTS; TG: MASS LOSS RATE CURVE



The first two thermal degradation events that occur at the temperatures of 90 and 138 °C are associated with loss of free water and volatile compounds (i.e. alcohols, aldehydes and organic acids) and absorbed water, respectively. A small variation (9%) in the total weight loss within this temperature range corresponds to the water content in fermented beans (JAKAB; FAIX; TILL, 1997). A study performed by Yeretzian et al. (2002) monitored the emission of volatile components during the roasting step of the fermented beans which observed that during the endothermic phase the loss of water and volatile compounds that are not derived from Maillard's reaction or non-volatile precursors is prominent. The latter thermal degradation events representing a significant loss in mass (68%) of the fermented beans correspond to a depolymerisation of hemicelluloses or pectin and cellulose decomposition which occurs at 240-315 ºC and 370-400 ºC, respectively (OUAJAI; SHANKS, 2005).

2.4. CONCLUSION

The results of the present study indicated that *Saccharomyces* sp. is a dominant, well- adapted yeast found in coffee fermentation at Brazilian Cerrado Mineiro region. In

addition, this study is the first to report the presence of aroma-producing yeast *Hanseniaspora vineae* in coffee beans fermentation. Physicochemical analyses showed that different organic compounds present in coffee bean samples may be derived from microbial metabolism during the fermentation process. Future studies should focus on the dynamic of diffusion of these compounds into the beans, and to determine the actual role of the microbial fermentation for beverage quality. Our findings are relevant as a support for the development of usual starter cultures and controlled batch processes.

CHAPTER III (RESEARCH RESULTS) – HIGH THROUGHPUT rRNA GENE SEQUENCING REVEALS HIGH AND COMPLEX BACTERIAL DIVERSITY ASSOCIATED WITH BRAZILIAN COFFEE BEAN FERMENTATION

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ABSTRACT

Coffee bean fermentation is a spontaneous, on-farm process involving the action of different microbial groups, including bacteria and fungi. In this study, high-throughput sequencing approach was employed to study the diversity and dynamics of bacteria associated with Brazilian coffee bean fermentation. The total DNA from fermenting coffee samples was extracted at different time points, and the 16S rRNA gene with segments around the V4 variable region was sequenced by Illumina high-throughput platform. Using this approach, the presence of over eighty bacterial genera was determined, many of which have been detected for the first time during coffee bean fermentation, including *Fructobacillus, Pseudonocardia*, *Pedobacter*, *Sphingomonas* and *Hymenobacter*. The presence of *Fructobacillus* suggests an influence of these bacteria on fructose metabolism during coffee fermentation. Temporal analysis showed a strong dominance of lactic acid bacteria with over 97 % of read sequences at the end of fermentation, mainly represented by the *Leuconostoc* and *Lactococcus*. Metabolism of lactic acid bacteria was associated with the high formation of lactic acid during fermentation, as determined by HPLC analysis. The results reported in this study confirm the underestimation of bacterial diversity associated with coffee fermentation. New microbial groups reported in this study may be explored as functional starter cultures for on-farm coffee processing.

Keywords: Lactic acid bacteria, coffee fermentation, bacterial dynamics, *Fructobacillus* sp.

3.1. INTRODUCTION

Coffee is one of the most appreciated beverages in the world, with a consumption of more than 500 billion coffee cups per year. Surpassing a global

production of 9 million tonnes, the coffee now stands as the second largest commodity in market value, after only petroleum (LEE et al., 2015). With an annual output of 3.02 million tonnes, Brazil is the main producer and exporter of coffee beans, followed by Vietnam, Colombia, Indonesia, Ethiopia, India and Honduras (ICO et al., 2017).

Coffee beans, unlike other fermented foods, require fermentation to facilitate the drying process. After harvesting and pulping, the residual mucilaginous layer that surrounds the coffee beans can be eliminated through microbial fermentation. This involves the action of complex microbial interactions, led mainly by yeasts (*e.g. Pichia guilliermondii, P. anomala, Kluvyeromyces marxianus* and *Saccharomyces cerevisae*) and lactic acid bacteria (*e.g. Erwinia herbicola, Klebsiella pneumoniae* and *Lactobacillus brevis*) (AVALLONE et al., 2002; EVANGELISTA et al., 2014a; SILVA et al., 2013). These fermentation organisms utilize the bean pulp as a carbon and nitrogen source and produce significant amounts of ethanol, lactic acid and other microbial metabolites, resulting in lowered pH (from 5.5–6.0 to 3.5–4.0) (AVALLONE et al., 2001; PEREIRA et al., 2014). In addition, some of these microbial metabolites, which are precursors of volatile compounds formed during roasting, help in improving beverage flavour (MUSSATO et al., 2011; PEREIRA et al., 2014).

Culture-independent techniques have helped to change the way to study food microbial ecology, leading to consideration of microbial populations as consortia (COCOLIN; ERCOLINI, 2015). The advent of the use of molecular techniques and, more specifically, the use of high-throughput sequencing (HTS), permitted to overcome the limitations of the cultivation-associated methods, allowing a breakthrough in understanding the diversity and composition of several food microbial ecosystems (DOYLE et al., 2017; GAROFALO et al., 2017; POŁKA et al., 2015; YANG et al., 2016). Illumina MiSeq® (Illumina Inc, San Diego, CA, USA) generates shorter reads (250 bp) than other HTS systems but gives a higher throughput, providing thousands of high-quality reads of the generated amplicons and allowing a superior taxonomical analysis (VASILEIADIS et al., 2012).

In this work, we report a diversity analysis aiming to characterize bacterial communities associated with coffee bean fermentation, using high-throughput sequencing, as part of a whole metagenome study of the microbiota associated with the Brazilian coffee processing chain.

3.2. MATERIAL AND METHODS

3.2.1. On-farm coffee fermentation and sampling

Spontaneous fermentations were performed at the Fazenda Apucarana located in the Cerrado Mineiro region (18°55'59.4" S, 46°50'41.5" W) at Minas Gerais, Brazil. Freshly harvested coffee (*Coffea arabica* var. Catuaí) cherries were depulped using a BDSV-04 depulper (Pinhalense, São Paulo, Brazil) obtaining beans with a surrounding layer of mucilage (PEREIRA et al., 2015). Fermentations were conducted for 24 h in cement tanks with a nominal volume of 4.5 m³, containing 20 kg of depulped beans and approx. 500 L of fresh water, in accordance with the local wet processing method. At the end of the process, fermented beans were sun-dried for 20 days until 11-12% moisture, as measured by a moisture meter (model AL-102 ECO; Agrologic, São Leopoldo, Brazil). Environmental temperature during the experimental procedure was 24-32 ºC (day) and 12-15 ºC (night). Samples (fermenting coffee pulp bean mass) were collected at random at 0, 12 and 24 h for HTS and target metabolic analysis.

3.2.2. Total DNA extraction

For extraction of total DNA from the samples, 1 mL of coffee pulp bean mass was centrifuged at 12 000×*g* for 1 min (centrifuge model 5430; Eppendorf, Hamburg, Germany). Cell pellet was resuspended in 500 µL of Tris-Ethylenediamine Tetraacetic Acid (EDTA), homogenized with 10 µL of lysozyme solution (20 mg/mL; Sigma-Aldrich, Arklow, Ireland) and incubated at 30 °C for 60 min. Then, 50 µL of Sodium dodecyl sulfate (SDS, 10 %; by mass per volume) and 10 µL of proteinase K solution at 20 mg/mL (Sigma-Aldrich) were added to the lysis solution, followed by homogenization and incubation at 60 ºC for 60 min. A volume of 150 µL of phenol/chloroform (25:24; Sigma-Aldrich) were added, homogenized by inversion and centrifuged at 12 000×*g* (model 5430R; Eppendorf) for 5 min. Supernatant was removed and the DNA was precipitated with 3× (by volume) absolute ethanol (Sigma-Aldrich). Pellets was washed with 80% ethanol, dried and resuspended in Mili-Q® ultrapure water (Merck, Kenilworth, NJ, USA). Total DNA was quantified with the Nanodrop 2000 instrument (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

3.2.3. Illumina high-throughput sequencing

A fragment of the 16S rRNA gene was amplified from the total DNA extracted using primers for the V4 region (bases 515 to 806), containing complementary adaptors for Illumina platform (CAPORASO et al., 2012) using KlenTAQ polymerase (Sigma-Aldrich). Amplification was performed using the degenerated primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), where M is A/C, H is A/C/T, V is A/C/G and W is A/T (CAPORASO et al., 2010). Bar-coded amplicons were generated by PCR under the following conditions: 95 ºC for 3 min, followed by 18 cycles at 95 ºC for 30 s, annealing at 50 ºC for 30 s, extension at 68 ºC for 60 s, final extension at 68 ºC for 10 min. Samples were sequenced in the MiSeq (Illumina Inc) platform using 500 V2 kit (Illumina Inc), following standard Illumina protocols.

3.2.4. Bioinformatics and data analysis

Data generated by sequencing went through a rigorous quality system that involved: (*i*) identification and removal of sequences containing more than one ambiguous base (N), and (*ii*) evaluation of the presence and complementarity of primer and barcode sequences. Chimeric sequence detection, removal of noises from pre-cluster and taxonomic attribution were also performed using standard parameters of QIIME (Quantitative Insights Into Microbial Ecology) software package, v. 1.9.0 (CAPORASO et al., 2010). Applying the UCLUST method (EDGARD, 2010), sequences presenting identity above 97% were considered the same operational taxonomic units (OTUs) according to the SILVA database (QUAST et al., 2013).

3.2.5. High-performance liquid chromatography

The concentration of reducing sugars (glucose and fructose), organic acids (acetic, succinic, lactic and propionic acids) and ethanol was determined during coffee bean fermentation by high-performance liquid chromatography (HPLC). Samples were centrifuged at 6000×*g* (centrifuge model CT-6000; Cientec, Porto Alegre, Brazil) and filtered through 0.22-µm pore size filter (Sartorius Stedim, Goettingen, Germany) in order to remove debris. Analysis parameters were performed according to de Carvalho

Neto et al. (2017). Filtered samples were injected into HPLC system equipped with an Aminex HPX 87 H column (300 mm × 7.8 mm; Bio-Rad, Richmond, CA, USA) and a refractive index (RI) detector (model HPG1362A; Hewlett-Packard Company, São Paulo, Brazil). The column was eluted in isocratic mode with a mobile phase of 5 mM H_2SO_4 at 60 °C and a flow rate of 0.6 mL/min.

3.3. RESULTS AND DISCUSSION

3.3.1. Sugar consumption and metabolite formation

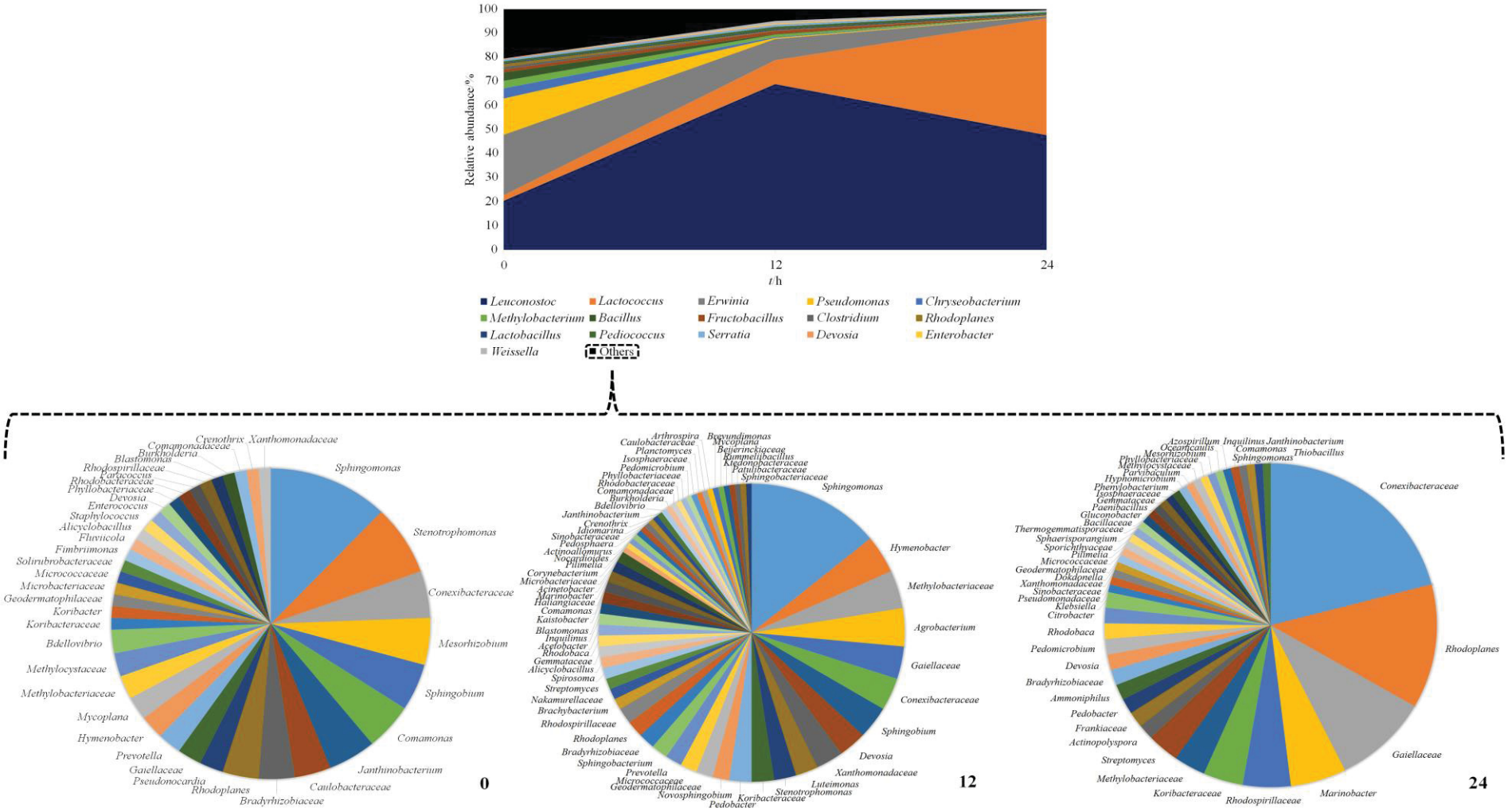
TABLE 3.1 shows the evolution of sugar consumption, metabolite formation and pH decrease during fermentation of coffee pulp bean. The observed increase in the concentration of reducing sugars (glucose and fructose) at 12 h of fermentation can be attributed to the hydrolysis of sucrose by the action of yeast invertase (MAGALHÃES et al., 2010). These sugars were partially consumed after 24 h of fermentation, with a final residual content of 3.2 and 4.5 g/L of glucose and fructose, respectively. Lactic acid (0.32 g/L) was the most important organic compound formed during fermentation, followed by succinic and acetic acids (0.08 and 0.05 g/L, respectively). Lactic acid is an important organic compound for coffee bean fermentation that assists in the coffee acidification process without interfering with the final product quality (PEREIRA et al., 2015). The accentuated production of lactic acid is in agreement with the strong dominance of lactic acid bacteria found in the present study (FIGURE 3.1), resulting in pH decrease from 5.3 to 4.0 at the end of fermentation (TABLE 3.1). The reduction of pH below 4.5 is a widely used method by coffee producers to determine the end of fermentation of coffee bean during wet processing (PEREIRA et al., 2016).

TABLE 3.1 – CONCENTRATION OF SUGARS, ORGANIC ACIDS AND ETHANOL DURING COFFEE BEAN FERMENTATION

γ/(g/L)	t(fermentation)/h		
	0	12	24
Glucose	2.7±0.3[a]	5.5±0.3[b]	3.3±0.1[a]
Fructose	3.4±0.3[a]	7.33±0.09[b]	4.5±0.2[c]
Succinic acid	n.d.	n.d.	0.08±0.01
Lactic acid	n.d.	n.d.	0.32±0.01
Acetic acid	n.d.	n.d.	0.051±0.004
Propionic acid	n.d.	n.d.	n.d.
Ethanol	n.d.	n.d.	n.d.
pH	5.30±0.03[a]	4.90±0.05[a]	4.00±0.10[b]

Mean values of triplicate measurements in each row with the same letter are not significantly different ($p>0.05$) from one another using Duncan's test (mean value±standard variation)
n.d.=not detected.

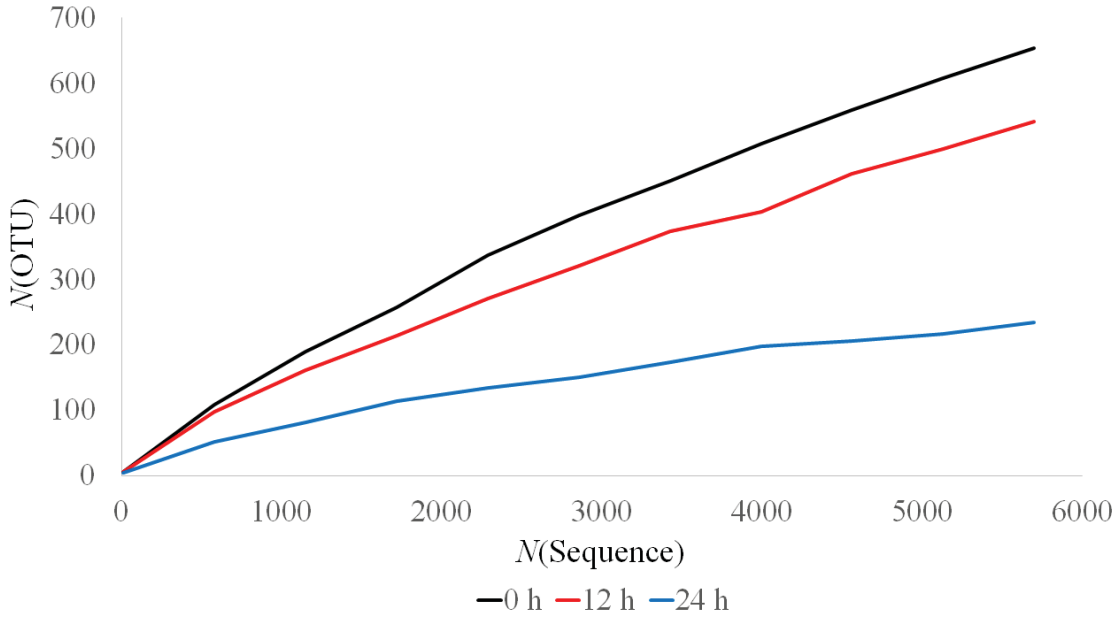
FIGURE 3.1 – BACTERIAL COMPOSITION AND DYNAMICS DURING COFFEE BEAN FERMENTATION AS SHOWN BY HIGH-THROUGHPUT rRNA GENE SEQUENCING



3.3.2. Characteristics of sample sequencing data

A total of 440 524 high-quality sequences of the hypervariable V3 region of the 16S rRNA gene region were obtained after trimming on the Illumina MiSeq sequencing, with an average length of 250 bp. A great coverage was obtained in all samples as demonstrated by the rarefaction curves (FIGURE 3.2).

FIGURE 3.2 – RAREFACTION ANALYSIS OF THE GENERA FOUND AT 0, 12 AND 24 H OF COFFEE BEAN FERMENTATION. OTU = OPERATIONAL TAXONOMIC UNIT



3.3.3. Bacterial diversity and dynamics

Studies evaluating the microbiology of coffee fermentation have been performed over the last 100 years in several coffee-producing regions, evidencing the dominant species during the post-harvest processing (AGATE; BHAT, 1966; AVALLONE et al., 2001; FRANK; LUM; DELACRUZ, 1965; MASOUD et al., 2004; PEDERSON; BREED, 1946; PEREIRA et al., 2014; SILVA et al., 2000, 2008). On average, nine bacterial genera had been reported in previous studies using culture-dependent methods (EVANGELISTA et al., 2015; FENG et al., 2016; HAMDOUCHE et al., 2016; NASANIT; SATAYAWUT, 2015; PEREIRA et al., 2014, 2015). Our work demonstrates that these findings are underestimate, since over eighty genera of bacteria have been identified by HTS. High frequency and abundance of readings corresponding to *Proteobacteria* (*e.g. Erwinia*, *Pseudomonas* and *Methylobacterium*) and *Firmicutes* (*e.g. Bacillus*, *Fructobacillus*, *Leuconostoc* and *Lactococcus*) were observed. The

possible habitat origins of these microbial groups are: human contact, *e.g. Pseudomonas* sp., *Enterobacter*, *Erwinia* and *Actinobacteria* (GRICE et al., 2008), soil or aerial parts of coffee plants, *e.g. Mesorhizobium*, *Methylobacterium*, *Stentrophomonas*, *Sphingobium* and *Sphingomonas* (CARREL; FRANK, 2014; MAI et al., 2013; VEGA et al., 2005), the water source used for wet processing, *e.g. Planctomyces*, *Luteimonas, Devosia* and *Brevundimonas* (MARTINY et al., 2005), and the air surrounding the fermentation tank, *e.g. Janthinobacterium*, *Pedobacter*, *Burkholderia* and *Kaistobacter* (FAHLGREN et al., 2010). These findings indicate the need for a program of research to understand the microbial ecology origin of coffee cherries and processing sites.

The rich and complex bacterial diversity revealed in this study demonstrates the potential of coffee *terroir* as a source of microorganism species with biotechnological application. An example is the first report of the presence of *Fructobacillus* in coffee fermentation. This LAB group has a unique biochemical metabolism when compared to other LAB, having preference consumption for fructose and the necessity of an electron acceptor when in presence of glucose (ENDO; DICKS, 2014a). *Fructobacillus* microorganisms were found in gastrointestinal tracts of insects feeding on fructose-rich diet and presented symbiotic interactions with its hosts (JANASHIA et al., 2016; JANASHIA; ALUX, 2016). A survey of previous studies demonstrates significant amount of residual pulp fructose at the end of coffee fermentations conducted under field conditions (CARVALHO NETO et al., 2017), even by using selected starter cultures (EVANGELISTA et al., 2014a, 2014b; PEREIRA et al., 2015). With these findings, the isolation and further implementation of *Fructobacillus* may assist in the fructose metabolism, contributing to drying of coffee beans.

Bacterial composition and dynamics shown in FIGURE 3.1 reveal that, despite the presence of a high bacterial diversity associated with coffee fermentation environment, several microorganisms are suppressed by the growth and dominance of LAB group. Reads assigned to LAB genera, including *Lactobacillus, Pediococcus, Enterococcus, Leuconostoc, Lactococcus* and *Fructobacillus,* corresponded to 26.32% at the start of the process and reached a total of 97.59% of the total operational taxonomic unity (OTU) at 24 h. The high availability of fermentable sugars coupled with the low presence of dissolved oxygen creates a propitious environment for the rapid growth and colonization of these species, which promote an efficient conversion of sugars into mainly lactic acid (ENDO; DICKS, 2014b).

Within the LAB group, *Leuconostoc* and *Lactococcus* shared dominance. Species of *Leuconostoc,* such as *L. mesenteroides*, *L. pseudomesenteroides* and *L. citreum*, have already been reported as dominant LAB in coffee fermentations performed in Mexico, Colombia, India and Taiwan (AVALLONE et al., 2001; LEONG et al., 2014; VELMOUROUGANE, 2013), while *Lactococcus* species dominates coffee fermentations performed in Taiwan and Brazil (LEONG et al., 2014; VILELA et al., 2010). Co-dominance of LAB enables the production of a wide range of organic compounds (*e.g.* acetate, acetaldehyde, ethanol, short-chain fatty acids) by heterofermentation (*e.g. Leuconostoc* sp.) and a high production of lactic acid through the homofermentation (*e.g. Lactococcus* sp.), which promotes yeast growth and reduces the prevalence of spoilage microorganisms.

3.5. CONCLUSION

The present study suggests that most of bacterial species involved in the coffee bean fermentation have not been determined. High-throughput 16S rRNA gene sequencing analysis allowed us to reveal in deepth the presence of several microbial groups with potential applications. A strong dominance of LAB was confirmed, proving the good adaptation of this microbial group to coffee fermentation environment. Further studies should focus on the isolation of some microbial groups first reported in this study for potential biotechnological applications.

CHAPTER IV (RESEARCH RESULTS) – EFFICIENT COFFEE BEANS MUCILAGE LAYER REMOVAL USING LACTIC ACID FERMENTATION IN A STIRRED-TANK BIOREACTOR: KINETIC, METABOLIC AND SENSORIAL STUDIES

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ABSTRACT

Post-harvest coffee processing involves a microbial process to remove the mucilage layer adhering to the fruits, prior to storage and transport of the coffee beans. In this study, coffee mucilage removal was done using lactic acid fermentation in a stirred-tank bioreactor (STR). Fermentation assays were done with or without the addition of starter culture (i.e., *Lactobacillus plantarum* LPBR01), and kinetic parameters, including microbial growth, sugar pulp consumption and metabolite production, were studied. High lactic acid bacteria (reaching 10.7 log CFU/mL at 10 h) were obtained in the STR process with the starter culture, which led to a high lactic acid productivity (0.366 g/L.h) and a pH decrease to below 4.0 during the initial 10 h. A temporal analysis using Illumina high-throughput 16S rRNA Gene Sequencing (HTS) showed the *Lactobacillus* genera's dominance in the inoculation process, as it reached over 88% of read sequences at the end of fermentation, while the Leuconostocaceae family was the dominant bacterial group in the spontaneous treatment. The STR fermentation process led to the production of coffee beans with richer aroma composition and beverages with a notable increase in the sensorial analysis of the coffee beverages compared to those resulting from the conventional process. This new fermentation model can be used to do controlled bean fermentation to supply the coffee industry with homogeneous and high-quality coffee beans.

Keywords: Lactic acid bacteria; coffee fermentation; coffee processing; stirred-tank reactor; *Lactobacillus plantarum.*

4.1. INTRODUCTION

Fermentation is one of the oldest food-processing technologies. Among the classical fermentation processes are winemaking, which has been documented from as early as 6000 B.C. in the Caucasus area, cheese making in northern Europe and soya sauce production in Japan and China (NIELSEN, 2002; SALQUE et al., 2013). It was, however, with penicillin production during World War II that large-scale fermentations were first introduced. Today a variety of fermented foods are produced using this technology in large commercial enterprises.

Coffee is one of the few globally produced food commodities where the fermentation process occurs spontaneously. The primary objective of coffee fermentation is to remove the mucilage layer adhering to the fruits during post-harvest processing, assisting in the drying process. The microbial species responsible for this process (i.e., yeast and lactic acid bacteria) originate from coffee processing sites, including fermentation tank, water, soil, air or the fruit itself (AVALLONE et al., 2001; MASOUD et al., 2004; VILELA et al., 2010; PEREIRA et al., 2014; VELMOUGRANE, 2013; LUDLOW et al., 2016; CARVALHO NETO et al., 2017, 2018). This uncontrolled, on-farm process results in the lack of predictability of final coffee beans quality because several microbial metabolites can diffuse into the beans and act as aroma precursors to the roasting process (MASOUD et al., 2005; PEREIRA et al., 2015; SILVA et al., 2013). Therefore, the use of selected yeasts has been suggested to have reproducible and predictable coffee beans by controlling the fermentation (EVANGELISTA et al., 2014a; EVANGELISTA et al., 2014b; PEREIRA et al., 2015; PEREIRA et al., 2016; LEE et al., 2017).

Recently, the removal of coffee mucilage using lactic acid fermentation was proposed by introducing a selected lactic acid bacteria, *Lactobacillus plantarum* LPBR01, into the field situation (PEREIRA et al., 2016). This bacterium strain was able to promote an accelerated coffee-pulp acidification process reducing the time required for mucilage removal. One of the limitations of introducing starter cultures into field systems is that coffee fermentations are done in open cement tanks that facilitate contamination by natural microbiota (PEREIRA et al., 2017). This means the added starter culture has to compete with a high load of indigenous microorganisms, decreasing its metabolic activity and effectiveness. The demand for hygienic production practices has increased the appeal of using stainless steel tanks in industrial

bioprocesses (e.g., the production of yogurt, beer, wine and cider) (STEINKRAUS, 2004). The use of bioreactors can provide a suitable environment for the development of controlled coffee beans fermentation.

The aim of this chapter was to study the kinetic parameters of coffee beans fermentation using a previously selected lactic acid bacteria (LAB) starter culture (i.e., *L. plantarum* LPBR01) and a stirred-tank bioreactor (STR) model. Additionally, the effects of this new process on the chemical and sensory quality of hot coffee were evaluated. The fermentation system used is part of a patented process (SOCCOL et al., 2016).

4.2. MATERIAL AND METHODS

4.2.1. STR fermentation

Coffee cherries of *Coffea arabica* var. Catuaí were manually harvested (2017 crop) at the mature stage from a farm 1270 m above sea level situated in Patrocínio in the Minas Gerais State, Brazil. The fruits were packed in plastic bags and transported at 4 ºC to the Bioprocess Engineering and Biotechnology Laboratory, Federal University of Paraná, Curitiba, Brazil. Coffee fruits (2 kg) were manually depulped 1 day after harvesting and immediately deposited into a 10.5 L New Brunswick™ BioFlo® 110 fermenter (Eppendorf, Hamburg, Germany), containing 2 L of sterilized water (pH 6.5) and equipped with pitched blade impellers. Two batch fermentations were done in triplicate: (i) spontaneous (non-inoculated control) and (ii) inoculated (lactic acid starter culture-added). The LAB strain used in this study, *L. plantarum* LPBR01, was previously selected as detailed in Pereira et al. (2016). The inoculum solution was added in the bioreactor with an initial concentration of 10^8 CFU/mL. For both fermentation processes, temperature (30 °C), agitation (200 rpm) and aeration (1 L/min) were controlled during the initial 12 h (aerobic phase). Then, aeration and agitation were interrupted and an anaerobic environment was formed by injecting $CO_{2(g)}$ into the fermenting coffee-pulp bean mass, allowing an anaerobic fermentation during the final 12 h (anaerobic phase). At the end of fermentation, coffee beans were dried in an air recirculation drying oven at 35 °C until a 12% a_w was reached. Relative humidity was measured at 6 h intervals using a portable grain moisture meter (Agrologic, São Leopoldo, RS, Brazil).

Samples (10 mL) of the fermenting coffee-pulp bean mass (liquid fraction) were randomly collected at intervals of 2 h to do microbiological and metabolite target analysis. At each sampling point, the pH was measured using a digital pH meter (Requipal, Curitiba, Brazil).

4.2.2. Microbial counts

Samples (100 µL) of the liquid fraction were homogenized in 900 µL of 0.1% saline-peptone water (10^{-1} solution) using a Vortex Mixer (Kasvi, Curitiba, Brazil) and diluted serially. Enumeration of total yeasts and LAB was done using the spread plate technique using Rose Bengal chloramphenicol agar (Oxoid, São Paulo, Brazil) containing 0.01% (w/v) chloramphenicol and MRS agar (Merck, Whitehouse Station, NJ, USA) containing 0.1% (w/v) nystatin, respectively. Plates were incubated at 30 ºC for 48 h and the number of colony-forming units (CFU) was quantified.

4.2.3. High performance liquid chromatography (HPLC)

Sugar consumption (glucose and fructose) and organic acids (citric, succinic, lactic, acetic and propionic acids) and ethanol formation were monitored from the liquid fraction of the fermenting coffee pulp–bean mass at 2 h intervals using the method of Pereira et al. (2016). Initially the liquid fraction (2 mL) was centrifuged at 6000 g for 5 min at 4 ºC using an Eppendorf centrifuge (SP Labor, SP, Brazil), and filtered using a 0.22-µm pore size filter (Millipore Corp., Billerica, MA, USA). Then, the samples were injected (50 µL) into a HPLC system (Aglient Technologies 1260 Infinity Series; Aglient Technologies, Santa Clara, CA, USA) equipped with an Aminex HPX 87 H column (300 x 7.8 mm; Bio-Rad, Richmond, CA, USA) and a refractive index (RI) detector, using H_2SO_4 (5 mM) at 60 ºC as the mobile phase to elute the column at a flow rate of 0.6 mL/min.

4.2.4. Kinetic parameters

The kinetic parameters determined in this study were maximum specific growth rate (μ_m) for yeast and LAB, and product formation rate (q) and specific product

formation rate (q_p) for organic acids and ethanol (LETTI et al., 2012). All parameters were calculated at 10 h of fermentation (time with maximum biomass accumulation), except μ_m values, which were calculated at intervals of 4-6 h in the inoculated treatment and 8-10 h in the spontaneous process. The following equations were used for the determination of kinetic parameters:

$$\mu = \frac{1}{X} \times \frac{dX}{dt} \quad (1)$$

$$\mu_{máx} = \frac{1}{X} \times \frac{dX}{dt} \quad (2)$$

$$q = \frac{dP}{dt} \quad (3)$$

$$q_p = \frac{1}{X} \times \frac{dP}{dt} \quad (4)$$

Where X and P are the final concentration of biomass and products (g/L), respectively, generated in the coffee-pulp bean mass during fermentation time (in h).

4.2.5. Illumina high-throughput 16S rRNA gene sequencing

Fermenting coffee-pulp bean mass samples were taken at 0, 12 and 24 h to access the total bacteria community composition and dynamics using Illumina high-throughput sequencing. Purified total DNA was obtained using the phenol-chloroform extraction method of Carvalho Neto et al. (2018). Total DNA was separated on a 0.8% (w/v) agarose gel at 40 V during 1 h using a horizontal electrophoresis apparatus (Loccus Biotecnologia, Cotia, Brazil). DNA fragments were stained with SYBR Green I (Life Technologies, Carlsbad, CA, USA) and visualized using 300 nm excitation and Polaroid DS-34 camera (Polaroid, Cambridge, MA, USA). The total DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Twenty ng of DNA was used as a template for the amplification of the V4 region of the 16S rRNA gene, using the primers 515F and 806R (CAPORASO et al., 2012) and KlenTaq Master Mix (Sigma-Aldrich, Saint Louis, MO, USA). The PCR products were quantified using the Qubit dsDNA HS kit (Invitrogen, Carlsbad, CA, USA) and sequenced using the 500V2 Sequencing Kit (Illumina, San Diego, CA, USA) on an Illumina MiSeq (Illumina). After sequencing, chimeric sequences detection, removal of noises from pre-cluster and taxonomic attribution were done using standard parameters with the QIIME software package, version 1.9.0

(http://qiime.org/). A similarity above 97% between the sequences was used as a parameter to group them as the same operational taxonomic unit (OTU) using the SILVA database (https://www.arb-silva.de/aligner/) (QUAST et al., 2013). The nucleotide sequences were deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank) with access numbers MG729835 to MG730031.

4.2.6. Fermented coffee beans quality assessment

4.2.6.1. Chemical analysis

Sugar and organic acids present in dried, fermented coffee beans from spontaneous and inoculated samples, as well as in green coffee beans (i.e., unfermented beans), were extracted using the method of Pereira et al. (2015a). For the samples preparations, 5 g of the dried, fermented coffee beans were ground using a domestic coffee grinder (Philco, Philadelphia, PA, USA) and mixed with 20 mL of ultrapure water (Merck Millipore, Burlington, MA, USA) using a Vortex Mixer. The concentration of the sugar and organic acids were obtained using high-performance liquid chromatograph (HPLC) as previously described in section 4.2.3. The volatile composition of samples was obtained using gas chromatography coupled to mass spectrometry (GC-MS) using the method of Carvalho Neto et al. (2017) with slight modifications. A carboxen/polydimethylsiloxilane (5%/95%) SPME fiber (Supelco, Saint Louis, MO, USA) was used to absorb the volatile compounds present in the coffee beans (fermented and unfermented samples). Samples (2.0 ± 0.1 g of grounded beans) were heated at 70 ºC for 10 min without agitation. The SPME fiber was put in the autosampler for 15 min. The compounds were thermally desorbed at 260 ºC and directly introduced into the gas chromatograph injection system. The analysis was done using a Shimadzu® - *GCMS2010 Plus* coupled to a mass spectrometer with a triple quadrupole *TQ8040*, equipped with an *AO 5000* autosampler (Shimadzu, Tokyo, Japan). The GC temperature program was as follows: the column oven temperature was maintained at 60 ºC for 10 min, followed by two heating ramps of 4 and 10 ºC/min until reaching the temperatures of 100 and 200 ºC, respectively. Mass spectra were obtained using electron impact at 70 eV and a start and end mass-to-charge ratio (m/z) of 30 and 200, respectively. The compounds were identified in full scan mode by comparison to the

mass spectra from library databases (Nist'98 (http://www.nist.gov) and Wiley7N (http://www.palisade.com)).

4.2.6.2. Coffee cup quality analysis

Fermented, dried coffee beans were roasted in a semi-industrial roaster (Leogap model, Probatino, Curitiba, Brazil) with nominal capacity of 1.3 kg. Roasted coffee was ground to 360-420 μm using a Coffee Grinder M-50 (Probat Leogap, Curitiba, Brazil). The samples for tasting were prepared using 105 g of roasted and ground coffee beans in 1,500 mL of filtered water (pH 6.5) (Aquasana, Austin, TX, USA) using a VP17-3 BLK coffee brewer (Bunn Corp., Springfield, IL, USA) using a bleached paper filter (Melitta orginal 1 × 4, Minden, Germany).

The beverages were evaluated by a panel of 4 expert coffee tasters with a Q-Grader Coffee Certificate (PEREIRA et al., 2018). A beverage prepared with coffee beans obtained by traditional processing from the same coffee farm shown in section 4.2.1 was included as a control. Cups were prepared and the attributes of acidity, aroma, balance, body, clean cup, finish, flavor, overall quality, uniformity and sweetness of the beverages were evaluated using the method of the Specialty Coffee Association of America Cupping Protocols (see 'http://www.scaa.org/?page=resourcesandd=cupping-protocols'). This protocol involves the determination of scores, on a scale from 6 to 10 at 0.25 point intervals, for each of the attributes. The samples were served in 240 mL snifters of 8 cm diameter to allow dispersion of volatile compounds to increase olfactory perception. Assessments started when the beverage temperature reached 65 °C for the olfactory step and 43 °C for the gustatory step. After the gustatory step, sensory descriptive terms were assigned by the coffee tasters for each beverage.

4.2.7. Statistical analysis

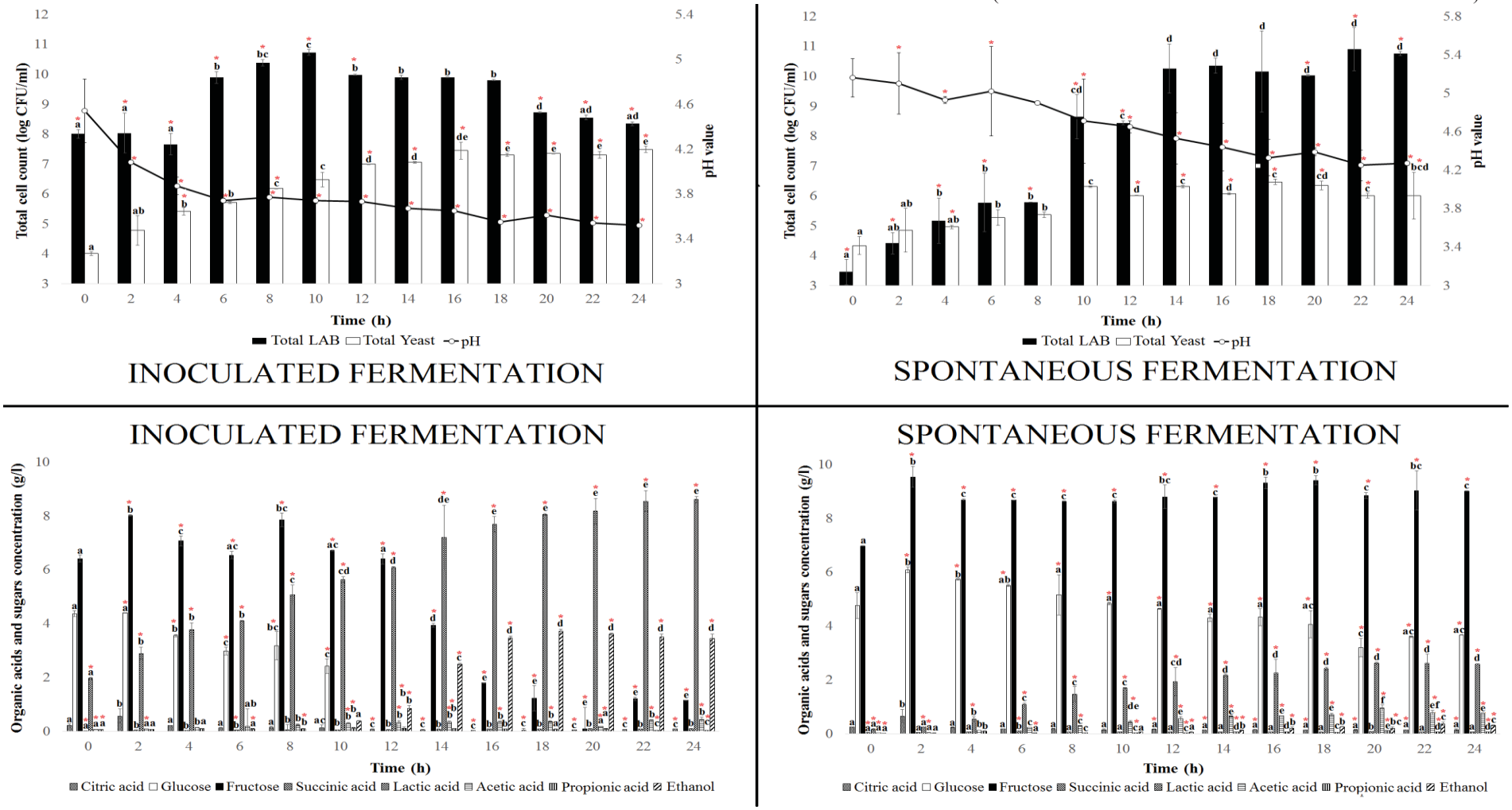
The data obtained using target metabolite analysis and sensory evaluation was analyzed using post-hoc comparison of means using Duncan's test. Statistical analyses were done using the SAS program, version 7.0 (Statistical Analysis System, Cary, NC, USA). Level of significance was established using a two-sided p-value <0.05.

4.3. RESULTS

4.3.1. Controlled bioreactor fermentations

The pH monitoring, microbial analysis (total yeast and LAB growth), sugar consumption (glucose and fructose) and organic acid production (citric, succinic, lactic, acetic and propionic acids) from inoculated and spontaneous STR processes are disposed in FIGURE 4.1. LAB counts remained high throughout the inoculated fermentation processes and peaked at 10 h. Conversely, initial LAB counts were low in the spontaneous process and peaked only after 22 h. In both processes, yeasts were present during the whole fermentation with maximum population sizes of 7.48 log CFU/mL at 24 h and 6.46 log CFU/mL at 10 h for inoculated and spontaneous processes, respectively.

FIGURE 4.1 – MICROBIAL GROWTH, PULP SUGAR CONSUMPTION AND METABOLITE FORMATION IN THE COURSE OF INOCULATED AND SPONTANEOUS COFFEE BEANS FERMENTATION IN A STIRRED-TANK BIOREACTOR. THE SIGNIFICANCE OF THE RESULTS WAS ASSESSED USING ANOVA WITH DUNCAN'S POST-HOC TEST AT $P<0.05$. DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES WITHIN THE SAME PROCESS OVER FERMENTATION TIME. ASTERISK INDICATE SIGNIFICANT DIFFERENCES BETWEEN TREATMENTS (SPONTANEOUS *VERSUS* INOCULATED PROCESS)



In the starter culture-added fermentation, efficient sugar consumption was observed through total glucose consumption in 12 h of fermentation (FIGURE 4.1). On the other hand, a considerable residual amount of both glucose and fructose was observed at the end of the spontaneous process. The level of lactic acid, the most abundant product of LAB metabolism, was higher in the inoculated treatment compared to the spontaneous, inducing a more pronounced mucilage acidification process (FIGURE 4.1).

The fermentative kinetic parameters of inoculated and spontaneous assays are shown in TABLE 4.1. In general, both processes showed similar LAB specific growth rates (0.05 and 0.06 h^{-1} in the spontaneous and inoculated assays, respectively). However, the inoculated treatment showed a higher biomass accumulation and lactic acid productivity than the spontaneous process (TABLE 4.1). The maximum specific LAB growth rate was achieved around 4-6 h in the inoculated treatment and 8-10 h in the spontaneous process (TABLE 4.1).

TABLE 4.1 – KINETIC PARAMETERS OF SPONTANEOUS AND INOCULATED COFFEE FERMENTATION PROCESSES DONE IN A STIRRED-TANK BIOREACTOR

Fermentation variable	**Kinetic Parameter**	**Fermentation assay***	
		Inoculated	Spontaneous
LAB	μ_m (h^{-1})	0.10	0.13
	Biomass accumulation (g)	3.84	3.09
Yeasts	μ_m (h^{-1})	0.05	0.07
	Biomass accumulation (g)	2.72	2.65
Lactic acid	q(g/L.h)	0.37	0.14
	q_p (h^{-1})	0.10	0.05
Acetic acid	q(g/L.h)	0.02	0.04
	q_p (h^{-1})	ND	ND
Ethanol	q (g/L.h)	0.04	0.01
	q_p (h^{-1})	0.01	0.002

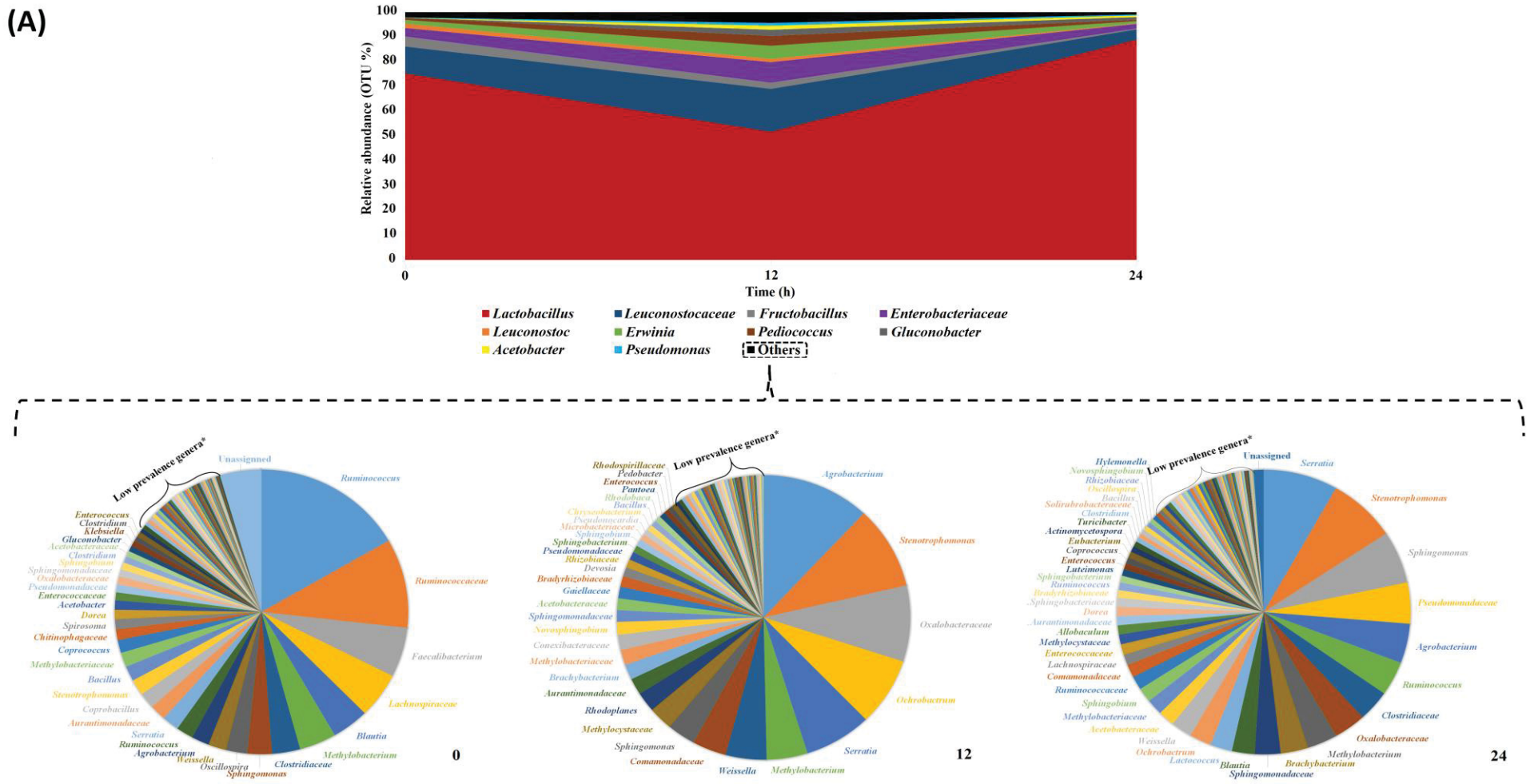
ND. = not detected. μ_m = maximum specific growth rate; q = product formation rate; q_p = specific product formation rate.
*All parameters were determined at 10 h of fermentation process, except maximum specific growth (μm) values, which were calculated at intervals of 4 to 6 h in the inoculated treatment and 8-10 h in the spontaneous process.

4.3.2. Bacterial population composition and dynamics

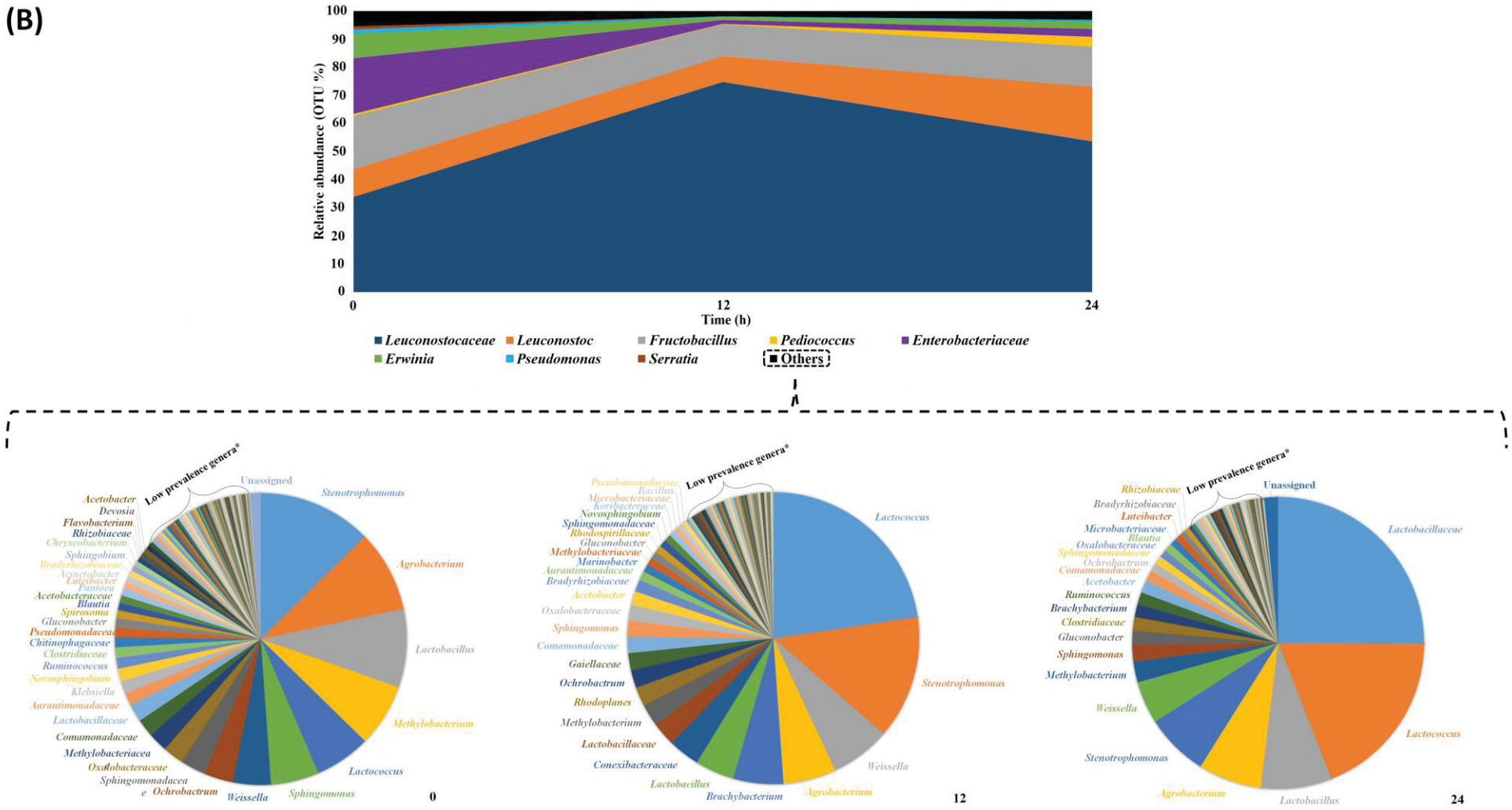
The number of high quality bacterial 16S rRNA gene sequences obtained by high-throughput sequencing was 369,101, resulting in more than 240 OTU at 97% sequence similarity. The rarefaction curves for all the samples did not reach a plateau at this sequencing depth (FIGURE A4.1), suggesting that major bacterial communities were largely covered.

The HTS analysis showed that LAB was the dominant group in both fermentation processes (FIGURE 4.2A and B). In the starter culture-added assay, reads assigned to the *Lactobacillus* genus reached from 75% at the start of the process to 88.8% of the total OTU at 24 h. Other LAB groups, including *Fructobacillus, Leuconostoc, Pediococcus* and Leuconostocaceae, as well as acetic acid bacteria belonging to *Acetobacter* and *Gluconobacter*, were also found but in very minor proportions. On the other hand, the Leuconostocaceae family dominated the STR spontaneous process, reaching 74.8% of the reads at the end of fermentation. Along with the Leuconostocaceae family, *Fructobacillus, Leuconostoc, Erwinia, Pseudomonas, Pediococcus, Serratia* and Enterobacteriaceae were found in significantly relative abundance.

FIGURE 4.2 – BACTERIAL COMMUNITY COMPOSITION AND DINAMYCS SHOWED BY HIGH-THROUGHPUT rRNA GENE SEQUENCNIG IN INOCULATED (A) AND SPONTANEOUS (B) COFFEE BEANS FERMENTATION DONE IN A STIRRED-TANK BIOREACTOR. *GENERA AND FAMILIES IDENTFIED WITH RELATIVE ABUNDANCE <0.5% AND GROUPED IN OTHERS ARE SHOWN IN ANEXES



(B)


Relative abundance (OTU %)
100
90
80
70
60
50
40
30
20
10
0
0
12
24
Time (h)
Leuconostocaceae
Leuconostoc
Fructobacillus
Pediococcus
Enterobacteriaceae
Erwinia
Pseudomonas
Serratia
Others
Low prevalence genera*
Unassigned
Stenotrophomonas
Agrobacterium
Lactobacillus
Methylobacterium
Lactococcus
Sphingomonas
Weissella
Ochrobactrum
0
Lactococcus
Stenotrophomonas
Weissella
Agrobacterium
Brachybacterium
Lactobacillus
12
Lactobacillaceae
Lactococcus
Stenotrophomonas
Agrobacterium
Lactobacillus
24

The HTS also showed the presence of over 100 bacterial genera in both inoculated and spontaneous processes, many of which detected for the first time in coffee beans fermentation, including *Agrobacterium, Aeromicobium*, *Pediococcus*, *Citrobacter*, *Methylobacterium* and *Fructobacillus*. Microorganisms that were characterized as "Others" in the HTS analysis (FIGURE 4.2) and whose prevalence was <0.5% are described in the supplementary data (TABLE A4.1).

4.3.3. Coffee bean and beverage quality assessment

The chemical composition of green beans (unfermented coffee sample) and fermented samples from inoculated and spontaneous treatments was determined using HPLC and GC-MS (TABLE 4.2). No differences ($p \geq 0.05$) in the concentration of sugars (glucose and fructose) and citric and succinic acids were observed in beans from any treatment. However, the lactic acid concentration was approximately twice as high in the inoculated process compared to spontaneous treatment (TABLE 4.2).

TABLE 4.2 – CONCENTRATION OF VOLATILE COMPOUNDS (AREA*10^6), ORGANIC ACIDS AND REDUCING SUGAR (mg/g OF COFFEE BEANS) IN COFFEE BEANS BEFORE (UNFERMENTED COFFEE BEANS) AND AFTER THE INOCULATED AND SPONTANEOUS STR FERMENTATION PROCESSES

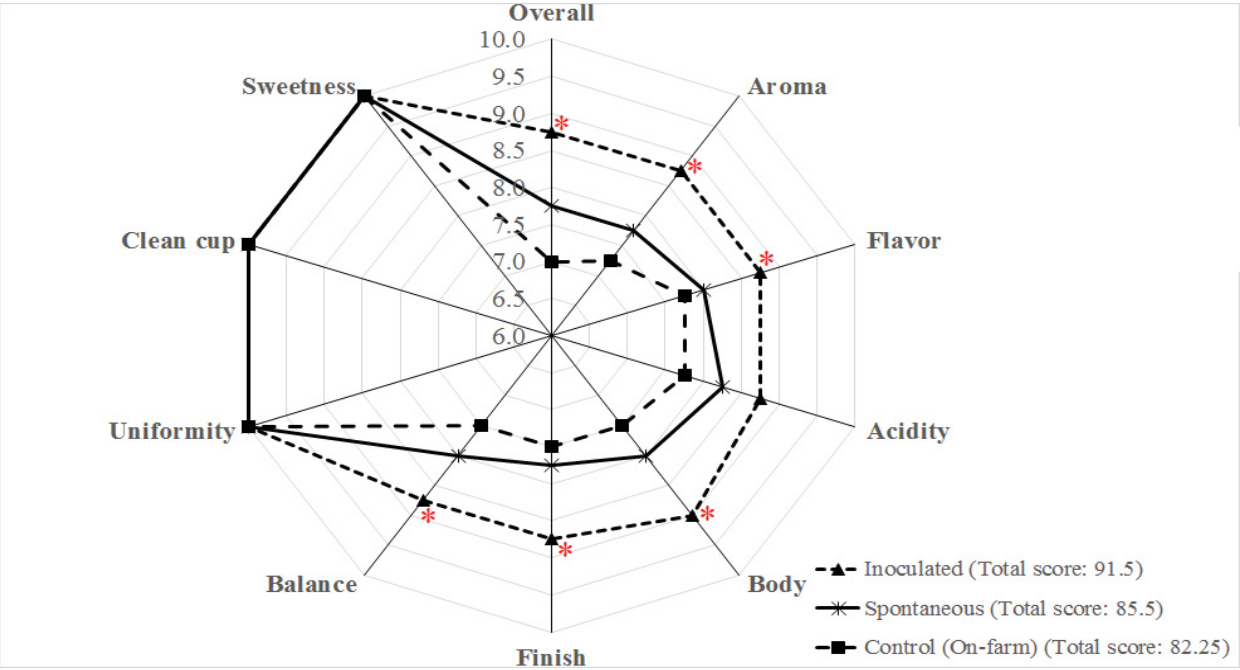
Compounds	**Aroma and taste description**	**Dried coffee beans**		
		Green beans	Inoculated assay (LAB)	Spontaneous assay
GC				
Hydrocarbons (6)				
Ethylbenzene	-	1.8 ± 0.5[a]	ND	1.5 ± 0.2[a]
Styrene	Sweet, foral; Almond	15 ± 3[a]	16 ± 4[a]	15 ± 6[a]
o-Xylene	Geranium	ND	ND	0.73 ± 0.21
n-Dodecane	-	ND	ND	1.1 ± 0.3
n-Heneicoisane	-	1.1 ± 0.3[a]	1.4 ± 0.1[a]	1.4 ± 0.5[a]
D-Limonene	Sweet, orange, citrus	4.5 ± 1.1[a]	14 ± 5[b]	9.5 ± 0.9[ab]
Aldehydes (9)				
Methional	Creamy tomato, potato skin, French fry	1.1 ± 0.1[a]	1.0 ± 0.1[a]	1.1 ± 0.1[a]
Benzaldehyde	Almond, fruity, powdery, nutty	6.3 ± 1.3[a]	10 ± 6[a]	13 ± 2[a]
Phenylacetaldehyde	Honey, chocolate and cocoa with a spicy nuance	21 ± 3[a]	38 ± 5[b]	18 ± 1[a]
2-Octenal	Fresh cucumber, banana, green, citrus	0.36 ± 0.03	ND	ND
Nonanal	Green lemon peel like nuance, citrus, melon rindy	2.4 ± 0.1[a]	4.7 ± 1.7[ab]	4.7 ± 0.3[b]
Decanal	Sweet, fatty, citrus and orange peel	1.9 ± 0.3[a]	2.3 ± 0.1[a]	4.3 ± 0.2[b]
Octanal	Aldehydic, green with a peely citrus orange note	ND	0.83 ± 0.27[a]	1.1 ± 0.1[a]
2-Phenyl-2-butenal	Green, vegetative, floral, cocoa and nutty	0.34 ± 0.04[a]	0.43 ± 0.07[a]	ND
Tetradecanal	Fatty, dairy, coconut	ND	0.40 ± 0.09	ND
Ketones (3)				
Butyrolactone	Milky, creamy with fruity peach-like afternotes	1.3 ± 0.1[a]	1.6 ± 0.7[a]	1.3 ± 0.6[a]
6-methyl-5-Hepten-2-one	Fruity, apple, musty	1.1 ± 0.1[a]	1.4 ± 0.6[a]	1.7 ± 0.3[a]
6,10,14-trimethyl-2-Pentadecanone	-	ND	ND	0.61 ± 0.24
Alcohols (5)				
1-Octen-3-ol	Earthy, green, oily, umami sensation	1.0 ± 0.2[a]	2.1 ± 1.0[a]	2.4 ± 0.4[a]
Benzyl alcohol	Sweet, fruity with balsamic nuances	8.3 ± 1.5[a]	4.9 ± 0.5[b]	3.6 ± 0.7[b]
Phenethyl alcohol	Floral, sweet and bready	8.9 ± 2.1[a]	12 ± 2[a]	7.2 ± 1.0[a]
1-Hexanol	Green, fruity, apple-skin and oily	ND	0.74 ± 0.05	ND
5-methyl-2-Hexanol	-	4.0 ± 1.2[a]	1.2 ± 0.7[a]	ND

Esters (6)				
Methyl salicylate	Sweet and root beer with aromatic nuances	4.5 ± 0.5[a]	3.1 ± 1.7[a]	2.1 ± 0.1[a]
Ethyl 3-hydroxybutyrate	Green, fruity and winey	ND	ND	0.60 ± 0.03
1-methoxy-2-Propyl acetate	-	ND	3.5 ± 1.6[a]	1.7 ± 0.2[b]
Phenylacetic acid ethyl ester	Floral honey, rosy, chocolate and cocoa notes	ND	ND	1.0 ± 0.1
2-Phenethyl acetate	Sweet, honey, floral, rosy with a slight fruity body	ND	5.1 ± 3.7	ND
Isoamyl acetate	Sweet, banana, fruity with a ripe estry nuance	ND	0.66 ± 0.61	ND
Terpene (1)				
Linalool	Citrus, orange, lemon and floral	3.4 ± 0.8[ab]	6.0 ± 1.4[a]	3.1 ± 0.9[b]
Furans (2)				
Furan, 2-pentyl	Fruity, green, cooked caramellic nuances	2.0 ± 0.2[a]	2.6 ± 1.2[a]	ND
Pyrazine (1)				
2-methoxy-3-(2-methylpropyl)-Pyrazine	Pepper, green pea and galbanum	2.4 ± 0.6[a]	2.1 ± 0.1[a]	1.8 ± 0.7[a]
Carboxylic acids (2)				
Oxalic acid, derivate	-	1.1 ± 0.1[a]	ND	1.6 ± 1.0[a]
2-methyl-Butanoic acid	Fruity, dirty, acidic with a dairy buttery	ND	4.4 ± 1.4	ND
HPLC				
Glucose		32 ± 8[a]	27 ± 3[a]	35 ± 3[a]
Fructose		26 ± 6[a]	23 ± 3[a]	25 ± 5[a]
Citric acid		14 ± 5[a]	10 ± 2[a]	16 ± 3[a]
Succinic acid		0.12 ± 0.09[a]	0.52 ± 0.28[a]	0.68 ± 0.13[b]
Lactic acid		0.25 ± 0.12[a]	4.6 ± 0.6[b]	1.8 ± 0.2[c]
Acetic acid		ND	ND	ND
Propionic acid		ND	ND	ND
Ethanol		ND	ND	ND

A wide variety of volatile compounds was identified in the dried beans, including aldehydes (9 compounds), esters (6 compounds) and hydrocarbons (6 compounds). Phenylacetaldehyde, styrene, phenethyl alcohol and D-limonene were the most important aromatic compounds quantified in the dried bean samples. The concentrations of phenylacetaldehyde and 1-methoxy-2-propyl acetate had a significant increase ($p<0.05$) in dried beans from inoculated treatment compared to the spontaneous process. In addition, the use of the starter culture promoted the formation of aroma compounds (e.g., 2-phenethyl acetate, 1-hexanol, 2-phenyl-2-butenal, tetradecanal, isoamyl acetate and 2-methyl-butanoic acid) that were not detected in the spontaneously fermented beans (TABLE 4.2).

Beverages, which were produced with roasted coffee beans from inoculated and spontaneous treatments, received different scores for several important sensory attributes (FIGURE 4.3). Aroma, flavor, acidity, body, and balance reached higher scores in the inoculated treatment compared to the spontaneous, while sweetness, clean cup, and uniformity were statistically similar for both treatments. Nonetheless, both beverages were scored over 80 points (91.5 and 85.5 for inoculated and spontaneous processes, respectively), being superior to those found from coffee beverage produced using a natural, on-farm processing.

FIGURE 4.3 – SENSORY EVALUATION OF BEVERAGES PRODUCED WITH FERMENTED, ROASTED COFFEE BEANS FROM SPONTANEOUS AND INOCULATED STR COFFEE FERMENTATIONS AND FROM A NATURAL, ON-FARM PROCESSING (CONTROL). COMPARISONS WERE MADE BY A PANEL OF FOUR EXPERIENCED COFFEE TASTERS. *ASTERISK* REPRESENT SIGNIFICANTLY DIFFERENCE IN A TWO-SIDED P-VALUE <0.05



According to the sensory descriptive terms selected by the coffee tasters, the coffee beverage produced with the inoculated treatment produced a cup with intense lactic perceptions (terms mentioned by the coffee tasters: “Aroma with lactic background”; “Taste with intense lactic character”; “Excellent combination between lactic and citric acidity”; “Perception of velvet-like body”; “Elegant finalization with medium-to-long persistence and lactic touch”) as well as a caramel-like taste and intense perception of ‘citric’ and “fruity” notes (data not shown). Terms relating to lactic perception were also mentioned from the beverages prepared with beans of spontaneous treatment, (*viz.,* “Perception of velvet-like body” and “Taste with slight lactic character”), in addition to the terms “Aroma with herbaceous notes, such as lemon grass and fennel” and “Caramel-like taste perception”. On the other hand, control (on-farm processed coffee beans) showed a beverage with basic sensory profile, with caramel-like taste and full bodied perception.

4.4. DISCUSSION

Over the last decade, disposable equipment has become an integral part of several biologic manufacturing processes, promoting greater consistency, predictability, and product value (STEINKRAUS, 2004). Coffee beans fermentation is still done as a natural process that brings inconsistent quality and depreciated product value (SCHWAN, 1998; SCHWAN et al., 2014). The present study is the first to set up coffee beans fermentation in a STR. Conditions of sanitization, aeration and temperature were controlled to achieve an improved process. The fermentation was done with the inoculation of a recently selected LAB strain, *L. plantarum* LPBR01, which has shown an ability to result in faster and improved on-farm coffee processing (PEREIRA et al., 2016).

It is known that coffee mucilage removal occurs three ways: (i) mucilage sugar consumption; (ii) pectinolytic enzymes production; and (iii) mucilage acidification process (MASOUD et al., 2004; MASOUD; JESPERSEN, 2006; PEREIRA et al., 2014). A survey of previous studies showed that significant residual sugars can be observed in the coffee mucilage after on-farm fermentation (CARVALHO NETO et al., 2017), even by using selected starter cultures (EVANGELISTA et al., 2014a; EVANGELISTA et al., 2014b; PEREIRA et al., 2015a). In this study, a high residual sugar content was also observed at the end of the spontaneous STR process, suggesting

that the initial microbial load found in coffee fruit is not sufficient for efficient mucilage layer degradation. On the other hand, the STR system combined with the use of the selected starter culture (i.e., *L. plantarum* LPBR01) efficiently promoted the consumption of fermentable sugars and, consequently, a faster acidification process (lower than 4.0 after 10 h of fermentation process) through lactic acid production. In previous studies pH levels lower than 4.0, an ideal parameter for signaling the end of the coffee fermentation process, were only reached after 24 h (JACKELS; JACKELS, 2005; VELMOUROUGANE, 2013). Therefore, taking into account these crucial aspects (i.e., pulp sugar consumption and pH level lower than 4.0), the use of the STR system combined with a selected LAB starter culture shows potential to reduce the time required for coffee fermentation from 24 to 10 h.

Consistent with the results of other coffee fermentation biodiversity studies, HTS showed that STR fermentation process supports a complex association of bacteria, mainly from the LAB group. However, the addition of starter culture changed drastically the dominant group, as showed by high sequence reads of the *Lactobacillus* genus in the inoculated process, while the Leuconostocaceae family dominated in the spontaneous treatment. The Leuconostocaceae family includes *Fructobacillus, Leuconostoc, Oenococcus* and *Weissella* genera, which may have shared this dominance. The microbial members of this family are obligatory heterofermentative and associated with the production of a vast array of compounds, including those that promote "*off-flavor*" in the final coffee beverage, such as volatile sulfur compounds (COMI; IACUMIN, 2012; PRIPIS-NICOLAU et al., 2004). This fact highlights the importance of adding starter culture to coffee fermentation to ensure process homogeneity and generation of desirable molecules.

The coffee industry has traditionally dedicated efforts in improving the final beverage quality using roasting and brewing steps (CAPRIOLI et al., 2015; LEE et al., 2015). However, recent studies have shown a significant increase in the quality of coffee beans that were subjected to fermentative processes using starter cultures (EVANGELISTA et al., 2014a; EVANGELISTA et al., 2014b; PEREIRA et al., 2015a; PEREIRA et al., 2016). In the present study, the STR coffee fermentation done with added starter culture enabled the production of coffee beans with richer aroma composition and beverages with increased quality compared to the conventional process. The stimulation of LAB growth and, consequently production of lactic acid and volatile organic compounds (i.e., 1-hexanol, nonanal, 2-phenethyl acetate, 2-methyl-

butanoic acid) positively influenced the final quality of the beverage. Previous studies showed that these compounds can be directly linked to the LAB metabolism (DAMIANI et al., 1996; MAICAS et al., 1999; MONTANARI et al., 2018). Although no experimental evidence has been given yet, it is often shown in the literature that the diffusion of such metabolites may occur and they modulate the chemical and sensorial profile of the coffee beans (PEREIRA et al., 2017).

4.5. CONCLUSION

In summary, the STR fermentation model used in this study (consecutive aerobic and anaerobic phases) was shown to be a positive environment for starter culture growth, removal of coffee-pulp sugar and formation of flavor-associated molecules. This new fermentation system showed a potential to reduce the time required for coffee fermentation from 24 to 10 h, taking into account important aspects such as pH and pulp-reducing sugars content. The kinetic parameters established can provide a basis for optimization and scaling-up of the proposed process. Finally, this new fermentation model can be used to supply the coffee industry with homogeneous and high-quality coffee beans.

CHAPTER V (RESEARCH RESULTS) – MICROBIOLOGICAL, PHYSICOCHEMICAL AND SENSORY STUDIES OF COFFEE BEANS FERMENTATION IN A YEAST BIOREACTOR MODEL

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ABSTRACT

Coffee fermentation refers to the on-farm, microbial process of removal of the mucilage layer adhered to the fruits, necessary for storage and transport of the coffee beans. This process is traditionally conducted spontaneously, thus leading to end-products of variable quality. The aim of this study was to evaluate the microbiological, physicochemical and sensory aspects of coffee beans fermentation conducted in a controlled yeast bioreactor model. Fermentations were conducted with or without the addition of a selected yeast starter culture (*viz., Pichia fermentans* YC5.2), and kinetic parameters, including microbial growth, pulp sugar consumption and metabolite formation, were studied. This fermentation system enabled an efficient yeast starter culture growth, which led to high ethanol (0.136 g/L.h) and ethyl acetate (0.383 μmol/L.h) formation rates. In the course of STR fermentation, an exponential lactic acid bacteria growth and, consequently, organic acid production and pH decreasing were reported. This bacteria population was mainly represented by *Pediococcus* sp. and Leuconostocaceae family, as reveled by Illumina-based metagenomic sequencing. The STR system combined with the use of the selected yeast starter culture also enabled the production of coffee beans with rich aroma composition and beverages with remarkable increase in quality compared to the conventional process. With further refinements, the STR model may be useful in designing novel bioreactors for the optimization of coffee fermentation with starter cultures.

Keywords: Bioreactor, coffee fermentation; coffee beverage; *Pichia fermentans*; starter culture

5.1. INTRODUCTION

After harvesting and pulping, the residual mucilaginous layer that surrounds the coffee beans is eliminated by microbial fermentation. This involves the action of complex microbial interactions, led mainly by yeasts (*e.g., Pichia guilliermondii, P. anomala, Kluyeromyces marxianus* and *Saccharomyces cerevisae*) and lactic acid bacteria (*e.g., Leuconostoc mesenteroides*, *Lactococcus lactis* and *Lactobacillus brevis*) (AVALLONE et al., 2002; EVANGELISTA et al., 2014a; SILVA et al., 2013). These fermenting organisms utilize the bean mucilage as a source of carbon and nitrogen to produce significant amounts of ethanol, lactic acid, and other microbial metabolites, resulting in lowered pH (from 5.5–6.0 to 3.5–4.0) (AVALLONE et al., 2001; PEREIRA et al., 2014). This process generates a range of microbial-derived volatile metabolites, which can diffuse into the seeds and have an impact on the final coffee quality (EVANGELISTA et al., 2014b; PEREIRA et al., 2015; SILVA et al., 2013). In this respect, yeasts have a pivotal influence through the generation of different aroma-influencing molecules (*e.g.,* esters, higher alcohols, aldehydes, ketone, and terpenoids) via central carbon metabolism (DZIALO et al., 2017; HIRST; RICHTER, 2016; PIRES et al., 2014).

Coffee is one of the few remaining beverages produced on a global scale where the fermentation occurs in a spontaneous way. Common problems involve levels of acidity of over-fermented coffee beans or incomplete mucilage removal by insufficient fermentation which hinders the drying process and encourages the growth of spoilage bacteria and fungi (PEREIRA et al., 2017). Thus, one of the current challenges for the coffee processing chain is to control the fermentation process in terms of both kinetics and quality of the resulting product. While experimental applications of defined yeast starter cultures have produced satisfactory results (EVANGELISTA et al., 2014a, 2014b; PEREIRA et al., 2015, 2016), this technique has not been implemented in the field. One limitation to the introduction of starter cultures is that coffee fermentations are conducted in cement tanks that facilitate contamination by natural microbiota (PEREIRA et al., 2017).

The demand for hygienic production practices has increased the appeal of using stainless steel tanks in industrial bioprocesses (*e.g.,* the production of yogurt, beer, wine, and cider) (STEINKRAUS, 2004). The use of designed bioreactors can provide a suitable environment for the development of controlled coffee bean fermentation. The

aim of this study was to determine the kinetic parameters of coffee bean fermentation conducted with a selected yeast starter culture (*viz., Pichia fermentans* YC5.2) in a stirred-tank bioreactor (STR) model, and to evaluate the effects of this new process on the chemical and sensory quality of coffee beverage.

5.2. MATERIAL AND METHODS

5.2.1. Controlled coffee beans fermentation in STR bioreactor

Coffee cherries of *Coffea arabica* var. Catuaí were obtained from a farm 1,270 m above sea level situated in Patrocínio in the Minas Gerais State, Brazil. The fruits were transported to the laboratory and manually depulped to obtain beans with mucilage. A total of 2 kg of manually depulped beans were deposited in a 10.5 l New Brunswick™ BioFlo® 110 fermenter (Eppendorf, Hamburg, Germany) equipped with pitched blade impellers, previously sterilized, with 2 L of sterile water (pH 6.5) (CARVALHO NETO et al., 2018). Two batch fermentations were conducted in triplicate: (i) spontaneous (non-inoculated control) and (ii) inoculated (yeast starter culture-added). The selected yeast starter culture used in this study, *Pichia fermentans* YC5.2, was previously isolated for its high ethyl- and isoamyl acetate production, as detailed in Pereira et al. (2014). The inoculum solution was added into the bioreactor reaching an initial concentration of 6 log CFU/mL. For both fermentation processes, conditions of temperature (30 °C), agitation (200 rpm) and aeration (1 L/min) were maintained controlled during the initial 12 h. After this time, aeration and agitation were interrupted and an anaerobic environment was created by injecting $CO_{2(g)}$ into the fermenting coffee-pulp bean mass, allowing a static fermentation during the final 12 h. At the end of fermentation, coffee beans were washed with water to remove the degraded mucilaginous layer and avoid secondary fermentations during the drying process. Then, coffee beans were dried in an air recirculation drying oven at 35 °C until the value of 12% of moisture was reached.

5.2.2. Sampling and pH measurement

Samples (5 mL) of the liquid fraction of the fermenting coffee-pulp bean mass were collected in triplicate at intervals of 2 h to perform microbial counts and

metabolite target analysis. At each sampling point, the pH was measured using a digital pH meter (Requipal, Curitiba, Brazil).

5.2.3. Microbial counts

Aliquots of 100 µL of the liquid fraction samples were homogenized with 900 µL of 0.1% saline-peptone water (10^{-1} solution) and diluted serially. Total yeasts and lactic acid bacteria (LAB) were enumerated according to Pereira et al. (2016), where 100 µL of the diluted solutions were inoculated on the surface of Rose Bengal Chloramphenicol agar (RBCA, Oxoid, São Paulo, Brazil) containing 0.01% (w/v) chloramphenicol and MRS agar containing 0.1% (w/v) natamycin, respectively. Plates were incubated at 35 ºC for 48 h and the number of cell-forming units (CFU) were recorded.

5.2.4. Verification of inoculum dominance

To verify inoculum dominance, colonies of *P. fermentans* YC5.2 were distinguished from the indigenous yeasts through colony morphology (i.e., cream color with furrowed appearance, membranous texture, oval shape, and absence of filaments) (CAPUTO et al., 2012). To confirm the identification of *P. fermentans*, representative yeast isolates were submitted to a *P. fermentans*-specific PCR primer protocol (PEREIRA et al., 2014) that contained 12.5 µL of Mix GoTaq® Green Master 1X (Promega, São Paulo, Brazil) and 0.3 µM of each *P. fermentans*-specific primer (PFF2 — 5′GAAGGAAACGACGCTCAGAC3′ and PFR2 — 5′ATCTCTTGGTTCTCGCATCG3′). A 136-bp amplification product pointed to the identification of *P. fermentans*.

5.2.5. High performance liquid chromatography (HPLC)

The concentration of reducing sugars (glucose and fructose), organic acids (citric, succinic, lactic, acetic, and propionic acids), and ethanol were determined in intervals of 2 h. Aliquots of 2 mL of the liquid fraction samples were centrifuged at 6000 ×g for 15 min and filtered through 0.22 µm pore size filter (Millipore Corp., Billerica, MA, USA). Analysis parameters were performed according to Carvalho Neto

et al. (2017). The filtered samples were injected into HPLC system equipped with an Aminex HPX 87 H column (300 x 7.8 mm; Bio-Rad, Richmond, CA, USA) and a refractive index (RI) detector (HPG1362A; Hewlett-Packard Company, Palo Alto, CA, USA). The column was eluted in an isocratic mode with a mobile phase of 5 mM H_2SO_4 at 60 ºC and a flow rate of 0.6 mL/min.

5.2.6. Ethyl acetate quantification by gas chromatography

Production of ethyl acetate, the major volatile organic compound produced by *P. fermentans* YC5.2 (PEREIRA et al., 2014), was monitored by gas chromatography. Every two hours, the gases formed in the bioreactor headspace were collected and injected into a gas chromatograph (model 17A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector at 230 °C. The operation conditions were as follows: a 30 m × 0.32 mm HP-5 capillary column, column temperature of 40 to 150 °C at a rate of 20 °C/min. A standard curve was constructed using an authentic standard purchased from Sigma and ethyl acetate concentration was expressed as μmol/L of headspace

5.2.7. Kinetic parameters

The kinetic parameters for bacteria and yeast growth [specific growth rate (μ)] and product formation [product formation rate (Q) and specific product formation rate (Q_p) of organic acids (acetic and lactic acids), ethanol, and ethyl acetate] were determined for both inoculated and spontaneous processes. To achieve these parameters the following equations were used:

$$\mu = \frac{1}{X} \times \frac{dX}{dt} \quad (1)$$

$$Q = \frac{dP}{dt} \quad (2)$$

$$Q_p = \frac{1}{X} \times \frac{dP}{dt} \quad (3)$$

Where X and P are the final concentration of biomass and products (g/L) generated in the coffee-pulp bean mass during fermentation time t (in hours).

5.2.8. Bacterial diversity by Illumina high-throughput sequencing

The bacteria community composition from liquid fraction samples withdrawn at 0, 12 and 24 h of spontaneous and inoculated STR fermentation was analyzed by Illumina high-throughput sequencing. For extraction of genomic DNA, 1 mL of each sample was centrifuged at 12,000 ×g for 1 min. The cell pellet was resuspended in 500 µL Tris-EDTA, homogenized with 10 µL of lysozyme solution at 20 mg/mL (Sigma Aldrich, Arklow, Ireland) and incubated at 30 °C for 60 min. Then, 50 µL of SDS 10% (w/v) and 10 µL of proteinase K solution at 20 mg/mL (Sigma Aldrich, Arklow, Ireland) were added to the lysis solution, followed by homogenization and incubation at 60 ºC during 60 min. Then, 150 µL of phenol-chloroform (25:24) were added, homogenized by inversion and centrifuged at 12,000 ×g for 5 min. The supernatant was collected and the DNA was precipitated with 3x (v/v) absolute ethanol. Pellets were washed with 80% ethanol, dried, and resuspended in ultrapure water. Total DNA was quantified with a Nanodrop 2000 instrument (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

A fragment of the of the 16S rRNA gene was amplified from the total extracted DNA using primers for the V4 region (bases 515 to 806) containing complementary adaptors for Illumina platform (CAPORASO et al., 2012) using KlenTAQ polymerase (Sigma Aldrich, Arklow, Ireland). Bar-coded amplicons were generated by PCR under the following conditions: 95 ºC for 3 min, followed by 18 cycles at 95 ºC for 30 s, annealing at 50 ºC for 30 s, extension at 68 ºC for 60 s, final extension at 68 ºC for 10 min. Samples were sequenced in the MiSeq platform using the 500 V2 kit, following standard Illumina protocols.

After sequencing, chimeric sequences detection, removal of noises from pre-cluster and taxonomic attribution were performed using standard parameters of QIIME software package, version 1.9.0. Applying the uclust method (EDGARD et al., 2010), sequences presenting identity above 97% were considered the same operational taxonomic units (OTUs) according to the SILVA database (QUAST et al., 2013).

5.2.9. Chemical analysis of dried, fermented coffee beans

Sugars and organic acids present in dried, fermented beans from spontaneous and inoculated processes, as well as in green coffee beans (namely unfermented beans),

were extracted in water and determined by high-performance liquid chromatography (HPLC). The HPLC procedure was performed as described in section 5.2.4.

The volatile aroma compound composition of spontaneous, inoculated and unfermented coffee beans was determined by gas chromatography coupled to mass spectrometry (GC-MS) according to Carvalho Neto et al. (2017). The extraction of volatile compounds from the beans was performed using a headspace vial coupled to a solid phase microextraction (SPME) fiber (5% Carboxen [CARB]/95% Polydimethylsiloxane [PDMS] df75 µm partially crosslinked) (Supelco, St. Louis, MI, USA). The flasks were heated at 70 ºC for 10 min without agitation, followed by 15 min of exposition of the fiber in a COMBI-PAL system. The compounds were desorbed into the gas chromatograph injection system gas phase (CGMS TQ Series 8040 and 2010 Plus GC-MS; Shimadzu, Tokyo, Japan) at 260 ºC. The column oven temperature was maintained at 60 ºC during 10 min, followed by two heating ramps of 4 and 10 ºC/min until reaching the temperatures of 100 and 200 ºC, respectively. The compounds were separated on a column 95% PDMS/5% PHENYL (30 m x 0.25 mm x 0.25 mm film thickness). The GC was equipped with an HP 5972 mass selective detector (Hewlett Packard, Palo Alto, CA, USA). Helium was used as carrier gas at a rate of 1.0 mL/min. Mass spectra were obtained by electron impact at 70 eV and a start and end mass-to-charge ratio (m/z) of 30 and 200, respectively. The compounds were identified by comparison to the mass spectra from library databases (Nist'98 and Wiley7N).

5.2.10. Coffee cup quality analysis

After dried, about 800 g of fermented coffee beans from spontaneous and inoculated STR processes were roasted in a semi-industrial roaster (Probatino, Leogap model, Brazil). A coffee beverage derived from conventional processing (i.e., on-farm fermentation condition) was included as a control. The roasting cycle was defined through the sensory markers technique according to the procedures described by Pereira et al. (2015). The roasted coffee was ground in a G3 Bulk Coffee Grinder (Bunn Corporation, São Paulo, Brazil) to an average particle size between 360 and 420 µm. The coffee samples for cupping were prepared using 105 g of roasted and ground coffee in 1500 mL of filtered water (Everpure Water Filter System, São Paulo, Brasil) using a VP17-3 BLK Coffee Brewer (Bunn Corporation) with paper filter method (Melitta original 1 x 4). The water was treated to avoid the influence of different solutes and

contained 90 ppm of total dissolved solids with a balance ratio of 1:4 sodium and calcium, and had a pH of 6.50. Cupping evaluation was performed by a panel of four expert coffee tasters with Q-Grader Coffee Certificate, as described in Pereira et al. (2015). An evaluation was conducted assessing the following attributes: aroma, taste, acidity, body, balance, aftertaste and overall quality according to the Specialty Coffee Association of America Cupping Protocols (see 'http://www.scaa.org/?page=resources&d=cupping-protocols'). The quality and intensity of each attribute were evaluated simultaneously using a scale varying from 0 to 10, with 0.25 increments, and a total score for each sample was assigned. After the gustatory step, coffee tasters assigned a score unfermented beans e to each sample and highlighted the remarkable attributes of each beverage in a descriptive analysis.

5.2.11. Statistical analysis

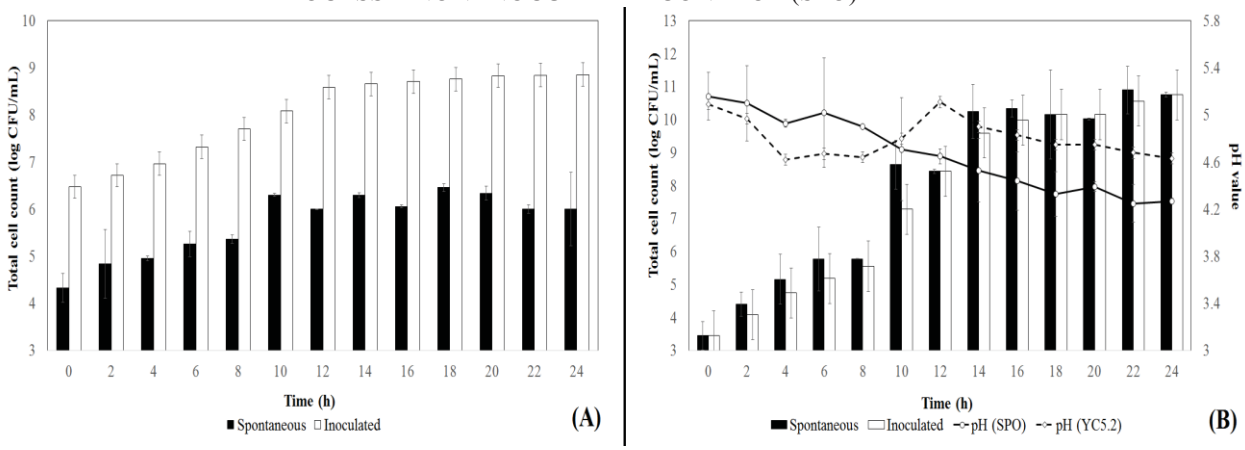
The data obtained of target metabolite analysis and sensory evaluations were analyzed by post-hoc comparison of means by Duncan's test. Statistical analyses were performed using the SAS program (Statistical Analysis System Cary, NC, USA). Level of significance was established in a two-sided p-value <0.05.

5.3. RESULTS AND DISCUSSION

5.3.1. Controlled bioreactor fermentation assay

The FIGURE 5.1 shows the time evolution of microbial growth and pH decrease during inoculated and spontaneous STR fermentation. For both processes, the bioreactor system enabled an ideal, continuous yeast and LAB growth. This led to a correct coffee-mucilage acidification, reaching final pHs of 4.63 and 4.27 in the inoculated and spontaneous process, respectively. The reduction in pH levels below 4.5 is a method widely used by coffee producers to determine the end of coffee beans fermentation (JACKELS; JACKELS, 2005). The acidification process assists in the promotion of pectin breakdown (the major carbohydrate polymer present in coffee mucilage), contributing to the removal of the fruit mucilage layer and drying of the beans (GERMANE et al., 2015; KIM et al., 2016; PEREIRA et al., 2017).

FIGURE 5.1 – GROWTH KINETICS OF TOTAL YEASTS (A) AND TOTAL LACTIC ACID BACTERIA (B) IN COFFEE FERMENTATIONS CONDUCTED IN A STR BIOREACTOR. INOCULATED PROCESS = YEAST STARTER CULTURE-ADDED (YC5.2); SPONTANEOUS PROCESS = NON-INOCULATED CONTROL (SPO)



The inoculation of *P. fermentans* resulted in high counts of total yeasts during entire fermentation process, reaching a peak of 8.85 log CFU/mL at 24 h of fermentation (FIGURE 5.1A). This yeast population was represented by over 75% of *P. fermentans* at the end of the fermentation process, as reveled by primer-specific PCR analysis (data not shown). On the other hand, the spontaneous fermentation showed a significantly lower yeast count, reaching a maximum of 6.46 log CFU/mL at 10 h of fermentation. The high yeast population present in the inoculated process led to an efficient coffee-mucilage sugar consumption, with glucose being totally consumed at 12 h (TABLE 5.1). A survey of previous studies demonstrates significant residual amounts of pulp sugars (approximately 6.93 g/L) at the end of coffee fermentations conducted under field conditions (CARVALHO NETO et al., 2017), even by using selected starter cultures (EVANGELISTA et al., 2014a, 2014b; PEREIRA et al., 2015). Unsatisfactory coffee bean demucilaging interferes with the drying process encouraging the growth of spoilage bacteria and fungi (AGATE; BHAT, 1996). Thus, the STR system combined with the use of selected yeast starter culture showed great potential for efficient removal of coffee beans mucilage layer.

TABLE 5.1 – PULP SUGAR CONSUMPTION AND METABOLITE FORMATION IN THE COURSE OF COFFEE BEANS FERMENTATION IN A STIRRED-TANK BIOREACTOR

Time (h)	Metabolites production and sugars consumption (g/L)																	
	Citric acid		Glucose		Fructose		Succinic acid		Lactic acid		Acetic acid		Propionic acid		Ethanol		Ethyl acetate	
	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S
0	0.19±0.01	0.24±0.01	4.75±1.51	4.75±0.47	6.62±1.21	6.97±0.01	0.03±0.00	0.03±0.00	0.04±0.04	0.15±0.04	0.03±0.01	0.02±0.00	0.01±0.01	0.01±0.00	0.37±0.01	0.000	4.92±1.20	ND
2	0.24±0.07	0.65±0.24	5.43±0.32	6.08±0.10	8.03±1.07	9.54±0.39	0.05±0.01	0.04±0.01	0.08±0.04	0.25±0.00	0.06±0.01	0.03±0.00	0.01±0.01	0.01±0.07	0.47±0.01	0.000	3.42±1.30	ND
4	0.16±0.01	0.22±0.01	3.69±0.15	5.72±0.03	7.44±1.98	8.69±0.02	0.11±0.01	0.05±0.01	0.48±0.04	0.54±0.09	0.16±0.01	0.11±0.00	0.02±0.00	0.09±0.00	1.01±0.11	0.000	23.01±3.90	ND
6	0.08±0.06	0.17±0.03	0.91±0.15	5.49±0.03	5.80±0.93	8.68±0.00	0.17±0.06	0.10±0.01	1.05±0.04	1.07±0.06	0.25±0.01	0.23±0.01	0.03±0.00	0.02±0.00	2.74±0.67	0.000	47.49±8.30	ND
8	0.07±0.09	0.16±0.03	0.40±0.11	5.15±0.74	2.01±0.22	8.63±0.01	0.14±0.02	0.08±0.00	2.22±0.04	1.45±0.29	0.18±0.01	0.29±0.00	0.04±0.01	0.03±0.00	4.23±0.88	0.000	97.92±12.10	ND
10	0.07±0.02	0.14±0.02	0.14±0.09	4.82±0.04	1.16±0.01	8.62±0.06	0.12±0.00	0.06±0.01	2.29±0.04	1.69±0.02	0.14±0.02	0.43±0.05	0.07±0.02	0.03±0.00	3.97±0.12	0.06±0.00	63.25±9.20	ND
12	0.18±0.01	0.14±0.02	0.00	4.63±0.01	0.94±0.11	8.79±0.44	0.06±0.01	0.05±0.00	2.41±0.04	1.92±0.52	0.23±0.10	0.56±0.07	0.11±0.01	0.03±0.00	3.60±0.63	0.04±0.04	37.45±7.30	ND
14	0.20±0.01	0.14±0.01	0.00	4.29±0.13	1.01±0.09	8.78±0.01	0.08±0.00	0.05±0.09	2.55±0.04	2.15±0.06	0.24±0.01	0.63±0.03	0.11±0.00	0.06±0.00	3.55±0.45	0.12±0.01	24.97±3.20	ND
16	0.21±0.01	0.14±0.01	0.00	4.31±0.31	1.05±0.09	9.31±0.22	0.09±0.02	0.06±0.00	2.63±0.04	2.25±0.50	0.25±0.01	0.66±0.00	0.08±0.01	0.06±0.00	3.58±0.83	0.19±0.05	27.35±2.93	ND
18	0.09±0.01	0.14±0.01	0.00	4.01±0.50	1.10±0.06	9.41±0.17	0.10±0.01	0.05±0.00	2.74±0.04	2.40±0.07	0.22±0.05	0.70±0.05	0.10±0.02	0.04±0.00	3.66±1.00	0.25±0.01	30.60±0.00	ND
20	0.16±0.02	0.13±0.01	0.00	3.19±0.34	1.11±0.12	8.84±1.11	0.09±0.01	0.08±0.02	2.79±0.04	2.61±0.04	0.23±0.08	0.94±0.10	0.10±0.02	0.11±0.00	3.68±1.02	0.20±0.05	18.51±4.3	ND
22	0.13±0.00	0.14±0.02	0.00	3.59±0.02	1.02±0.26	9.03±1.73	0.10±0.00	0.06±0.01	2.81±0.04	2.61±0.35	0.24±0.08	0.77±0.08	0.09±0.02	0.07±0.00	3.55±0.22	0.36±0.07	8.37±2.20	ND
24	0.15±0.01	0.13±0.04	0.00	3.66±0.01	0.97±0.12	9.01±2.00	0.10±0.01	0.06±0.01	2.88±0.04	2.58±0.01	0.23±0.09	0.75±0.01	0.10±0.01	0.07±0.00	3.64±0.19	0.30±0.01	14.12±3.40	ND

I = Inoculated; S = Spontaneous

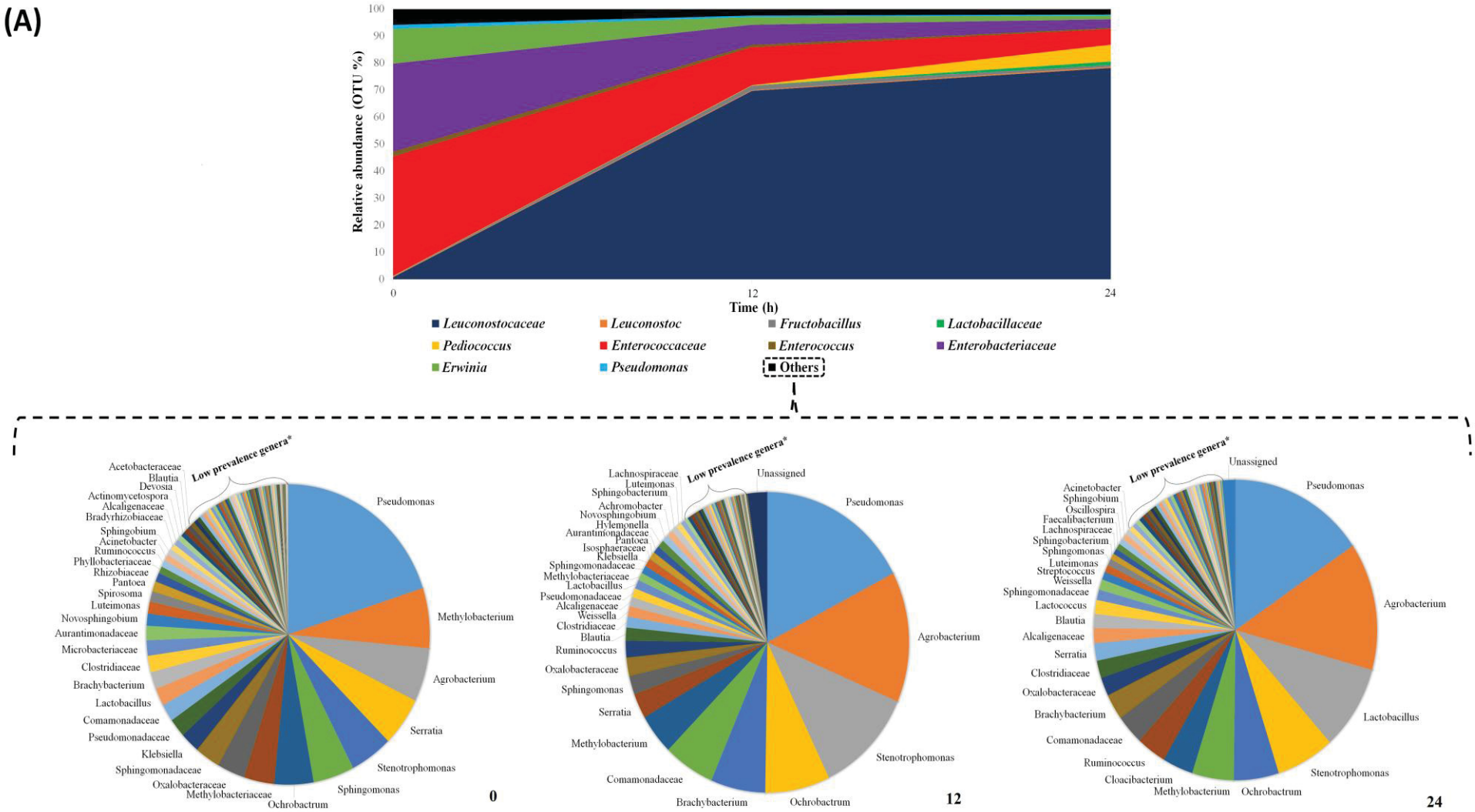
The fermentative kinetic parameters for both inoculated and spontaneous assays are disposed in TABLE 5.2. In general, the efficient yeast biomass accumulation in the inoculated process (3.72 g/L) led to higher ethanol (0.136 g/L.h) and ethyl acetate (0.383 g/L.h) formation rates when compared to spontaneous treatment. A similar lactic acid bacteria growth rate (0.028 h^{-1}) and lactic acid formation rate (~0.11 h^{-1}) was observed in the course of both fermentation processes (TABLE 5.2). Lactic acid is an important organic compound to coffee beans fermentation, which assists in the coffee pulp acidification process without interfering in the final product quality (PEREIRA et al., 2017). On the other hand, acetic acid content (a common coffee-transmitting off-flavor) was higher in the spontaneous process (0.752 and 0.098 g/L in spontaneous and inoculated assays, respectively), indicating a possible difference in the bacterial composition between treatments. To prove this hypothesis, a high throughput 16S rDNA gene sequencing analysis was performed at 0, 12, and 24 h of fermentation (FIGURE 5.2). In both inoculated and spontaneous processes, a constant increase in the population of LAB was observed, reaching over 85% of the total read sequences at the end of the fermentation process. A great diversity of LAB genera was reported, including *Fructobacillus, Leuconostoc, Pediococcus, Erwinia*, and *Lactobacillus*, with strong dominance of Leuconostocaceae family. However, 16S rDNA gene sequencing revealed differences in the minor bacteria composition between the treatments. At the end of the spontaneous process, it was detected the presence of acetic acid bacteria (AAB) belonging to genera *Gluconobacter*, *Roseomonas*, *Roseococcus*, and *Acetobacter,* that were not detected in the inoculated treatment. AAB are known to have unique fermentation ability, so called “oxidative fermentation” of acetic acid from ethanol (WAGNER et al., 2005), indicating the probable origin of this organic acid in the spontaneous process. Thus, the use of yeast starter culture was seen to be essential to decrease acetic acid production in coffee beans fermentation.

TABLE 5.2 – ESTIMATED KINETIC PARAMETERS OF THE SPONTANEOUS AND INCULATED FERMENTATIONS

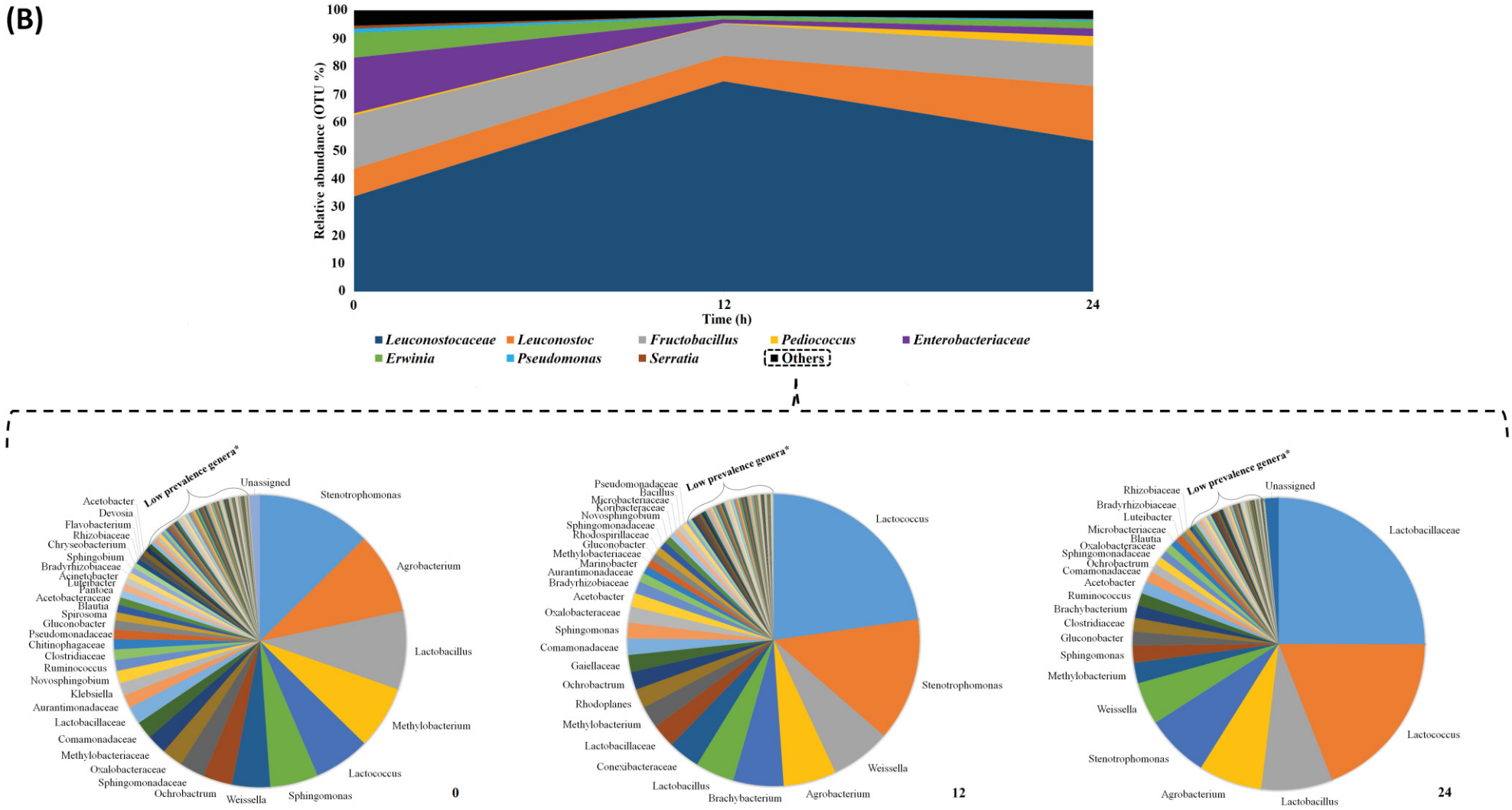
Kinetic parameters		**Fermentation assay**	
Fermentation variable	Parameter	Inoculated	Spontaneous
LAB	μ (h^{-1})	0.028	0.028
	Biomass accumulation (g)	3.844	3.844
Yeasts	μ (h^{-1})	0.011	0.012
	Biomass accumulation (g)	3.720	2.520
Lactic acid	Q (g/L.h)	0.119	0.101
	Q_p (h^{-1})	0.031	0.026
Acetic acid	Q (g/L.h)	0.008	0.030
	Q_p (h^{-1})	ND	ND
Ethanol	Q (g/L.h)	0.136	0.012
	Q_p (h^{-1})	0.037	0.004
Ethyl acetate	Q (µmol/L.h)	0.383	ND
	Q_p (h^{-1})	0.103	ND

µ, specific growth rate; Q, product formation rate; Qp specific product formation rate.

FIGURE 5.2 – BACTERIAL COMMUNITY COMPOSITION AND DYNAMICS PERFORMED BY HIGH-THROUGHPUT 16S RRNA GENE SEQUENCING THROUGHOUT THE INOCULATED (A) AND SPONTANEOUS (B) FERMENTATIONS CONDUCTED IN A STR BIOREACTOR



(B)


100
90
80
70
60
50
40
30
20
10
0
Relative abundance (OTU %)
0
12
24
Time (h)
Leuconostocaceae
Leuconostoc
Fructobacillus
Pediococcus
Enterobacteriaceae
Erwinia
Pseudomonas
Serratia
Others
Low prevalence genera*
Unassigned
Stenotrophomonas
Agrobacterium
Lactobacillus
Methylobacterium
Lactococcus
Sphingomonas
Weissella
Ochrobactrum
Sphingomonadaceae
Oxalobacteraceae
Methylobacteriaceae
Comamonadaceae
Lactobacillaceae
Aurantimonadaceae
Klebsiella
Novosphingobium
Ruminococcus
Clostridiaceae
Chitinophagaceae
Pseudomonadaceae
Gluconobacter
Spirosoma
Blautia
Acetobacteraceae
Pantoea
Lutibacter
Acinetobacter
Bradyrhizobiaceae
Sphingobium
Chryseobacterium
Rhizobiaceae
Flavobacterium
Devosia
Acetobacter
0
Low prevalence genera*
Pseudomonadaceae
Bacillus
Microbacteriaceae
Korlbacteraceae
Novosphingobium
Sphingomonadaceae
Rhodospirillaceae
Gluconobacter
Methylobacteriaceae
Marinobacter
Aurantimonadaceae
Bradyrhizobiaceae
Acetobacter
Oxalobacteraceae
Sphingomonas
Comamonadaceae
Gaiellaceae
Ochrobactrum
Rhodoplanes
Methylobacterium
Lactobacillaceae
Conexibacteraceae
Lactobacillus
Brachybacterium
Agrobacterium
Weissella
Stenotrophomonas
Lactococcus
12
Low prevalence genera*
Unassigned
Rhizobiaceae
Bradyrhizobiaceae
Luteibacter
Microbacteriaceae
Blautia
Oxalobacteraceae
Sphingomonadaceae
Ochrobactrum
Comamonadaceae
Acetobacter
Ruminococcus
Brachybacterium
Clostridiaceae
Gluconobacter
Sphingomonas
Methylobacterium
Weissella
Stenotrophomonas
Agrobacterium
Lactobacillus
Lactococcus
Lactobacillaceae
24

5.3.1. Determination of coffee beans and beverage quality

The chemical composition of coffee beans before (unfermented beans) and after inoculated and spontaneous processes was analyzed by HPLC and GC-MS (TABLE 5.3). HPLC analysis demonstrated that glucose, fructose and citric acid were unaltered in the seeds after fermentation processes, while lactic acid, acetic acid and succinic acid increased significantly ($p<0.05$) due to the microbial activity. In addition, acetic acid content was higher in the beans originated from spontaneous process, probably derived from AAB metabolism occurred during this treatment.

A total of 36 volatile organic compounds were identified in the analyzed coffee beans by SPME-GC-MS analysis, including hydrocarbons, aldehyde, ketones, alcohols, esters, furans, terpenes, pyrazine, and carboxylic acids (TABLE 5.3). Phenyl-acetaldehyde, styrene, phenylethyl alcohol, and D-limonene were the most important aromatic compounds quantified in both unfermented and fermented beans. Interestingly, coffee beans generated from *Pichia fermentans*-inoculated treatment had significantly higher concentrations ($p<0.05$) of specific volatile compounds, such as D-limonene, phenyl-acetaldehyde, and phenylethyl alcohol. In addition, the use of the starter culture promoted specific formation of some compounds, including phenylethyl acetate, ethylsalycilate, butanoic acid, 2-ethyl, and furfural, 5-methyl, that were not detected in beans from spontaneous process. Many of these compounds are typically reported in the literature as attributable to *Pichia* metabolism (i.e., benzaldehyde, ethyl acetate, phenylethyl alcohol, phenyl-acetaldehyde) (KONÉ et al., 2016; MANTZOURIDOU; PARASKEVOPOULOU, 2013; ROJAS et al., 2003), while others may have been originated from the beans itself (*i.e.,* linalool and β-myrcene). This indicated that the use of selected starter culture in bioreactor promoted a modification in the aromatic chemical compounds composition of coffee beans, although more studies on these kinetics should be performed.

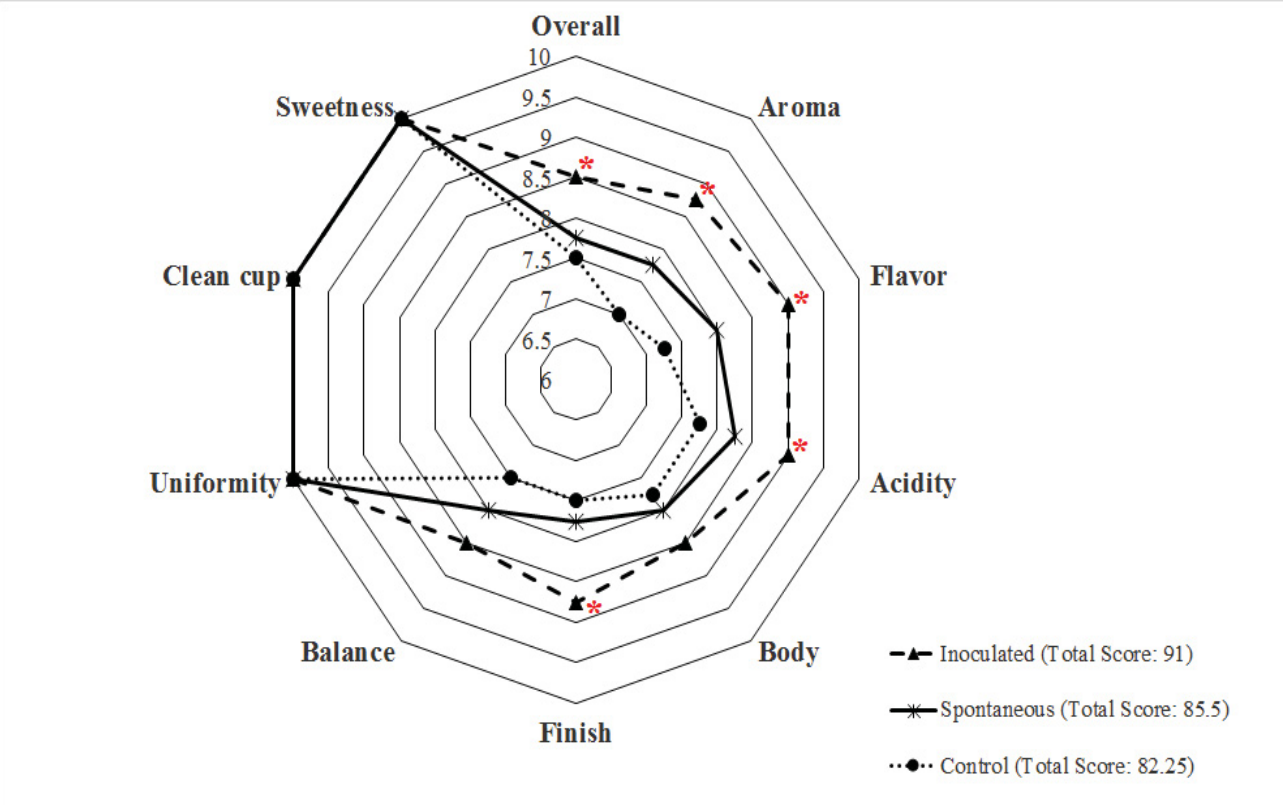
TABLE 5.3 – CONCENTRATION OF VOLATILE COMPOUNDS, ORGANIC ACIDS AND REDUCING SUGAR IN COFFEE BEANS BEFORE (UNFERMENTED BEANS) AND AFTER THE FERMENTATION PROCESS

Compound (retention time)	Aroma and taste description	Dried fermented coffee beans		
		Unfermented assay	Inoculated assay	Spontaneous assay
GC				
Hydrocarbons (7)				
Ethylbenzene (4.990)	-	1.81 ± 0.50[a]	ND	1.46 ± 0.16[a]
Styrene (5.990)	Sweet, foral; Almond	15.23 ± 2.73[a]	15.93 ± 4.81[a]	14.62 ± 5.48[a]
o-Xylene (5.283)	Geranium	ND	ND	0.73 ± 0.21
n-Heneicoisane (25.909)	-	1.12 ± 0.31[a]	1.31 ± 0.39[a]	1.42 ± 0.48[a]
D-Limonene (13.528)	Sweet, orange, citrus	4.51 ± 1.12[a]	19.18 ± 5.16[b]	11.91 ± 4.27[b]
n-Dodecane (11.720)	-	ND	1.37 ± 0.17[a]	1.12 ± 0.32[a]
Tridecane, 2,6,10-trimethyl- (26.863)	-	ND	0.90 ± 0.25	ND
Aldehydes (8)				
Methional (6.521)	Creamy tomato, potato skin, French fry	1.06 ± 0.09[a]	0.91 ± 0.304[a]	1.14 ± 0.09[a]
Benzaldehyde (9.275)	Almond, fruity, powdery, nutty	6.27 ± 1.33[a]	6.22 ± 1.09[a]	12.63 ± 1.66[b]
Acetaldehyde, phenyl- (14.408)	Honey, chocolate and cocoa with a spicy nuance	20.60 ± 3.38[a]	29.65 ± 1.35[b]	25.89 ± 12.89[a]
2-Octenal (15.218)	Fresh cucumber, banana, green, citrus	0.36 ± 0.03	ND	ND
Nonanal (17.587)	Green lemon peel like nuance, citrus, melon rindy	2.37 ± 0.11[a]	4.98 ± 0.29[b]	4.73 ± 0.29[b]
Decanal (21.749)	Sweet, fatty, citrus and orange peel	1.94 ± 0.30[a]	2.24 ± 0.33[a]	4.33 ± 0.17[b]
Octanal (12.055)	Aldehydic, green with a peely citrus orange note	ND	1.47 ± 0.01[a]	0.96 ± 0.27[b]
2-Phenyl-2-butenal (23.580)	Green, vegetative, floral, cocoa and nutty	0.34 ± 0.04[a]	0.36 ± 0.02[a]	ND
Ketones (3)				
Butyrolactone (6.901)	Milky, creamy with fruity peach-like afternotes	1.33 ± 0.05[a]	1.66 ± 0.28[a]	1.29 ± 0.57[a]
5-Hepten-2-one, 6-methyl- (11.066)	Fruity, apple, musty	1.08 ± 0.12[a]	1.32 ± 0.48[a]	1.71 ± 0.29[a]
2-Pentadecanone, 6,10,14-trimethyl- (28.439)	-	ND	0.42 ± 0.18[a]	0.61 ± 0.23[b]
Alcohols (5)				
1-Octen-3-ol (10.549)	Earthy, green, oily, umami sensation	1.04 ± 0.17[a]	1.20 ± 0.12[a]	2.37 ± 0.41[b]
Benzyl alcohol (13.935)	Sweet, fruity with balsamic nuances	8.31 ± 1.51[a]	3.12 ± 0.52[b]	3.62 ± 0.72[b]
Phenethyl alcohol (17.990)	Floral, sweet and bready	8.89 ± 2.09[a]	17.15 ± 3.19[b]	7.22 ± 1.02[a]
1-Hexanol (5.201)	Green, fruity, apple-skin and oily	ND	ND	2.00 ± 0.19

2-Hexanol, 5-methyl- (6.221)	-	3.97 ± 1.23	ND	ND
Esters (6)				
Methyl salicylate (21.439)	Sweet and root beer with aromatic nuances	4.46 ± 0.50[a]	4.22 ± 0.20[a]	2.12 ± 0.06[b]
Hydroxybutyrate, 3-ethyl- (7.485)	Green, fruity and winey	ND	ND	0.59 ± 0.03
Methoxyisopropyl acetate (7.826)	-	ND	1.26 ± 0.21[a]	1.69 ± 0.23[a]
Benzeneacetic acid, ethyl ester (22.904)	Floral honey, rosy, chocolate and cocoa notes	ND	ND	0.95 ± 0.13
Phenylethyl acetate (23.195)	Sweet, honey, floral, rosy with a slight fruity body	ND	3.49 ± 0.16	ND
Ethyl salycilate (23.523)	Sweet, wintergreen, tutti frutti, cooling, spicy	ND	0.34 ± 0.18	ND
Terpene (1)				
Linalool (17.364)	Citrus, orange, lemon and floral	3.41 ± 0.77[a]	5.69 ± 0.21[b]	3.11 ± 0.93[a]
Furans (2)				
Furan, 2-pentyl (11.328)	Fruity, green, cooked caramellic nuances	2.02 ± 0.22[a]	2.36 ± 0.53[a]	3.08 ± 0.48[a]
Furfural, 5-methyl- (9.517)	Sweet, brown, caramellic, maple-like	ND	0.47 ± 0.15	ND
Pyrazine (1)				
Pyrazine, 2-methoxy-3-(2-methylpropyl) (20.975)	Pepper, green pea and galbanum	2.39 ± 0.57[a]	1.97 ± 0.01[a]	1.84 ± 0.73[a]
Carboxylic acids (1)				
Butanoic acid, 2-ethyl- (22.245)	Acidic, fruity, tropical with a creamy aftertaste	ND	0.51 ± 0.06	ND
HPLC				
Glucose		32.13 ± 7.50[a]	26.09 ± 4.52[a]	35.46 ± 3.53[a]
Fructose		26.09 ± 5.94[a]	18.31 ± 4.61[a]	25.50 ± 5.48[a]
Citric acid		11.13 ± 3.45[a]	9.02 ± 1.38[a]	16.30 ± 3.31[a]
Succinic acid		0.12 ± 0.09[a]	0.31 ± 0.08[ab]	0.68 ± 0.13[b]
Lactic acid		0.30 ± 0.11[a]	3.96 ± 0.30[b]	1.80 ± 0.19[c]
Acetic acid		ND	0.52 ± 0.07[a]	1.35 ± 0.28[a]
Propionic acid		ND	ND	ND
Ethanol		ND	ND	ND

Sensory analysis of beverages produced with roasted coffee beans from STR fermentations and on-farm processing (control) is shown in FIGURE 5.3. The beverage produced from *Pichia fermentans*-inoculated treatment received scores above 90 points, which indicate a very high coffee quality according to Specialty Coffee Association of America Cupping Protocol. This beverage had higher scores in overall, aroma, flavor, acidity, body, finish and balance aspects when compared to STR spontaneous processes and control fermentation. This indicates the positive influence of yeast metabolism in final coffee beverage quality.

FIGURE 5.3 - SENSORIAL DIFFERENCES OF COFFEE BEVERAGES GENERATED FROM FERMENTED, ROASTED COFFEE BEANS OF THE CONTROL (SPONTANEOUS) AND INOCULATED PROCESSES *ASTERISK* REPRESENT SIGNIFICANTLY DIFFERENCE IN A TWO-SIDED P-VALUE <0.05



Comparisons were made by a panel of four experienced coffee tasters.
Control indicates a coffee beverage derived from conventional processing (i.e., on-farm fermentation condition). Asterisk= significantly higher in a two-sided p-value<0.05.

5.4. CONCLUSION

In summary, for the first time, a yeast bioreactor model was applied to the coffee fermentation process. The fermentation system adopted in this work (consecutive aerobic and anaerobic phases) proved to be a conducive environment for starter culture

growth, coffee-pulp sugar consumption and formation of metabolite compounds that can improve coffee beverage quality. The inoculation with high titers of selected yeast cultures modulates the overall fermentation, with efficient sugar mucilage consumption and aroma compounds formation. In addition, very high-quality coffee beans and beverages were produced from bioreactor processes. The use of STR may be of great interest for those who seek improved control over the coffee beans fermentation process and/or to optimize coffee fermentation through the use of starter cultures.

GENERAL CONCLUSION

In summary, the application of the next generation sequencing (Illumina platform) in the present study allowed a good representation of both high- and low-prevalent bacterial population, obtaining a total of 440,524 OTUs and over 80 families and genera identified. Among these, it is possible to highlight the first report of *Fructobacillus, Pseudonocardia, Pedobacter, Sphingomonas, Hymenobacter* and other 52 microbial groups, showing the potential of the *terroir* in the isolation of microorganisms with potential biotechnological applications. In parallel, the proposition of a new fermentation model in stirred-tank bioreactors under controlled parameters allowed the creation of a favorable environment for the *Lactobacillus plantarum* LPBR01 and *Pichia fermentans* YC5.2 development. The starter culture growth resulted in an effective consumption of sugars, with glucose being totally consumed at 12 h and the presence of a low concentration of residual fructose (1.14 and 0.98 g/L, repectively) at the end of the fermentation, and the production of important metabolites such as lactic acid, ethyl acetate, D-limonene and 2-phenylethyl acetate. The diffusion of lactic acid and volatile organic compounds into the beans promoted the sensorial modulation of the coffee beverages, which reached scores above 90 points in the SCAA scale and were significantly superior to the spontaneous fermentations conducted in bioreactors and on-farm conditions. In addition, the LAB bioreactor model showed a pronounced acidification of the coffee pulp-bean mass, achieving a pH bellow 4.0 during the initial 6 h of fermentation and significantly reducing the time required for the fermentation. Thus, the present study allowed an in-deepth temporal analysis of the microbial populations' dynamics associated with coffee fermentation and suggested a new segment for the technological advance of the coffee post-harvest processing.

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ANNEXES

CHAPTER IV

TABLE A4.1 – LIST OF MICROBIAL FAMILIES AND GENERA FOUND DURING INOCULATED AND SPONTANEOUS COFFEE BEANS FERMENTATIONS CONDUCTED IN STR BIOREACTOR

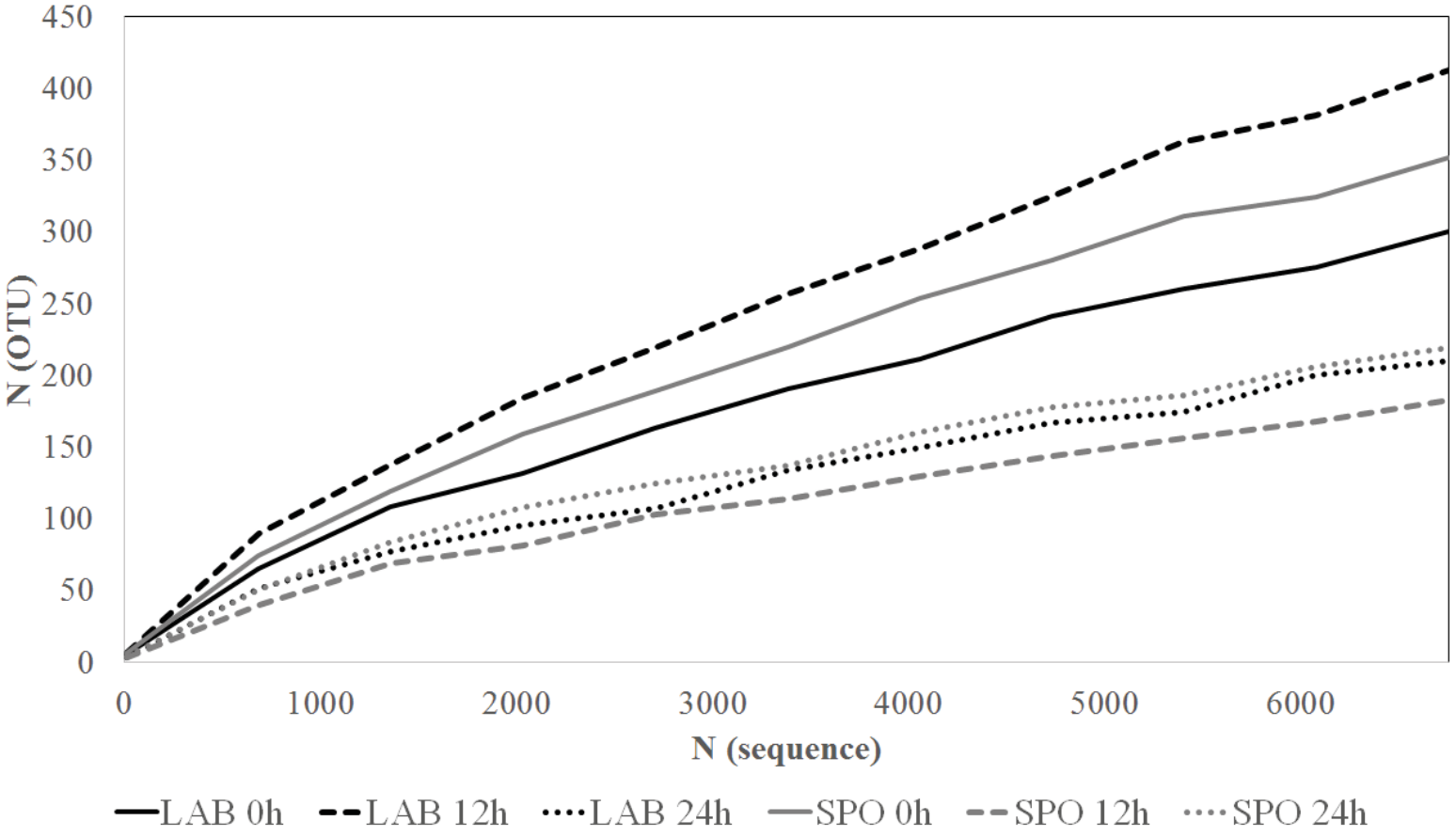
Fermentation time (h)	Microorganisms	Relative prevalence (OTU %)
Inoculated		
0	*Bradyrhizobiaceae, Lactococcus*	0.490
	Erysipelotrichaceae, Eggerthella, Fimbriimonas, Chryseobacterium	0.408
	Luteibacter, Bacillaceae, Ochrobactrum, Rhizobiaceae, Acinetobacter	0.327
	Anaeroplasma, Dyadobacter, Larkinella, Allobaculum, Gemmata, Devosia, Methylocystaceae, Novosphingobium, Alcaligenaceae, Comamonadaceae, Pantoea	0.245
	Micromonosporaceae, Actinomycetospora, Prevotella, Pedobacter, Sphingobacterium, Acaryochloris, Peptostreptococcaceae, Mycoplana, Rhodoplanes, Rubellimicrobium, Comamonas, Polaromonas, Hydrogenophilus, Luteimonas, Catenibacterium	0.163
	Hylemonella, Kaistobacter, Phenylobacterium, Brevundimonas, Chondromyces, Eubacterium, Mogibacteriaceae, Peptococcus, Enhydrobacter, Turicibacter, Pseudonocardia, Bifidobacterium, Intrasporangiaceae, Kineococcus, Nocardioidaceae, Bacteroides, Hymenobacter, Sphingobacteriaceae, Aerococcus	0.082
12	*Sanguibacter, Chitinophagaceae, Lactococcus, Rhodobacteraceae, Luteibacter, Luteimonas, Alcaligenaceae, Marinobacter*	0.398
	Arsenicicoccus, Streptomyces, Solirubrobacteraceae, Fluviicola, Bacillaceae, Phyllobacteriaceae, Mesorhizobium, Kaisobacter, Syntrophobacteraceae, Acinetobacter, Xantomonadaceae	0.266
	Brevibacterium, Frankiaceae, Kineococcus, Dactylosporangium, Pilimelia, Nakamurellaceae, Rhodococcus, Nocardioides, Acrinomycetospora, Saccharopolyspora, Actinoallomurus, Patulibacteraceae, Fimbriimonas, Dyadobacter, Larkinela, Balneolaceae, Phormidiaceae, Ammoniphilus, Planococcaceae, Caulobacteraceae, Beijerinckiaceae, Hyphomicrobium, Burkholderia, Comamonas,	0.133

	Methylibium, Rhodocyclaceae, Haliangaceae, Enterobacter, Klevsiella, Unassinged	
24	*Bifidobacterium, Hymenobacter, Nocardioidaceae, Chitinophagaceae, Caulobacteraceae, Erythrobacteraceae*	0.431
	Prevotella, Pedobacter, Faecalibacterium, Catenibacterium, Coprobacillus, Fusobacterium, Brevundimonas, Klebsiella, Acinetobacter, Luteibacter	0.345
	Nocardioides, Pseudonocardia, Bacteroides, Clostridium, Gemmata, Pedomicrobium, Pantoea	0.259
	Georgenia, Cellulomonas, Frankiaceae, Microbacteriaceae, Sanguibacter, Collinsella, Fimbriimonas, Dyadobacter, Spirosoma, Bacillaceae, Paenibacillus, Rummeliibacillus, Peptostreptococcaceae, Erysipelotrichaceae, Beijerinckiaceae, Alcaligenaceae, Nannocystis, Luteolibacter	0.173
	Geodermatophilaceae, Arthrobacter, Rhodococcus *Parabacteroides, Nostoc, Staphylococcus, Phyllobacteriaceae, Rhizobium, Azohydromonas, Methylibium, Janthinobacterium, Chondromyces*	0.863
Spontaneous		
0	*Lachnospiraceae*	0.490
	Gemmata	0.461
	Pedobacter	0.432
	Ruminococcaceae	0.403
	Sphingobacteriaceae	0.375
	Brachybacterium; Microbacteriaceae	0.346
	Alcaligenaceae, Hylemonella	0.317
	Clostridium	0.288
	Cytophagaceae, Dyadobacter, Sphingobacterium, Ruminococcus, Methylocystaceae	0.259
	Kaistobacter, Coprococcus, Caulobacteraceae, Luteimonas	0.230

	Rhodococcus, Paenibacillus, Dorea, Faecalibacterium, Clostridium, Isosphaeraceae, Mycoplana	0.202
	Nocardioidaceae	0.173
	Prevotella, Solirubrobacteraceae, Fimbriimonas, Larkinella, Bifidobacterium, Eubacterium, Phyllobacteriaceae, Erythrobacteraceae, Cystobacterineae	0.144
	Actinomycetospora, Hymenobacter, Enterococcaceae, Beijerinckiaceae, Janthinobacterium, Legionella, Rubellimicrobium, Rhodospirillaceae	0.115
	Solibacteraceae, Kineococcus, Cellulomonas, Micrococcaceae, Micromonosporaceae, Sediminibacterium, Armatimonadaceae, Oscillospira, Fusobacteriaceae, Balneimonas, Bradyrhizobium, Rhodoplanes, Achromobacter, Methylibium	0.086
	Beutenbergiaceae, Geodermatophilaceae, Aeromicrobium, Pseudonocardia, Flavisolibacter, Segetibacter, Nostoc, Bacillus, Enterococcus, Turicibacter, Peptostreptococcaceae, Allobaculum, Catenibacterium, Coprobacillus, Gemmataceae, Pirellula, Brevundimonas, Phenylobacterium, Rhizobiaceae, Rhizobium, Roseococcus, Roseomonas, Burkholderia, Azohydromonas, Comamonas, Polaromonas, Bdellovibrio, Polyangiaceae, Chondromyces, Sinobacteraceae	0.058
	Collinsella, Bacteroides, Parabacteroides, Acaryochloris, Aerococcus, Peptococcus, Phascolarctobacterium, Mogibacteriaceae, Erysipelotrichaceae, Rhodobacteraceae, Paracoccus, Sutterella, Trabulsiella, Enhydrobacter	0.029
12	*Kaistobacter, Luteibacter, Streptomyces*	0.351
	Rhodobaca, Alcaligenaceae, Syntrophobacteraceae, Xanthomonadaceae	0.293
	Thermogemmatisporaceae, Devosia, Methylocystaceae, Acinetobacter	0.234
	Sinobacteraceae, Pedomicrobium, Blastomonas, Oceanicaulis, Acetobacteraceae, Rhizobiaceae, Nitrospira, Gemmataceae, Paenibacillus, Ammoniphilus, Phormidiaceae, Spirosoma, Actinomycetospora, Pseudonocardia, Micromonosporaceae, Mycobacterium	0.176
	Sphingobium, Catellatospora, Rhodococcus, Solirubrobacteraceae, Cyclobacteriaceae, Cyclobacteriaceae, Fluviicola, Chitinophagaceae, Alicyclobacillus, Bacillaceae, Planococcaceae, Staphylococcus, Mycoplana, Hyphomicrobium, Phyllobacteriaceae, Mesorhizobium, Myxococcaceae, Klebsiella, Puniceicoccaceae	0.117
	Hyphomonadaceae, Rhodobacteraceae, Paracoccus, Rubellimicrobium, Inquilinus, Methylibium, Janthinobacterium, Haliangiaceae, Nannocystis, Citrobacter, Enterobacter, Pantoea, Luteimonas, Pedosphaera, Solibacteraceae, Actinopolyspora, Brevibacterium, Cellulomonas, Frankiaceae, Geodermatophilaceae, Kineococcus, Micrococcaceae, Aeromicrobium, Patulibacteraceae,	0.058

	Saccharopolyspora, Actinoallomurus, Ktedonobacteraceae, Chroococcidiopsis, Cohnella, Gemmata, Planctomyces, Caulobacteraceae, Arthrospira, Beijerinckiaceae	
	Klebsiella	0.458
	Lachnospiraceae, Ruminococcaceae, Methylobacteriaceae	0.382
	Aurantimonadaceae	0.343
	Eubacterium	0.305
	Turicibacter, Chitinophagaceae, Pseudomonadaceae, Novosphingobium, Alcaligenaceae	0.267
	Prevotella, Hylemonella	0.229
24	*Sphingobacterium, Ruminococcus, Dorea, Clostridium, Pantoea, Clostridium*	0.191
	Collinsella, Coprococcus, Faecalibacterium, Oscillospira, Catenibacterium, Sphingobium	0.153
	Coprobacillus, Devosia, Acetobacteraceae, Azohydromonas, Acinetobacter, Nocardioidaceae, Actinomycetospora	0.114
	Kineococcus, Mycobacterium, Pseudonocardia, Bifidobacterium, Pedobacter, Acaryochloris, Enterococcaceae, Phascolarctobacterium, Erysipelotrichaceae, Allobaculum, Caulobacteraceae, Methylocystaceae	0.076
	Cellulomonas, Geodermatophilaceae, Intrasporangiaceae, Arsenicicoccus, Rhodococcus, Solirubrobacteraceae, Fimbriimonas, Cytophagaceae, Dyadobacter, Spirosoma, Flavobacteriaceae, Chryseobacterium, Wautersiella, Sphingobacteriaceae, Nostoc, Enterococcus, Peptostreptococcaceae, Isosphaeraceae, Brevundimonas, Mycoplana, Bradyrhizobium, Rhodoplanes, Phyllobacteriaceae, Paracoccus, Roseomonas, Erythrobacteraceae, Polaromonas, Polyangiaceae, Trabulsiella, Luteimonas	0.038

FIGURE A4.1 –RAREFACTION CURVES OF THE DIFFERENT SAMPLES. X-AXIS = SEQUENCING DEPTH; Y-AXIS = AMOUNT OF OTUS RETURNED BY EACH PIPELINE



CHAPTER V

TABLE A5.1 – LIST OF MICROBIAL FAMILIES AND GENERA FOUND DURING INOCULATED AND SPONTANEOUS COFFEE BEANS FERMENTATIONS CONDUCTED IN STR BIOREACTOR.

Fermentation time (h)	Microorganisms	Relative prevalence (OTU %)
Inoculated		
0	Lachnospiraceae, *Sphingobacterium*	0.458
	Chitinophagaceae, *Prevotella,* Ruminococcaceae	0.401
	Achromobacter, Nocardioidaceae, *Pseudonocardia,* Methylocystaceae	0.344
	Burkholderia, Fusobacterium, Paenibacillus, Sphingobacteriaceae, *Hymenobacter*	0.287
	Dorea, Caulobacteraceae, *Chryseobacterium, Dyadobacter, Fimbriimonas, Collinsella, Pedobacter*	0.229
	Peptostreptococcaceae, *Faecalibacterium, Oscillospira, Turicibacter, Clostridium, Eubacterium, Mycoplana,* Solirubrobacteraceae, Intrasporangiaceae, Frankiaceae, *Azohydromonas, Hylemonella, Xanthomonadaceae*	0.172
	Terriglobus, Microbacteriaceae, *Rhodococcus, Propionibacterium, Bifidobacterium, Rudanella, Staphylococcus, Lactococcus, Coprococcus, Ruminococcus, Allobaculum, Bulleidia, Clostridium, Brevundimonas, Phenylobacterium, Roseomonas, Legionella*	0.115
	Geodermatophilaceae, Beutenbergiaceae, *Arthrobacter, Aeromicrobium, Sanguibacter,* Armatimonadaceae, *Bacteroides, Larkinella,* Flavobacteriaceae, *Wautersiella, Flavisolibacter, Sediminibacterium, Weissella, Catenibacterium, Coprobacillus,* Isosphaeraceae, *Bradyrhizobium, Rhodoplanes, Roseococcus, Kaistobacter, Polaromonas, Janthinobacterium, Cystobacterineae*	0.057
12	*Acinetobacter,* Ruminococcaceae	0.402
	Microbacteriaceae, *Sphingobium*	0.361
	Dorea, Turicibacter, Bradyrhizobiaceae, Rhizobiaceae, *Burkholderia*	0.281
	Actinomycetospora, Eubacterium, Pedobacter	0.241
	Clostridium, Flavobacteriaceae, *Oscillospira, Ruminococcus*	0.201
	Lactococcus, Clostridium, Faecalibacterium, Sphingobacteriaceae	0.161
	Gluconobacter, Pseudonocardia, Bifidobacterium, Dyadobacter, Chryseobacterium, Paenibacillus, Erysipelotrichaceae, *Catenibacterium*	0.120

	Kineococcus, Rhodococcus, Nocardioidaceae, *Bacteroides, Bacillus, Coprococcus,* Phyllobacteriaceae, *Brevundimonas, Allobaculum*	0.080
	Beutenbergiaceae, *Arsenicicoccus, Arthrobacter, Collinsella, Prevotella, Larkinella,* Chitinophagaceae, Bacillaceae, *Staphylococcus, Peptococcus, Coprobacillus,* Fusobacteriaceae, *Fusobacterium, Gemmata,* Caulobacteraceae, *Mycoplana,* Acetobacteraceae, *Acetobacter,* Erythrobacteraceae, *Janthinobacterium, Enhydrobacter, Anaeroplasma*	0.040
24	*Eubacterium, Novosphingobium,* Ruminococcaceae, Pseudomonadaceae	0.500
	Dyadobacter, Bacillus, Pedobacter, Erysipelotrichaceae, *Clostridium, Gemmata,* Aurantimonadaceae, Bradyrhizobiaceae, Phyllobacteriaceae, *Achromobacter, Hylemonella, Klebsiella*	0.375
	Cellulomonas, Corynebacterium, Bifidobacterium, Turicibacter, Clostridium, Ruminococcus, Fusobacterium, Caulobacteraceae, *Devosia,* Methylobacteriaceae, Methylocystaceae, *Pantoea, Steroidobacter, Xanthomonadaceae*	0.250
	Beutenbergiaceae, Nocardioidaceae, *Actinomycetospora, Bacteroides, Spirosoma, Chryseobacterium,* Bacillaceae, *Dorea, Peptococcus, Allobaculum, Coprobacillus,* Fusobacteriaceae, Rhizobiaceae, Acetobacteraceae, *Azohydromonas*	0.125
Spontaneous		
0	Lachnospiraceae	0.490
	Gemmata	0.461
	Pedobacter	0.432
	Ruminococcaceae	0.403
	Sphingobacteriaceae	0.375
	Brachybacterium, Microbacteriaceae	0.346
	Alcaligenaceae, *Hylemonella*	0.317
	Clostridium	0.288
	Cytophagaceae, *Dyadobacter, Sphingobacterium, Ruminococcus,* Methylocystaceae	0.259
	Kaistobacter, Coprococcus, Caulobacteraceae, *Luteimonas*	0.230
	Rhodococcus, Paenibacillus, Dorea, Faecalibacterium, Clostridium, Isosphaeraceae, *Mycoplana*	0.202
	Nocardioidaceae	0.173

	Prevotella, Solirubrobacteraceae, *Fimbriimonas, Larkinella, Bifidobacterium, Eubacterium,* Phyllobacteriaceae, Erythrobacteraceae, Cystobacterineae	0.144
	Actinomycetospora, Hymenobacter, Enterococcaceae, Beijerinckiaceae, *Janthinobacterium, Legionella, Rubellimicrobium,* Rhodospirillaceae	0.115
	Solibacteraceae, *Kineococcus, Cellulomonas,* Micrococcaceae, Micromonosporaceae, *Sediminibacterium,* Armatimonadaceae, *Oscillospira,* Fusobacteriaceae, *Balneimonas, Bradyrhizobium, Rhodoplanes, Achromobacter, Methylibium*	0.086
	Beutenbergiaceae, Geodermatophilaceae, *Aeromicrobium, Pseudonocardia, Flavisolibacter, Segetibacter, Nostoc, Bacillus, Enterococcus, Turicibacter,* Peptostreptococcaceae, *Allobaculum, Catenibacterium, Coprobacillus,* Gemmataceae, *Pirellula, Brevundimonas, Phenylobacterium*, Rhizobiaceae, *Rhizobium, Roseococcus, Roseomonas, Burkholderia, Azohydromonas, Comamonas, Polaromonas, Bdellovibrio,* Polyangiaceae, *Chondromyces,* Sinobacteraceae	0.058
	Collinsella, Bacteroides, Parabacteroides, Acaryochloris, Aerococcus, Peptococcus, Phascolarctobacterium, Mogibacteriaceae, Erysipelotrichaceae, Rhodobacteraceae, *Paracoccus, Sutterella, Trabulsiella, Enhydrobacter*	0.029
12	*Kaistobacter, Luteibacter, Streptomyces*	0.351
	Rhodobaca, Alcaligenaceae, Syntrophobacteraceae, Xanthomonadaceae	0.293
	Thermogemmatisporaceae, *Devosia,* Methylocystaceae, *Acinetobacter*	0.234
	Sinobacteraceae, *Pedomicrobium, Blastomonas, Oceanicaulis,* Acetobacteraceae, Rhizobiaceae, *Nitrospira,* Gemmataceae, *Paenibacillus, Ammoniphilus,* Phormidiaceae, *Spirosoma, Actinomycetospora, Pseudonocardia,* Micromonosporaceae, *Mycobacterium*	0.176
	Sphingobium, Catellatospora, Rhodococcus, Solirubrobacteraceae, Cyclobacteriaceae, Cyclobacteriaceae, *Fluviicola,* Chitinophagaceae, *Alicyclobacillus,* Bacillaceae, Planococcaceae, *Staphylococcus, Mycoplana, Hyphomicrobium,* Phyllobacteriaceae, *Mesorhizobium,* Myxococcaceae, *Klebsiella,* Puniceicoccaceae	0.117
	Hyphomonadaceae, Rhodobacteraceae, *Paracoccus, Rubellimicrobium, Inquilinus, Methylibium, Janthinobacterium,* Haliangiaceae, *Nannocystis, Citrobacter, Enterobacter, Pantoea, Luteimonas, Pedosphaera,* Solibacteraceae, *Actinopolyspora, Brevibacterium, Cellulomonas,* Frankiaceae, Geodermatophilaceae, *Kineococcus,* Micrococcaceae, *Aeromicrobium,* Patulibacteraceae, *Saccharopolyspora, Actinoallomurus,* Ktedonobacteraceae, *Chroococcidiopsis, Cohnella, Gemmata, Planctomyces,* Caulobacteraceae, *Arthrospira,* Beijerinckiaceae	0.058
24	*Klebsiella*	0.458
	Lachnospiraceae, Ruminococcaceae, Methylobacteriaceae	0.382
	Aurantimonadaceae	0.343
	Eubacterium	0.305

Turicibacter, Chitinophagaceae, Pseudomonadaceae, *Novosphingobium,* Alcaligenaceae	0.267
Prevotella, Hylemonella	0.229
Sphingobacterium, Ruminococcus, Dorea, Clostridium, Pantoea, Clostridium	0.191
Collinsella, Coprococcus, Faecalibacterium, Oscillospira, Catenibacterium, Sphingobium	0.153
Coprobacillus, Devosia, Acetobacteraceae, *Azohydromonas, Acinetobacter*, Nocardioidaceae, *Actinomycetospora*	0.114
Kineococcus, Mycobacterium, Pseudonocardia, Bifidobacterium, Pedobacter, Acaryochloris, Enterococcaceae, *Phascolarctobacterium,* Erysipelotrichaceae, *Allobaculum,* Caulobacteraceae, Methylocystaceae	0.076
Cellulomonas, Geodermatophilaceae, Intrasporangiaceae, *Arsenicicoccus, Rhodococcus,* Solirubrobacteraceae, *Fimbriimonas, Cytophagaceae, Dyadobacter, Spirosoma,* Flavobacteriaceae, *Chryseobacterium, Wautersiella,* Sphingobacteriaceae, *Nostoc, Enterococcus,* Peptostreptococcaceae, Isosphaeraceae, *Brevundimonas, Mycoplana, Bradyrhizobium, Rhodoplanes,* Phyllobacteriaceae, *Paracoccus, Roseomonas,* Erythrobacteraceae, *Polaromonas,* Polyangiaceae, *Trabulsiella, Luteimonas*	0.038

FIGURE A5.1 – RAREFACTION CURVES OF THE DIFFERENT SAMPLES. X-AXIS = SEQUENCING DEPTH; Y-AXIS = AMOUNT OF OTUS RETURNED BY EACH PIPELINE