UNIVERSIDADE FEDERAL DO PARANÁ

GILBERTO VINÍCIUS DE MELO PEREIRA

THE POTENTIAL USE OF YEAST STARTER CULTURES TO IMPROVE THE FERMENTATION AND QUALITY OF COFFEE DURING WET PROCESSING: SELECTION, IMPLEMENTATION AND SENSORIAL EFFECTS

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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. Carlos Ricardo Soccol

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UNIVERSIDADE FEDERAL DO PARANÁ
Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia
Setor de Tecnologia

UFPR

RELATÓRIO DE DEFESA DE TESE DE DOUTORADO

Aos quatorze dias do mês de janeiro de 2015, no Salão Nobre do Setor de Tecnologia, do Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pelo Prof. Dr. Júlio Cesar de Carvalho, Coordenador do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, a banca examinadora para a Centésima Segunda Defesa de Tese de Doutorado, Área de Concentração: Biotecnologia Agroalimentar. Estiveram presentes no Ato, além do Coordenador do Curso de Pós–Graduação, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do Colegiado do Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, ficou constituída pelos Professores Doutores Juliano De Dea Lindner (UFSC), Maria Giovana Binder Pagnoncelli (UFRN), Adenise Lorenci Woiciechowski (UFPR), Adriane Bianchi Pedroni Medeiros (UFPR), e Carlos Ricardo Soccol (UFPR – orientador da tese).

Às 14h00, a banca iniciou os trabalhos, convidando o candidato **Gilberto Vinícius de Melo Pereira** a fazer a apresentação da Tese intitulada: **"THE POTENTIAL USE OF YEAST STARTER CULTURES TO IMPROVE THE FERMENTATION WET PROCESSING: SELECTION, IMPLEMENTATION AND SENSORIAL EFFECTS"**. Encerrada a apresentação, iniciou-se a fase de arguição pelos membros participantes.

Tendo em vista a tese e a arguição, a banca composta pelos professores doutores Juliano De Dea Lindner, Maria Giovana Binder Pagnoncelli, Adenise Lorenci Woiciechowski, Adriane Bianchi Pedroni Medeiros e Carlos Ricardo Soccol declarou o candidato Aprovado (de acordo com a determinação dos Artigos 59 a 68 da resolução 65/09 de 30.10.09).

Curitiba, 14 de janeiro de 2015.

Prof. Dr. Juliano De Dea Lindner

Profª Drª Adenise Lorenci Woiciechowski

Profª Drª Maria Giovana B. Pagnoncelli

Profª Drª Adriane Bianchi Pedroni Medeiros

Prof Dr. Carlos Ricardo Soccol

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Obrigado!

RESUMO

Durante o processamento via úmida de café os frutos são despolpados e as sementes são submetidas ao processo de fermentação para a retirada do restante da mucilagem aderida ao pergaminho. A fermentação é uma etapa crítica no processamento, devido seu impacto sobre a qualidade final do produto. Este estudo teve como objetivo isolar, selecionar e implementar leveduras para controle e otimização do processo fermentativo de café. Na primeira etapa deste estudo, um total de 144 leveduras foram isoladas e identificadas por métodos moleculares. *Pichia fermentans* and *P. kluyveri* foram as espécies dominantes durante o processo, seguido por *Candida glabrata, C. quercitrusa, Saccharomyces* sp., *P. guilliermondii, P. caribbica* e *Hanseniaspora opuntiae*. Uma linhagem da espécie *P. fermentans*, denominada YC5.2, foi então selecionada devido sua capacidade de (i) resistir as condições de estresse impostas pelo ambiente de fermentação de café, (ii) produzir enzimas pectinolíticas para a aceleração do processo fermentativo e (iii) produzir quantidades significativas de compostos aromáticos (como por exemplo, acetato de etila e acetato de isoamila). Na segunda etapa deste estudo, *P. fermentans* YC5.2 foi inoculada em condições de campo de fermentação de café e comparada ao processo espontâneo (tratamento controle). Esta levedura mostrou-se apta para dominar o processo fermentativo e aumentou a eficiência do consumo de açúcares da polpa comparado ao tratamento controle. A inoculação da levedura também aumentou a produção de alguns compostos voláteis (como por exemplo, etanol, acetaldeído, acetato de etila e acetato de isoamila) e reduziu a produção de ácido láctico durante o processo fermentativo. Em relação aos grãos obtidos após o processo de torra, os teores de açúcares (glicose e frutose) e ácidos orgânicos (ácidos láctico, acético, cítrico, fumárico e málico) foram estatisticamente semelhantes ($p < 0,05$) em ambos os tratamentos. No entanto, a inoculação provocou um aumento na fração de voláteis oriundos do metabolismo da levedura nos grãos torrados obtidos por este processo. Além disso, a análise sensorial da bebida mostrou que a inoculação produziu um café com características distintas em relação ao tratamento controle, como por exemplo, intensa percepção de sabor de baunilha e notas florais. Em conclusão, o uso de *P. fermentans* YC5.2 como uma cultura iniciadora para a fermentacão de café mostrou ser uma alternativa viável para melhor controle do processo de fermentação de café visando obter bebidas de sabor diferenciado e com alta qualidade.

Palavras-chave: processamento de café, cultura iniciadora, levedura, *Pichia fermentans*, processamento por via úmida.

ABSTRACT

During wet processing of coffee, the ripe cherries are pulped, then fermented and dried. The fermentation is considered to be a critical step of processing due to its impact on the final quality of the product. This study aimed to isolate, select and implement yeasts in fermentation of coffee beans by the wet method. In the first stage of the study, a total of 144 yeast isolates originating from spontaneously fermenting coffee beans were identified by molecular approaches and screened for their capacity to grow under coffee-associated stress conditions. *Pichia fermentans* and *P. kluyveri* were the most frequent isolates, followed by *Candida glabrata*, *C. quercitrusa*, *Saccharomyces* sp., *P. guilliermondii*, *P. caribbica* and *Hanseniaspora opuntiae*. *Pichia fermentans* YC5.2 strain was selected due to its ability to (i) grow under coffee-associated stress conditions, (ii) produce pectinolytic enzymes and (iii) produce significant amounts of volatile aroma compounds (e.g., ethyl acetate and isoamyl acetate). In the second stage of the study, *P. fermentans* YC5.2 was inoculated into coffee beans fermentation under field conditions and compared with spontaneous (control) fermentation. This yeast strain prevailed over wild bacteria and yeast populations and increased the efficiency of pulp sugar consumption compared with control. The inoculation also increased the production of specific volatile aroma compounds (e.g., ethanol, acetaldehyde, ethyl acetate, and isoamyl acetate) and decreased the production of lactic acid. In roasted beans, the content of sugars (glucose and fructose) and organic acids (lactic, acetic, citric, fumaric, and malic acids) were statistically ($p<0.05$) similar for both treatments. However, the inoculated fermentation was shown to influence the volatile fraction of roasted coffee beans by increasing the concentration of yeast-derived metabolites compared to control. Sensory analysis of coffee beverages demonstrated that the use of the YC5.2 strain was favorable for the production of coffee with distinctive sensory profiles, presenting characteristics such as ‘vanilla’ taste and ‘floral’ aromas. In conclusion, the use of *P. fermentans* YC5.5 in wet processing of coffee beans was shown to be a viable alternative for those who seek improved control over the fermentation process and to obtain beverages of distinctive flavor and high quality.

Keywords: coffee processing, starter culture, yeast, *Pichia fermentans*, wet method.

SUMÁRIO

CHAPTER I (LITERATURE REVIEW) - MICROBIAL ECOLOGY AND STARTER CULTURE TECHNOLOGY IN COFFEE FERMENTATION

Manuscript submitted for publication in the *Critical Reviews in Food Science and Nutrition*.

1.1. ABSTRACT

Coffee has been for decades the most commercialized food product and most widely consumed beverage in the world, with over 600 billion cups served per year. Before coffee cherries can be traded and processed into a final industrial product, they have to undergo postharvest processing on farms, which have a direct impact on the cost and quality of a coffee. Three different methods can be used for transforming the coffee cherries into beans, know as wet, dry and semi-dry methods. In all these processing methods, a spontaneous fermentation is carried out in order to eliminate any mucilage still stuck to the beans and helps improve beverage flavor by microbial metabolites. The microorganisms responsible for the fermentation (e.g., yeasts and lactic acid bacteria) can play a number of roles, such as degradation of mucilage (pectinolytic activity), inhibition of mycotoxin-producing fungi growth and production of flavor-active components. The use of starter cultures (mainly yeast strains) has emerged in recent years as a promising alternative to control the fermentation process and to promote quality development of coffee product. However, scarce information is still available about the effects of controlled starter cultures in coffee fermentation performance and bean quality, making it impossible to use this technology in actual field conditions. A broader knowledge about the ecology, biochemistry and molecular biology could facilitate the understanding and application of starter cultures for coffee fermentation process. This review provides a

comprehensive coverage of these issues, while pointing out new directions for exploiting starter cultures in coffee processing.

1.2. INTRODUCTION

Since the opening of the first coffee house in Mecca at the end of the fifteenth century, coffee consumption has greatly increased all around the world. The reasons for this continuous increase include, for example, improved cup quality and a change in coffee's image as a functional food (FARAH, 2012). A critical step determining the coffee beverage quality is the postharvest practices to make the beans suitable for transport and roasting. Three different methods are employed in producing countries to process coffee fruit, referred to as dry, wet and semi-dry (PANDEY et al., 2000). These methods involve removal of the waste from the crop and taking off the outer layers of the beans. Following postharvest processing on farms, coffee beans can be transported to industrial plants, where semi-manufactured or finished products are obtained for commercialization.

Fermentation in coffee refers to the process during which the pulp and mucilage surrounding the seeds are broken down through microbial action. This process produces a vast array of metabolites, such as organic acids, higher alcohols and esters, which will later add complexity and depth to a coffee (MUSSATTO et al., 2011; PEREIRA et al., 2014). The microorganisms responsible for the fermentation are indigenous species that originate as natural contaminants of the process, including yeasts (e.g., Pichia, Debaryomyces, Sacharomyces and Candida species), bacteria (e.g., members of the family Enterobacteriaceae, lactic acid bacteria and Bacillus spp.) and filamentous fungi (e.g., Aspergillus, Penicillium and Fusarium species) (VAUGHN et al., 1958; FRANK et al., 1965; VAN PEE and CASTELEIN, 1972; GAIME-PERRA et al., 1993; MASOUD et al., 2004; SILVA et al., 2008; VILELA et al., 2010; LOEW, 2014, PEREIRA et al., 2014).

The microbial ecology of coffee bean fermentation has been reorganized for over 100 years, and numerous studies have been conducted in different countries to determine the microbial species associated with this process. However, although

recent progress has been made in defining the diversity and role of microbial species associated with coffee fermentation, scarce information on the growth and activities of specific microbial groups and their impacts upon final product quality and process efficiency, are available. Consequently, what specific species are essential for the fermentation and development of a good beverage are the questions to be raised and responded. This article reviews the composition and metabolism of coffee fermentation microflora, its impact on the quality of commercial coffee beans, and new directions for exploiting starter cultures in coffee processing

1.3. BOTANY AND PRODUCTION OF COFFEE

Coffea is a genus of flowering plants whose seeds are used to make coffee beverage. The *Coffea* genus contains around 100 species (CHARRIER and BERTHAUD, 1985), all being native to the inter-tropical forest of Africa and Madagascar (BRIDSON and VERDCOURT, 1988). Within these species, *Coffea arabica* (Arabica) and *C. canephora* (Robusta) represent respectively about 70 % and 30 % of the coffee market (COLTRO et al., 2006).

Coffee tree is grown primarily in the globeencircling geographic region between latitudes 30° N and 30° S, known as “the coffee belt” (FIGURE 1). The coffee belt provides the ideal tropical climate for coffee production, where coffee trees grow from sea level to 2000 m (ANDERSON and SMITH, 2002). Summarized by regions, the main coffee-producing countries are: South American (e.g., Brazil, Colombian and Peru), Central American (e.g., Honduras and Guatemala), North American (e.g., Mexico), Africa (e.g., Ethiopia), Indonesia and India (FIGURE 1).

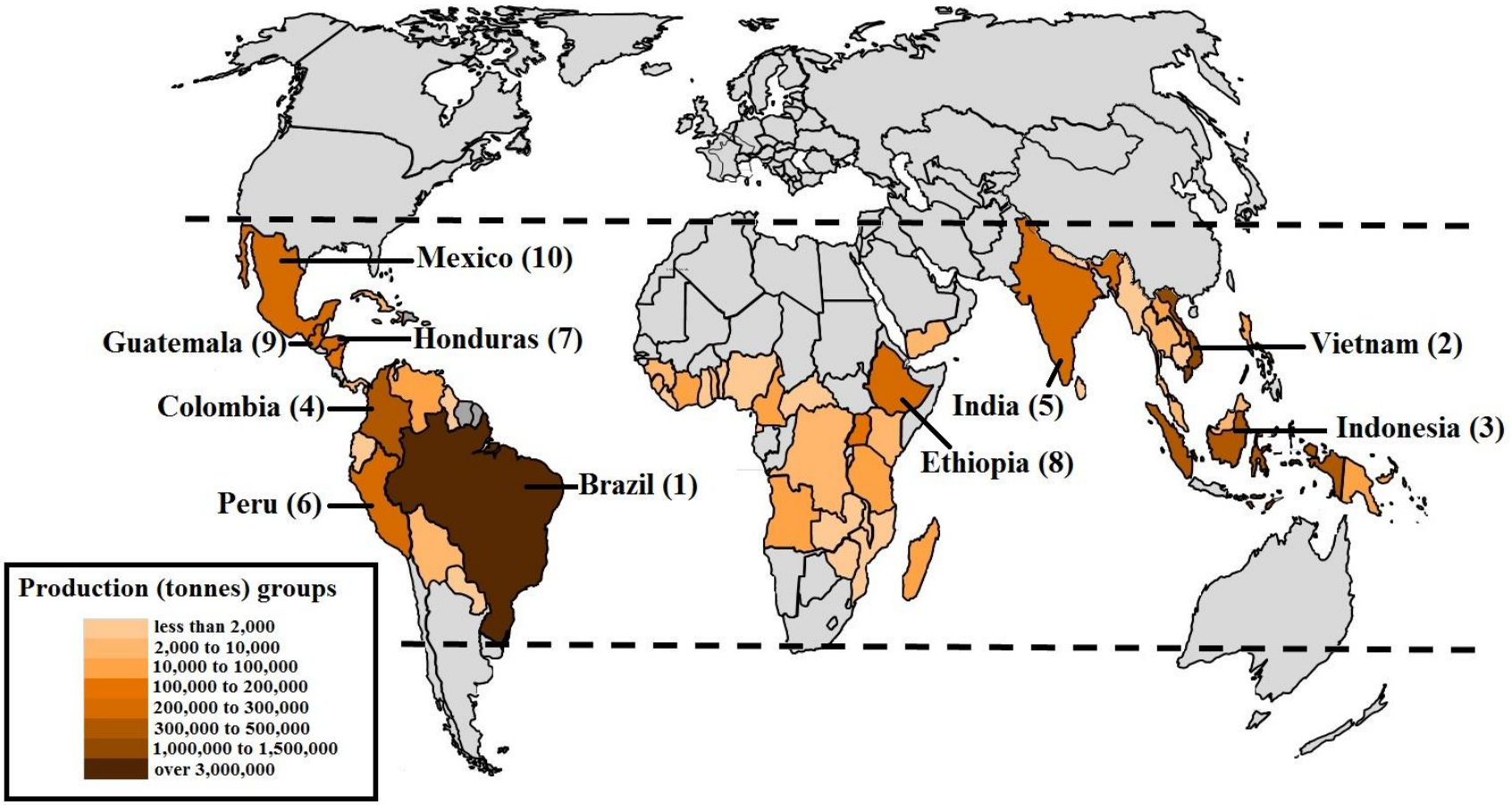

FIGURE 1 - GEOGRAPHICAL DISTRIBUITION OF COFFEE PRODUCTION (FAO, 2013). THE DASHED LINES INDICATE “THE COFFEE BELT” ZONE. THE HEATMAP WAS GENERATED BY USING VISUAL BASIC FOR APPLICATIONS (VBA) IN CONJUNCTION WITH MICROSOFT® OFFICE EXCEL VERSION 7.0

The coffee cherry is a bilocular ovoid drupe that contains two plane-convex seeds called flat seeds (DE CASTRO and MARRACCINI, 2006). A coffee fruit is shown in FIGURE 2.

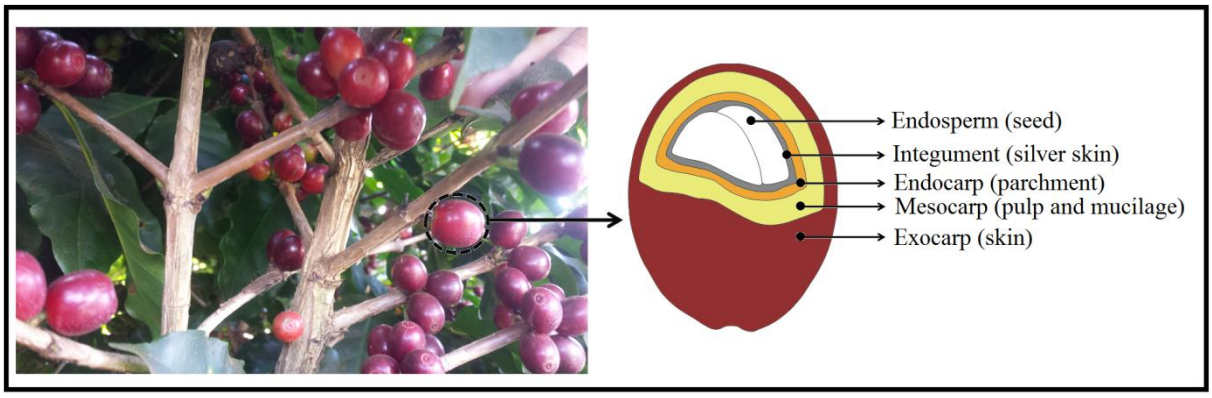

FIGURE 2 - PHOTOGRAPHS OF COFFEE FRUIT AND ITS SECTION

In a ripe cherry the exocarp is red or yellow. There is no scientific evidence that exocarp color has any influence on the beverage quality or any other agronomic characteristic of coffee (SAKIYAMA and FERRÃO, 2014). The mesocarp is subdivided in outer mesocarp (pulp) and inner mesocarp (mucilage). It is a gelatinous, translucent and sweet substance, which is richer in sugar (ELIAS, 1978). The polysaccharide constitution of mucilage is 30% pectic substances, 8% cellulose and 18% of non-cellulosic polysaccharides (ELIAS, 1978; AVALLONE et al., 2001). The presence of the pectic mucilage layer is a serious obstacle to the rapid drying of the beans; also, it is a very favorable medium for the development of different microorganism groups. The endocarp is a hard and lignified tissue with pale yellow color. The silver skin (integument) envelops the seed and is adherent and brown in robusta coffee. The seed (endosperm) is a storage tissue of nutrients for the embryo development. Many volatile and non-volatile components such as acids, aldehydes, ketones, sugars, proteins, amino acids, fatty acids, carbohydrates, trigonelline, caffeine, phenolic compounds and enzymes are found in the seed (BIOSCI, 1993; MENEZES, 1994). There is an active transport of sugars and caffeine between the pericarp and the endosperm; however, the diffusion of these biochemical compounds is limited by the presence of the endocarp, which is a physical barrier. The transport

should first occur from the pericarp to the perisperm (future silver skin), where the biochemical compounds should be accumulated before being further transported to the endosperm (DE CASTRO and MARRACCINI, 2006; SAKIYAMA and FERRÃO, 2014).

Coffee is an important plantation crop grown in more than 70 developing countries and is one of the most traded and consumed agricultural products worldwide, at times only surpassed by oil (SELVAMURUGAN et al., 2010; FAO, 2014). Its annual production has reached 8.4 million metric tons, with a turnover close to US $10 billion (ICO, 2011). Brazil is leading producer of coffee, supplying about a third of total production, followed by Vietnam, Indonesia, Colombia, India, Peru, Honduras, Ethiopia, Guatemala, Mexico and another 60 countries (FIGURE 1). The top five consumers are the USA, Brazil, Germany, Japan and France, while the Nordic countries have the world's highest coffee consumption per capita (PETIT, 2007). The most economically relevant Brazilian coffee cultivars are *Coffea arabica* cv. Mundo Novo, *C. arabica* cv. Catuaı´ Vermelho , *C. arabica* cv. Bourbon and *Coffea canephora* cv. Conillon (PERRONE et al., 2008). These cultivars are used in most commercial blends around the world (DUARTE et al., 2010).

1.4. POSTHARVEST PROCESSING

The quality of coffee beverage is strongly related to the chemical composition of the roasted beans but is also dependent on the postharvest processing (ILLY and VIANI, 2005). To produce coffee beans suitable for transport and roasting, there is a need to separate the seeds from the outer layers (skin, pulp, mucilage and parchment) enabling the reduction of 65% moisture content to 10-12%. Thus, the postharvest processes aim basically to remove the components surrounding the beans, so that the beans may be stored for many months without significant change in quality (TRUGO and MACRAE, 1984; TEXEIRA et al., 1995).

After harvesting, three different systems are used for processing of coffee fruit: dry, wet and semi-dry methods (FIGURE 3). The coffee fruits must undergo

either dry, semidry or wet processing as soon as possible after harvesting, otherwise uncontrolled natural fermentation may have negative impacts on the coffee quality.

The dry process, which results in so-called unwashed or natural coffee, is the oldest and simplest method of coffee processing. In this process, after harvest, the fruits are separated from impurities (sticks, stones, leaves), subsequently washed and classified according their maturation. The entire coffee fruits are then spread on the ground (earth, platforms, concrete or tarmac) in layers approximately 10-cm thick, heaped at night and respread each day. Drying is continued until the beans moisture decreases to 11–12% (FIGURE 3). To reduce dry time, mechanical dryer can be used after pre-drying in the sun for a few days. During the course of 10–25 days of sun drying a natural microbial fermentation occurs during which secreted enzymes break down the pulp and mucilage (SILVA et al., 2008). The dry process is manly used in countries where rainfall is scarce and long periods of sunshine are available to dry the coffee properly, such as Brazil and Ethiopia (SILVA et al., 2000). The drying operation is the most important step because it affects the final quality of coffee. While the over-dried fruits become brittle and produce too many broken (defective) seeds during hulling, on the other hand, fruits that are not sufficiently dried become more vulnerable to deterioration caused by filamentous fungi and bacteria attacks. Natural coffees that have been properly dry processed can be a quality product with their own market (ROTHFOS, 1980; DUARTE et al., 2010).

In wet processing, 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 allowed microbial degradation of remaining mesocarp layer (called mucilage) adhering to the parchment. The beans are then sun-dried to 10-12 % moisture content (FIGURE 3). The wet method decreased the time and area required for drying. However, the requirement of specific equipment and substantial amounts of water cause additional costs for the process (BÁRTHOLO and GUIMARÃES, 1997). This processing method emerged when arabica coffee began to be cultivated in tropical regions, such as Colombia, Central America and Hawaii, where the abundant rainfall and warm temperatures caused an immediate undesirable fermentation after harvest.

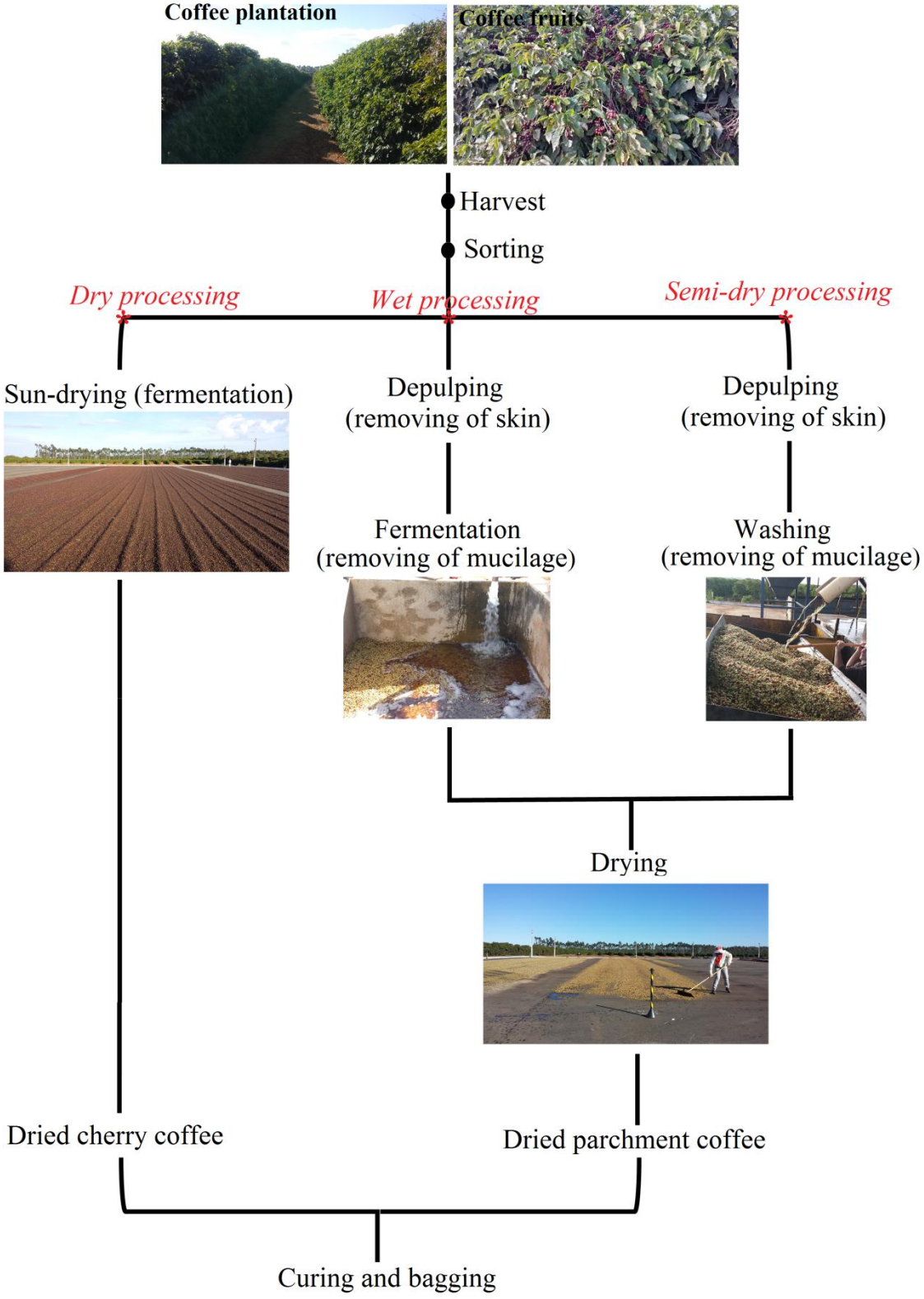

FIGURE 3 - STEPS OF THE DIFFERENT METHODS EMPLOYED TO PROCESS COFFEE FRUITS

The most practical way to avoid such detrimental fermentation was to remove the outer mesocarp tissue, rich in sugars, and submit the depulped fruits to an underwater tank process to achieve desirable fermentations (BRANDO, 1999). By this method, the fermentation can be controlled in terms of time, temperature and exchange of the water so that spontaneous development of microorganisms can be better managed to minimize any adverse impacts on coffee quality or to encourage desirable quality impacts (SILVA, 2014). The final product resulting from the wet processing method is called ‘‘washed” or ‘‘wet” coffee.

The semi-dry processing, also called pulped natural method, presents stages of both dry and the wet methods. This process started to be used in Brazil in the early 1990s (DUARTE et al., 2010; VILELA et al., 2010). The coffee fruits are selected (only mature red cherries) and depulped by mechanical process. After removing the pulp, the berries are transferred to concrete platforms for the drying stage during which fermentation also occurs to remove mucilage that is still adhered to the beans (VILELA et al., 2010). Thus, similar to the dry processing method, the depulped fruit is exposed to the local temperature and other environmental conditions. The aims of the semi-dry process is to go one step beyond the dry process and to mechanically separate unripe cherries from ripe ones in order to treat the ripe ones separately and improve coffee quality (BRANDO, 2010). This processing method can only occur in countries where the humidity is low and the coffee covered in the sweet mucilage can be dried rapidly without undesirable fermentation. Brazil has made this method famous and produces some of the best pulped natural coffees. The final product resulting from this processing method are called pulped natural coffees (TEXEIRA et al., 1995).

It is well accepted that green coffees resulting from the wet method yield roasted beans and coffee beverages, respectively, that are characteristically different from those produced with the dry method. Coffees from the wet processing are known to present better quality, less body, higher acidity and more aroma than the ‘unwashed` coffees (MAZZAFERA and PADILHA-PURCINO, 2004). There is no doubt that these flavour differences in part have to be attributed to differences in the thoroughness applied during either method of post-harvest treatment and the fact that only fully ripe coffee cherries are used for wet processing, whereas fruits of all stages of ripeness are utilized for dry processing (KNOPP et al., 2006). Bytof et al.

(2005) demonstrated that during post-harvest treatment various metabolic processes occur inside the coffee seeds which significantly alter the chemical composition of the green beans. This metabolism becomes evident by the conversion of glutamic acid into γ-aminobutyric acid mediated by an enzymatic α-decarboxylation. The reaction is related to a physiological drought stress situation and is specific to the mode of processing applied (BYTOF et al., 2005). Suchlike alterations in the pool of free amino acids— components which are considered as essential precursors of flavour and colour of the coffee brew (HOMMA, 2002) — may be suitable to explain some of the sensorial differences between washed and unwashed coffees. In addition, it have been suggested that the intense perception of ‘‘floral” and ‘‘fruity” aromas and high acidity in coffee processed by wet method can be the results of microbial metabolites (yeast and bacteria) produced during fermentation stage (MUSSATTO et al., 2011; PEREIRA et al., 2014).

The pulped natural coffees (semi-dry processed coffees) present an intermediate body between washed and unwashed ones. It is often sweeter than wet-processed coffees, has some of the body of a dry-processed coffee, but also retains some of the acidity of a wet-processed coffee. Because of this, pulped natural coffees are also strongly appreciated in blends for espresso coffee (TEIXEIRA et al., 1995).

1.5. OVERVIEW OF COFFEE FERMENTATION PROCESS

Coffee fermentation can be defined as the process where the coffee mucilage layer is degraded by combined action of enzymes that occur naturally in coffee or are produced by its natural microbiota (ROTHFOS, 1985; SCHWAN and WHEALS, 2003). Presently, the fermentation of coffee beans is conducted as a simple, traditional process. The process generally lasts 24 to 216 h according to cultivar or method employed (VILELA et al., 2010; VELMOUROUGANE, 2013; PEREIRA et al., 2014). The main chemical changes occurring during coffee fermentation are pectin degradation by pectinase enzyme present in the mucilage and microbial production of organic acids, ethanol, esters and other metabolites from the carbohydrates (WOOTTON, 1963; SIVETZ 1963; PEREIRA et al., 2014).

Although the characteristic flavor of coffee originates from the chemical composition of the bean, 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 (FRANK, 1965; SILVA, 2014; Pereira et al., 2014).

In wet processing, depulped coffee beans are held in fermentation tanks till the mucilage is completely digested and ready for drying. Fermentation times can vary substantially, from 12 to 48 h, depending on the environmental temperature. Higher temperatures and thicker mucilage layers accelerate fermentation (SIVETZ and DESROSIER, 1979). Throughout fermentation process, approximately 60% of the sugars are utilized as substrate for microbial growth which produces significant amounts of ethanol and acetic and lactic acids, resulting in lowered pH (from 5.5-6.0 to 3.5-4.0) (AVALLONE et al., 2001). In general, the wet process can be characterized as a mixed bacterial and yeast fermentation (AVALLONE et al., 2001; PEREIRA et al., 2014).

The dry process involves fermentation of whole fruit on the ground (earth, platforms, concreteor asphalt). Over the course of 10–25 days of sun drying, natural microbial fermentation occurs and produces ethanol and acetic, lactic, butyric and higher carboxylic acids (AMORIM and AMORIM, 1977). The microbiota involved in dry fermentation (encompassing bacteria, yeasts and filamentous fungi) are much more varied and complex than those found during wet fermentation. At the beginning of fermentation, the high water activity of the pulp-mucilage (~0.9) and a pH value of 6.5 favor the growth of bacteria. As fermentation progresses, the pulp–mucilage composition changes due to endogenous metabolic activity of the beans and the metabolic activity of bacteria. These changes reduce the pH to 5.5–5.8 and water activity to 0.7–0.8 and encourage the growth of yeasts (SILVA et al., 2000, 2008, 2014).

The fermentation in semi-dry processing occurs when the depulped fruits are exposed to the local temperature and other environmental conditions (relative humidity, sunlight, rain). The microbial succession is similar to the ecological succession described in dry coffee fermentations (SILVA et al., 2008), except that the presence of filamentous fungi was rarely observed (VILELA et al., 2010).

1.6. SOURCE OF MICROORGANISMS THAT CONDUCT THE FERMENTATION

The microorganisms responsible for the fermentation of coffee beans are indigenous species that originate as natural contaminants of the process. During the stages of harvest, transport and depulping, the pulp and beans become contaminated with a variety of microorganisms, many of which contribute to the subsequent fermentation (AGATE and BHAT, 1966; SILVA et al., 2000; AVALLONI et al., 2001). The different sources that microorganisms can access the fermenting coffee pulp–bean mass are summarized in FIGURE 4.

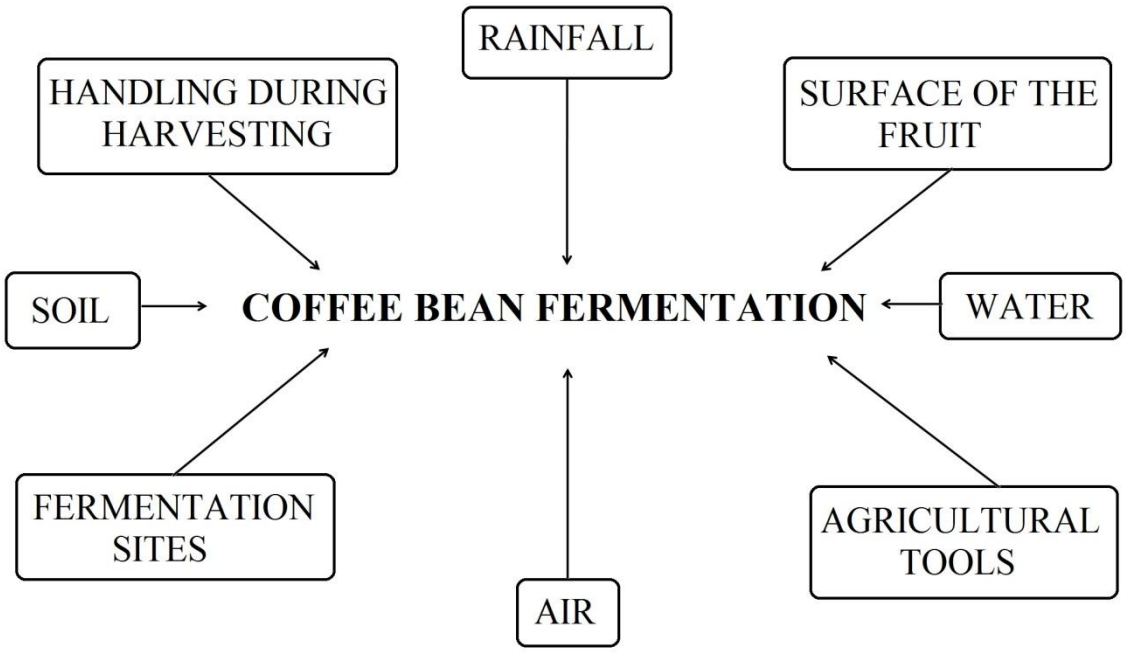

FIGURE 4 - DIFFERENT SOURCES THAT MICROORGANISMS ACCESS COFFEE FERMENTATION

Frank et al. (1965) demonstrated that certain bacteria species belonging to the family enterobacteria associated with coffee fermentations in Hawaii came from the surfaces of the cherries and the coffee plantation soil, while Avalloni et al. (2001) observed that the water used for pulping was usually highly contaminated by aerobic

mesophilic microflora (5.2 x10^6 cfu/ml), mainly composed of enterobacteria (2.3 x 10^4 cfu/ml).

Lactic acid bacteria have been associated mainly with fresh coffee cherries (DJOSSOU et al., 2012; LEONG et al., 2014). With regard to the habitat origins of yeast, Agate and Bhat (1965) reported the presence of certain species on the cherry surfaces, and evidence was adduced to show that the natural fermentation of coffee was the result of activity of microflora from the cherry surface itself rather than that of flora of air or water.

Research on the microbiota of wine fermentations may suggest mechanisms by which microorganisms access coffee bean fermentations. The surfaces of grape berries are considered to be a primary source of microorganisms associated with wine production. Many factors affect the microbial ecology of this surface, including degree of fruit maturity, climatic conditions, applications of agrichemicals and damage to the grape berry (insect, bird or animal attack, mechanical damage). Consequently, the proportion of damaged raw material has important influences on the microbial ecology of the overall fermentation process and product quality (FLEET et al., 2003; FLEET, 2008; BARATA et al., 2012). These conclusions can be applied to coffee bean fermentations, and indicate the need for a program of research to understand the microbial ecology of coffee cherries and processing sites.

1.7. PULP PECTIN DEGRADATION DURING FERMENTATION

In coffee processing, pulping of the coffee cherries removes their skin and pulp, leaving a viscous mucilage adhering to the parchment. This highly hydrated tissue, rich in pectic substances, is an obstacle to further drying of the beans. Pectin is a complex heteropolysaccharide composed of D-galacturonic acid residues joined by α-1.4-linkages, which form homogalacturonan chains (BE MILLER, 1986). The main enzymes involved in coffee fermentation is poligalacturonase (PG) which catalyses the hydrolysis of a-1.4 glycosidic bonds into pectic acid (poligalacturonic acid); pectin lyase (PL) which acts catalysing pectin breakage by transelimination, releasing insaturated galacturonic acids. The third enzyme is pectin methylesterase

(PME) responsible for the de-esterification of the methoxil group of the pectin forming pectic acid and methanol (SILVA et al., 2012).

A common assertion to explain the pectin degradation during coffee fermentation is that mucilage pectic substances are extensively degraded by the natural microflora and/or endogenous coffee enzymes; however, there are still conflicting views in reaching this. Many studies have shown the pectinolytic activity in yeasts (e.g., *Candida, Pichia, Kluyveromyces, Schizosaccharomyces* and *Saccharomyces* species) and bacteria (e.g., *Bacillus, Aerobacter, Escherichia* and *Erwinia* species) isolated from coffee fermentation process, and suggested their involvement in the degradation of the mucilage (Vaughn et al., 1958; Frank and DE LA CRUZ, 1964; FRANK et al., 1965; AGATE and BHAT, 1966, MASOUD and JESPERSEN, 2006; SILVA et al., 2012; PEREIRA et al., 2014). However, contrary to this hypothesis, Avallone et al. (2001) showed that the number of pectolytic microorganisms did not increase during fermentation of coffee beans by the wet method. Furthermore, the isolated pectolytic bacterial strains produced only pectate lyase activities that are unable to degrade highly methylesterified coffee pectic substances (CASTELEIN et al., 1976; AVALLONE et al., 2001). Finally, a histological examination of mucilage cells showed that, after fermentation, their cell walls still contained pectic substances (AVALLONE et al., 1999). These results lead to the conclusion that depolymerization of pectic substances by pectolytic microorganisms does not occur or is negligible during fermentation.

A third hypothesis suggests that the pectin-rich mucilage is degraded neither by endogenous pectolytic enzymes nor by pectolytic microorganisms, but due to the acidification process (CALLE, 1965; WOOTTON, 1965; AVALLONE et al., 2002). Microbial growth is necessary but the microflora does not directly participate in mucilage degradation by enzyme production. Its role is to produce metabolites such as organic acids (lactic and acetic acids) inducing a pH decrease (CALLE, 1965; LOPEZ et al., 1989). As a consequence, the mucilage cell walls swelling capacity in water is modified as well as their bound calcium (AVALLONE et al., 2002). These alterations loosen the polysaccharide network with a clear textural change. These mechanisms are well known in the cellular response to microbial attack observed in plant/pathogen interactions (D'AUZAC, 1996).

1.8. MICROBIOLOGICAL STUDIES EVOLUTION OF COFFEE FERMENTATION

Studies on the microbiology of coffee fermentations have been performed over the lasts 100 years in many coffee-producing countries like Brazil, Mexico, Colombia, Tanzania, India, Ethiopia, Hawaii, Taiwan and Thailand (VAUGHN et al., 1958; SILVA et al., 2000, 2008; MASOUD et al., 2004; AVALLONE et al., 2001; DE BRUYNE et al., 2007; VILELA et al., 2010; VELMOUROUGANE, 2013; LEONG et al., 2014; PEREIRA et al., 2014). Questions about this microbial action in coffee processing started in 1907 when Loew attributed the fermentation process to yeasts that formed ethyl alcohol, carbon dioxide, and later acetic acid. Later, Lilienfeld-Toal (1931) isolated several species of yeast and bacteria from coffee fermentation conducted in Brazil. The bacteria of coliform group was the dominant and it was thought that they were the cause of acid production during fermentation process. The author observed that the mucilage adhering to coffee beans was loosened before a high production of acid, and believed that the pulp may be decomposed by the nature enzymes of the mature coffee.

Vaughn et al. (1958) was the first to confirm the presence pectinolytic coliform bacteria associated with coffee fermentation. They observed that during the first 12 to 24 hr of fermentation the pectic material in Brazilian coffee cherries was degraded by coliform-like bacteria, resembling *Aerobacter* and *Escherichia*. These microorganisms were abundantly present on the cherry surfaces. Other pectinolytic bacteria (*Bacillus*) as well as filamentous fungi were also isolated.

Frank and Dela Cruz (1964) and Frank et al. (1965) isolated bacteria from wet fermentation trials at laboratory scale in Hawaii. They observed that the predominant population responsible for the degradation of the mucilage was *Enterobacter dissolvens*. The fermentation process was faster after the inoculation from decomposing whole cherries proving the microorganism action. Species of *Paracolobactrum* and *Escherichia* were also isolated but were not able to degrade the mucilage. Agate and Bhat (1966) reported the yeasts *Kluveromyces marxianus, Saccharomyces bayanus, S. cerevisiae var. ellipsoideus*, and *Schizosaccharomyces* sp., as predominates during coffee fermentation in India. Bacteria belonging to the genera *Streptococcus, Pseudomonas, Flavobacterium* and *Proteus* were also

isolated. The presence of yeast and bacteria in the surface of the mucilage of Congo coffee fruits was reported by Van Pee and Castelein (1972), who observed the presence of *Enterobacter* ssp., *Hafnia* spp. and *Klebsiella* spp, in the bacteria group and *Candida* ssp., *Saccharomyces* ssp., *Rhodotorula mucilaginosa* and *Torulopsis fumata* in the yeast group.

All these earlier studies, while valuable in their extension of knowledge, were limited in their contributions because of inadequacies within the ecological and taxonomic methodologies available at the time. The recent applications of newly developed molecular methods to the study of microbial ecology overcomes many of these limitations and have now lead to major advances in understanding the roles of specific yeasts, bacteria and fungi in coffee fermentations (MASOUD et al., 2004; SCHILLINGER et al., 2008; VILELA et al., 2010; PEREIRA et al., 2014). However, the overall microbiology and biochemistry of coffee fermentation is poorly studied when compared to other fermentation processes and are, therefore, complex and, still, little understood.

A survey on the microbial diversity present in different types of coffee processing and producing countries (TABLE 2, 3, and 4) shown that the distribution of the taxa of yeast and bacteria is highly variable from one coffee ecosystem to another. It demonstrates that growth rate and yield of microorganisms are governed by a multitude of ecological factors, such as temperature, pH, oxygen availability, plant variety and quality of the fruit, method of processing and management after harvesting (ARUNGA 1982; JONES and JONES, 1984; SCHWAN and WHEALS, 2003). An important variable is the load of contaminating microorganisms at the start of fermentation and their maximum growth during the process (TABLE 1). This has been reported to range between 1.0×10^4 to 1.0×10^9 cfu/g for total bacteria and 5.1×10^2 to 5.0×10^7 cfu/g for yeast, depending on the study. Factors affecting this initial 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. Microorganisms grow very fast in coffee pulp at the ambient temperatures (25-30°C) of tropical climates (PEREIRA et al., 2014). Consequently, short delays between bean removal and transport to fermentation can have a major impact on the levels of microorganisms at the start of fermentation.

TABLE 1 - INITIAL AND MAXIMUM POPULATION OF YEAST AND BACTERIA IN COFFEE FERMENTATION BY THE DIFFERENT METHODS

Processing method	Country	Yeast cfu/g		Bacteria cfu/g		Reference
		Initial population	Maximum population	Initial population	Maximum population	
Wet	India	4.3×10^4	5.5×10^6	3.2×10^4	6.59×10^5	Agate and Bhat (1966)
Wet	Hawaii	6.2×10^4	6.6×10^4	NA	NA	Frank et al. (1965)
Wet	Mexico	3.9×10^4	1.0×10^6	1.0×10^7	1.0×10^8	Avallone et al. (2001)
Wet	Tanzania	4.0×10^4	5.0×10^7	NA	NA	Masoud et al. (2004)
Wet	Brazil	5.01×10^2	1.4×10^7	NA	NA	Pereira et al. (2014)
Semi-dry (Arabica)	India	3.9×10^5	7.9×10^5	1.9×10^5	1.2×10^6	Velmourougane (2003)
Semi-dry (Robusta)	India	2.51×10^5	1.3×10^6	1.3×10^5	6.3×10^5	Velmourougane (2003)
Semi-dry	Brazil	5.0×10^3	7.9×10^6	5.0×10^5	1.0×10^7	Vilela et al. (2010)
Dry	Brazil	1.0×10^3	1.0×10^6	1.0×10^4	1.0×10^9	Silva et al. (2008)

NA.: Not analyzed

1.8.1. Diversity and function of yeast

Yeasts are among the most frequently isolated microorganisms from fermenting coffee beans. They are considered to be essential to the fermentation process and development of coffee beverage flavor. Consequently, yeast are the microbial group most widely studied in coffee fermentations which metabolic function has been elucidated in recent studies (EVANGELISTA et al., 2014 a,b; PEREIRA et al., 2014). Even so, this knowledge was not sufficient to enable their use as starter culture for this process.

Early studies of coffee yeasts were conducted by Agate and Bhat (1966) and Van Pee and Castelein (1971) in wet fermentations in India and Congo, respectively. A diversity of yeast was found, including species of *Saccharomyces marxianus* (*Kluyveromyces marxianus* (EC Hansen) van der Walt (1971)), *S. bayanus*, *S. cerevisiae* var. ellipsoideus and *Schizosaccharomyces* spp. (AGATE and BHAT 1966) and *Candida guilliermondii* var. *membranifaciens*, *C. parapsilosis, C. pelliculosa*, *Saccharomyces cerevisiae, S. marxianus, Rhodotorula mucilaginosa* and *Torulopsis fumata* (VAN PEE and CASTELEIN, 1971).

More detailed ecological studies of coffee yeasts have now been conducted in most coffee producing-countries and the main findings of these studies are summarized in TABLE 2. Avallone et al. (2001) found that yeast isolated from wet fermentation in Mexico were varied and consisted of classical strains found in plants, like *Cryptococcus laurentii, Kloeckera apis apicuata, Cryptocccus albidus* and *Candida guilliermondii*. Detailed study of yeasts involved in fermentation of coffee beans in East Africa was reported by Masoud et al. (2004) who used both traditional culture methods (viz., genotyping using ITS–PCR and sequence analysis of the D1/D2 domain of the 26S rRNA gene) and culture independent molecular method (viz., denaturing gradient gel electrophoresis (PCR-DGGE)) for the isolation and characterization of yeasts. *Hanseniaspora uvarum* was the predominant yeast with population size of $2.6 \times 10^5 - 1.5 \times 10^7$ cfu/g, while *Kluyveromyces marxianus, Candida pseudointermedia, Issatchenkia orientalis, Pichia ohmeri* and *Torulaspora delbrueckii* occurred in concentrations of 10^3 cfu/g. Although the authors reported that a good agreement was found between the profiles obtained by the DGGE and

the findings obtained by traditional isolation and further identification of yeasts, the species *Saccharomyces cerevisiae* and *Candida xestobii* were not identified by cultivation, but by the DGGE technique. The authors concluded that DGGE was seems to be an efficient tool for studying yeast diversity during natural coffee fermentation. In addition, DGGE is a fast technique compared to time-consuming cultivation and isolation methods, especially when investigating large numbers of samples.

In Brazil, studies on yeast diversity have been performed for all three coffee processing methods, i.e., dry (SILVA et al., 2000, 2008), semi-dry (VILELA et al., 2010) and wet processing (PEREIRA et al., 2014). Silva et al. (2000, 2008) reported the isolation of 200 yeasts during fermentation of coffee by the dry method. A rich diversity was found encompassing 15 species, with *Debaryomyces hansenii, Pichia guilliermondii, P. ofunaensis* and *Arxula adeninivorans* being the most prevalent (TABLE 2). These species were isolated throughout the process but were most prevalent at 14–18 days when the total yeast population had increased to maximum values of about 10^6 cfu/g. Later, Silva et al. (2012) demonstrated that seven species (viz., *Debaryomyces hansenii, D. polymorphus, Pichia anomala, P. holstii, P. burtonii, P. guilliermondii* and *Arxula adeninivoran*s) were pectinolytic and may function to break down pulp and mucilage pectin. Also in Brazil, Vilela et al. (2010) applied the frist comprehensive polyphasic approach in coffee fermentation, encompassing culture dependent and culture independent methods, to study the diversity of yeast during the fermentation of semi-dry processing method*. Pichia anomala* was the dominant throughout the fermentation process followed by *Rhodotorula mucilaginosa, Saccharomyces bayanus, Saccharomyces* sp., and *Torulaspora delbrueckii*. Other species less frequently isolated were *Arxula* sp., *C. ernobii*, *C. fukuyamaensis, C. membranifaciens, C. carpophila, Hanseniaspora uvarum, Kloeckera* sp., *Kluyveromyces* sp., *Pichia caribbica* and *S. cerevisiae*.

TABLE 2 - YEAST DIVERSITY IN COFFEE FERMENTATION FROM DIFFERENT COUNTRIES

Country	Processing	Species isolated*	Reference
Brazil	Dry	***Arxula adeninivorans***, ***Pichia ofunaensis***, *P. acaciae*, *P. anomala, P. ciferii*, *P. jadinii*, *P. lynferdii, P. sydowiorium*, *Blastobotrys proliferans, Candida auringiensis,C. glucosophila, C. incommunis, C. paludigena, C. schatarii, C. vartiovaarae, Citeromyces matritensis, Geotrichum fermentans, Saccharomyces cerevisiae*, *Saccharomycopsis fermentans*, *S. fibuligera*, *Schizosaccharomyces pombe*, *Sporopachydermia cereana*, *Trichosporonoides oedocephales, Williopsis saturnus* var. *sargentensis.*	Silva et al. (2000)
Brazil	Dry	***Debaryomyces hansenii***, *D. polymorphus, D. polymorphus,* ***Pichia guilliermondii***, *P. guilliermondii, P. burtonii, P. anomala, S. smithiae, P. Burtonii, P. sydowiorum, P. subpelliculosa, Candida saitoana, C. fermentati, C. membranifaciens, Stephanoascus smithiae, Saccharomyces cerevisiae, Arxula adeninivorans. A. adeninivorans.*	Silva et al.(2008)
Brazil	Semi-dry	***Pichia anomala***, *P. caribbica,* ***Rhodotorula mucilaginosa***, ***Saccharomyces sp., S. bayanus,*** *S. cerevisiae,* ***Torulaspora delbrueckii***, *Arxula sp., Candida ernobii, C. fukuyamaensis, C. membranifaciens, C. carpophila, Hanseniaspora uvarum, Kloeckera sp., Kluyveromyces* sp.	Vilela et al. (2010)
Brazil	Wet	***Pichia fermentans***, *P. guilliermondii, P. caribbica, Hanseniaspora opuntiae. Candida glabrata, C. quercitrusa, Saccharomyces* sp.	Pereira et al. (2014)
Mexico	Wet	***Kloeckera apis apicuata***, *Cryptococcus laurentii, C. albidus, Candida guilliermondii.*	Avallone et al. (2001)
Tanzania	Wet	***Pichia kluyveri***, ***P. Anomala***, ***Hanseniaspora uvarum***, *Candida pseudointermedia, Kluyveromyces marxianus, Issatchenkia orientalis, Torulaspora delbrueckii, Eremothecium coryli.*	Masoud et al. (2004)
India	Wet	***Saccharomyces marxianus*** (*Kluyveromyces marxianus*), *S. bayanus, S. cerevisiae* var. *ellipsoideus*, *Schizosaccharomyces* spp.	Agate and Bhat (1966)
India	Wet	***Saccharomyces marxianus* (*Kluyveromyces marxianus*)**, *S. bayanus, S. cerevisiae var. ellipsoideus, Schizosaccharomyces* sp.	Agate et al. (1965)
India	Semi-dry	***Saccharomyces* sp., *Shizosaccharomyces* sp.**	Velmourou. (2013)

*Predominant species indicated in bold type.

Recently, Pereira et al. (2014) reported the dominance of *Pichia fermentans* in coffee fermentation by the wet method in Brazil, followed by *Candida glabrata, quercitrusa, Saccharomyces* sp., *Pichia guilliermondii, Pichia caribbica* and *Hanseniaspora opuntiae*. The authors reported that the phylogenetic position of some isolates belonging to the genus *Saccharomyces* had less than 97% similarity to known species, and suggested the isolation of a new candidate species of the genus *Saccharomyces*.

The metabolic activity of yeasts is undoubtedly the most studied between the coffee-related microbial groups. Primarily, the yeasts initiate an alcoholic fermentation of pulp sugars to produce mainly ethanol and carbon dioxide, and a vast array of secondary metabolites such as higher alcohols, organic acids, esters, aldehydes, ketones, sulphur and nitrogenvolatiles, as has been well established for other fermented commodities (ROMANO et al., 2003; UGLIANO and HENSCHKE, 2009; PEREIRA et al., 2012; SCHWAN et al., 2014). These secondary metabolites generally have high flavor impact and will also diffuse into the bean to affect coffee beverage character, although little research has been done on this topic (EVANGELISTA et al., 2014 a,b; PEREIRA et al., 2014). The ability to decrease the numbers of mycotoxin-producing fungi as well as producing pectinolytic enzymes during coffee fermentation are other mechanisms by which yeasts are considered to impact on coffee bean quality. These aspects will be better described in the Starter Culture Technology for Coffee Bean Fermentation section.

1.8.2. Diversity and function of lactic acid bacteria

Lactic acid bacteria are generally isolated in high populations during wet and semi-dry processing. However, this group of bacteria does not participate in dry coffee processing. Silva et al. (2000) was the only one study that isolated lactic acid bacteria from dry processing, but found a very low population and did not identify them to species level. It is possible that the anaerobic or low oxygen conditions present in wet fermentation favor the development of lactic acid bacteria (Silva, 2014).

Pederson and Breed (1946) reported the first study of lactic acid bacteria associated with coffee fermentation. From coffee cherry samples shipped by air from Mexico and Colombia to Geneva, N.Y., these authors isolated *Leuconostoc mesenteroides, Lactobacillus* ssp. and *Streptococcus faecalis*. Although their isolates were probably responsible for the acid detectable in later stages of fermentation, Pederson and Breed doubted the possible involvement of these bacteria in mucilage-layer decomposition. Years later, Frank et al. (1965) presented a similar hypothesis, which assigns the very low incidence of lactic acid bacteria in coffee fermentation in Hawaii due to their inability to decompose the cherry mucilage layer. Since then, various authors have reported the isolation and identification of lactic acid bacteria from coffee fermentation and these data are summarized in TABLE 1.

In wet processing, a range of studies have been applied to identify lactic acid bacteria in different producing countries. Avallone et al. (2001) identified the heterofermentative lactic acid bacteria *Leuconostoc mesenteroides dextranicum* and *Lactobacillus brevis* in Mexico. *Leuconostoc mesenteroides* grew primarily during the early phase of fermentation, however, at the final stage, a change of lactic acid population was observed with the *Lactobacillus brevis* appearance.

Schillinger et al. (2008) studied the involvement of *Leuconostoc* and *Weissella* species in coffee fermentation in Ethiopia and Tanzania through a molecular approach polyphasic encompassing genus-specific PCR method, repetitive extragenic palindromic-PCR (rep-PCR) and sequencing of the 16S rRNA gene. According to the results of the molecular methods, the 71 strains isolated analyzed belonged to the species *Leuconostoc citreum, L. mesenteroides, L. pseudomesenteroides, Weissella cibaria* and *W. soli*, and one strain consisted of the novel species *Leuconostoc holzapfelii*. Leong et al. (2014) also reported that species of *Leuconostoc* (such as *L. pseudomesenteroides* and *L. citreum*) and *Weissella* (such as *W. confusa* and *W. thailandensis*) were found in fresh coffee cherries from three different coffee farms in Taiwan. Other species related in this study were *Lactobacillus plantarum*, *Lactococcus lactis* subsp. *lactis, Enterococcus* sp., and *Enterococcus faecalis*. Besides, *Leuconostoc* and *Weissella* species, and *Lactobacillus plantarum* were also found in the silage of fresh coffee pulp collected from Ivory Coast (DJOSSOU et al., 2011). These findings indicate that the lactic acid

bacteria present in coffee fermentation probably originated from the fresh coffee cherries.

Vilela et al. (2010) reported the presence of lactic acid bacteria in coffee fermentation by the semi-dry method based on a combination of both traditional culture methods (viz., amplified ribosomal DNA restriction analysis (ARDRA) and rDNA 16S sequencing) and culture independent molecular method (viz., denaturing gradient gel electrophoresis (PCR-DGGE)). *Lactobacillus plantarum* was found as the predominant species followed by *Lactobacillus brevis*, *Leuconostoc mesenteroides* and *Lactococcus lactis*.

So far, little research has investigated the diversity of lactic acid bacteria in coffee fermentation while some studies have only focused on the isolation of this group from the coffee fruit. Thus, the function of lactic acid bacteria is not yet known. However, some species are encountered frequently and probably play some role in the fermentation. To date, the only function assigned to this group was its apparent antifungal activity and bacteriocin-like inhibitory substance-producing capability (DJOSSOU et al., 2011; LEONG et al., 2014). Therefore, the potential use of artificial inoculation of lactic acid bacteria to inhibit mould growth could be exploited during coffee processing. In addition, the action of lactic acid bacteria allows the pH to drop, preventing the proliferation of other bacteria and favoring the growth of yeast (MASSAWE and LIFA, 2010).

Metabolically, homofermentative lactic acid bacteria convert the available energy source (sugar) almost completely into lactic acid via pyruvate to produce energy and to equilibrate the redox balance. However, pyruvate can lead to the generation of many other metabolites such as acetate, ethanol, diacetyl and acetaldehyde. In addition, heterofermentative lactic acid bacteria produce lactic acid and ethanol as well as several short-chain fatty acids such as acetic acid and formic acid (HELINCK et al., 2004). These chemical compounds may contribute to the acidity and off-flavours of fermented coffee beans. In addition, although the diffusion of lactic acid into the coffee beans during fermentation process has not been reported, because it is nonvolatile, its excess may not be reduced during drying and impart sour flavor to the beverage. Similar phenomenon has been demonstrated for the cocoa fermentation in order to produce chocolate (PEREIRA et al., 2012; SCHWAN et al., 2014).

TABLE 3 - LACTIC ACID BACTERIA DIVERSITY IN COFFEE FERMENTATION FROM DIFFERENT COUNTRIES

Country	Processing	Species isolated	Reference
Brazil	Dry	*Lactobacillus* sp.	Silva et al. (2000)
Brazil	Semi-dry	***Lactobacillus plantarum*****,** *L. brevis, , Lactococcus lactis, Leuconostoc mesenteroides.*	Vilela et al. (2010)
Mexico	Wet	***Leuconostoc mesenteroides dextranicum****, Lactobacillus brevis*	Avallone et al. (2001)
Mexico/ Colombia	Not mentioned	***Leuconostoc mesenteroides****, Lactobacillus ssp., Streptococcus faecalis*	Pederson and Breed (1946)
India	Semi-dry	***Lactobacillus*** **sp.,** ***Leuconostoc*** **sp.**	Velmourou. (2013)
Ethiopia and Tanzania	Not mentioned	***Leuconostoc citreum****, L. holzapfelii L. mesenteroides, L. pseudomesenteroides, Weissella cibaria, W. soli.*	Schillinger et al. (2008)
Hawaii	Wet	*Leuconostoc mesenteroides*	Frank et al. (1965)
Taiwan	Not mentioned	***Leuconostoc pseudomesenteroi, L. citreum****, Weissella confusa, W. thailandensi, Lactobacillus plantarum, Lactococcus lactis subsp. lactis, Enterococcus sp., Enterococcus faecalis*	Leong et al. (2014)

*Predominant species indicated in bold type.

1.8.3. Other bacterial species

A broad bacterial diversity other than those of lactic acid bacteria have been isolated from coffee fermentations in the different processing methods and identified in genera such as *Bacillus, Paenibacillus, Acinetobacter, Streptococcus, Pseudomonas, Flavobacterium, Proteus, Aerobacter, Escherichia, Hafnia, Klebsiella, Tatumella, Paracolobactrum* and *Serratia* (TABLE 4). Generally, these bacteria are detected in freshly extracted beans and are thought to originate from exocarp (skin plus pulp), water, the surfaces of fermentation tanks and soil (AVALLONE et al., 2001; SILVA et al., 2008).

In dry processing, bacterial diversity have been only reported through of standard cultural methods (VAUGHN et al., 1958; VAN PEE and CASTELEIN 1972; SILVA et al., 2000, 2008). The presence of bacteria in the exocarp (skin plus pulp) was reported by Van Pee and Castelein (1972), who observed an initial population of 2.5×10^5 cfu/g belonging to the Enterobacteriaceae, nominally *Enterobacter dissolvens, E. aerogenes, E. cloacae, Hafnia* spp., *Klebsiella* spp. This population increased to about 1.5×10^7 cfu/g during the first 24 h and then decreased to 3.5×10^6 cfu/g by 72 h. Silva et al. (2000, 2008) found a very high bacterial density (10^6 and 10^9 cfu/g) in dry fermentation process in Brazil. Bacterial populations were predominant in coffee cherries sampled on the bush (time 0) where they represented 96.3% of the total isolated microorganisms. The bacterial population was greater than or equal to 50% of the microorganisms isolated until the 8th day and then decreased to <10% of the total isolates by the 14th day of fermentation. The diversity of bacteria isolated from these fermentations encompassed the species *Tatumella ptyseos, Pseudomonas putrefaciens, Proteus mirabilis, E. aerogenes, Acinetobacter* spp., *Bacillus subtilis*, *B. macerans* and *B. megaterium*. The *Bacillus* species predominated, representing some 50% in the bacterial isolates obtained. A number of these bacteria had the ability to degrade pectin, especially the *Bacillus* species (SILVA et al., 2013).

In wet processing, early studies reported the presence of species of *Erwinia, Paracolobactrum* and *Escherichia* (FRANK et al., 1965) and *Streptococcus*, *Pseudomonas, Flavobacterium* and *Proteus* (AGATE and BHAT, 1966) in coffee

fermentations in Hawaii and India, respectively. Avallone et al. (2001) studied the microbiota during wet processing of coffee in Mexico. Populations of 10^7–10^8 cfu/mL were observed throughout the fermentation, being manly attributed for the high microbial contamination from water used in the fermentation tanks. Aerobic, Gram-negative bacteria, represented by *Enterobacter herbicola*, *Klebsiella pneumonia*, *K. ozaenae* and *K. oxytoca* were the main bacteria species isolated during fermentation.

Vilela et al. (2010) found that bacterial counts in semi-dry fermentation of coffee processing in Brazil varied from 10^2 to 10^7 cfu/g. This population was largest during the first 24 h of fermentation (10^7 cfu/g) and decreased with the progress of fermentation (reaching to 10^2 log cfu/g). *Escherichia coli, Bacillus cereus, Bacillus megaterium, Bacillus macerans* and *Klebsiella pneumoniae* were the predominant bacterial species identified during the fermentation process. Other identified species were *Acinetobacter* sp., *Bacillus subtilis*, *Bacillus* sp., *Enterobacter herbicola*, *Escherichia coli*, *Klebsiella pneumoniae* and *Serratia* sp.

Further research is needed to understand how these “other” bacteria might impact on the fermentation process and coffee bean quality. It is clear that their growth occurs at high levels in the early stages of fermentation, but the only one function attributed for these bacterial groups is the pectinolytic enzymes production and possible contribution on the decomposition of the mucilage layer. Thus, the occurrence and significance of these bacteria species needs more consideration. In the case of those species that can lead to beans with grossly unacceptable odours and flavours, good hygienic management throughout the harvesting-fermentation chain is required to avoid this potential problem.

TABLE 4. BACTERIAL DIVERSITY IN COFFEE FERMENTATION FROM DIFFERENT COUNTRIES

Country	Processing	Species isolated (Predominant species indicated in bold type)	Reference
Brazil	Dry	***Enterobacter cloacae,*** *E. aerogenes, E. sakazakii, E. gergoviae,* ***Pseudomonad paucimobilis****, P. pseudoalcaligenes, P. cepacia, P. vesicularis, P. fluorescens, P. aeruginosa, Serratia liquefaciens, S. plymuthica, S. marcescens, Cedecea* sp., *Chromobacter violaceum, Citrobacter freundi, Flavobacterium odoratum, Hafnia alvei, Klebsiella oxytoca, K. ozaenae, Pasteurella haemolytica, Salmonella choleraesuis, S. enterica var. arizonae, S. paratyphi, Shigella dysenteriae, Tatumella ptyseos, Bacillus subtilis, B. cereus, B. anthracis, B. megaterium, B. stearothermophilus, B. laterosporus,* ***Cellulomonas*** **spp.***, Arthrobacter* spp.*, Microbacterium* spp.*, Brochothrix* spp., *Dermabacter* spp.	Silva et al. (2000)
Brazil	Dry	*Enterobacter agglomerans, Yersinia* sp., *Arthrobacter,* ***Bacillus cereus****, B. megaterium,* ***B. subtilis****, B. macerans, Acinetobacter* sp., *B. polymyxa.*	Silva et al. 2008
Brazil	Semi-dry	***Enterobacter agglomerans****, Erwinia herbicola,* ***Escherichia coli****, Klebsiella pneumoniae, Serratia* sp., *Bacillus* sp., ***B. cereus****,* ***B.macerans****,* ***B. megaterium****, B.subtilis, Acinetobacter* spp.	Vilela et al. (2010)
Mexico	Wet	***Klebsiella pneumoniae****,* ***K. ozaenae****,* ***K. oxytoca, Enterobacter herbicola****, Pseudomonas cepaciae, Chrysomonas luteola.* *Streptococcus* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Proteus* sp.	Avallone et al.(2001)
India	Wet	*Pseudomonas* sp., *Flavobacterium* sp., *Bacillus* sp.	Agate et al. (1965)
India	Semi-dry	*Erwinia dissolvens, Paracolobactrum aerogenoides, P. coliforme, P. intermedium, Escherichia intermedium.*	Velmourou.(2013)
Hawaii	wet	***Enterobacter cloacae,*** *E. aerogenes, E. sakazakii, E. gergoviae,* ***Pseudomonad paucimobilis****, P. pseudoalcaligenes, P. cepacia, P. vesicularis, P. fluorescens, P. aeruginosa, Serratia liquefaciens, S. plymuthica, S. marcescens, Cedecea* sp., *Chromobacter violaceum, Citrobacter freundi, Flavobacterium odoratum, Hafnia alvei, Klebsiella oxytoca, K. ozaenae, Pasteurella haemolytica, Salmonella choleraesuis, S. enterica var. arizonae, S. paratyphi, Shigella dysenteriae, Tatumella ptyseos, Bacillus subtilis, B. cereus, B. anthracis, B. megaterium, B. stearothermophilus, B. laterosporus,* ***Cellulomonas*** **spp.***, Arthrobacter* spp.*, Microbacterium* spp.*, Brochothrix* spp., *Dermabacter* spp.	Frank et al., 1965

1.8.4. Filamentous fungi

Detailed investigations of the involvement of filamentous fungi in coffee fermentation during dry and semi-dry processing have been reported by Silva et al. (2008) and Vilela et al. (2010), respectively, while no reports related to the involvement of filamentous fungi during wet processing. This is not unexpected given that the process only lasts for about 48 h and is essentially a high water activity environment that is more conducive for yeast and bacterial growth (SILVA, 2014). Thus, although it was suggested the presence of endophytic fungi in cherry fruits and also in the production environment, bacteria and yeast grow faster than filamentous fungi in wet fermentation environment conditions, competing for nutrient and site of colonization.

In semi-dry processing, Vilela et al. (2014) reported that the population of filamentous fungi was always less numerous than the bacterial and yeast populations. However, it was found a greater diversity of filamentous fungal species (14 different species) in the washed fruits sample, which demonstrated that the washing process was likely insufficient for the physical removal of fungi. In this study, the most frequently identified species during semi-dry processing were *Aspergillus tubingensis, Aspergillus versicolor, Cladosporium cladosporioides, Aspergillus* sp. and *Penicillium decumbens* with counts of 10^3-10^5 log cfu/g (VILELA et al., 2010). Some of the filamentous fungal species are associated with good quality coffee, such as *Cladosporium cladosporioides* (LICCIARDI *et al.,* 2005), while others depreciate the quality, including *Fusarium* and *Penicillium*, or may have safety concerns (*Aspergillus ochraceus*) (BATISTA et al., 2003; 2009). Among the mycotoxigenic species already reported (BATISTA et al., 2003, 2009), only *Aspergillus ochraceus* was detected in semi-dry processing method, indicating that this process minimised the colonization of toxigenic fungi (VILELA et al., 2010).

The dry process allows more fungal contamination and increases the possibility of mycotoxin production. Silva et al. (2001, 2008) isolated a total of 292 and 363 filamentous fungi colonies, respectively, from dry processing in Brazil. In general, *Aspergillus* was the most abundant genus besides *Penicillium, Fusarium* and *Cladosporium*, with 42.6% of the total fungi isolates (SILVA et al., 2008).

Aspergillus flavus, A. niger, A. ochraceus, A. tamarii, A. sydowii, A. foetidius and *A. dimorphicus* were the most frequent species, being detected mainly starting on the 8th fermentation day for coffee cherries on the ground, but were more abundant during storage, where they represented 59.6% of the total isolates.

The presence o filamentous fungi in coffee processing have been studied due to two main aspects, such as their potential to adversely affect coffee flavor (LIARDON et al., 1989; IAMANAKA et al., 2011) and produce toxins that adversely affect consumer health and safety (LEVI et al., 1974; MISLIVEC et al., 1983; NAKAJIMA et al., 1997). Levi et al. (1974) were the first to report occurrence of OTA in coffee beans. Since then, several studies have detected both OTA-producing fungi and OTA in green coffee beans (LEVI et al., 1974; MISLIVEC et al., 1983; NAKAJIMA et al., 1997; PITTET et al., 2001; TANIWAKI et al., 2003; PARDO et al., 2004; CHALFOUN et al., 2007). The main filamentous fungi that have been found in coffee with the potential to produce OTA were *Aspergillus ochraceus, A. carbonarius and A. niger* (NAKAJIMA et al., 1997; JOOSTEN et al., 2001; PITT et al., 2001).

1.9. THE FERMENTATION IMPACT ON COFFEE QUALITY

Over the years many studies have reported that the coffee bean fermentation process needs to be well controlled to ensure the development of microorganisms that give a high-quality beverage with good coffee aroma (AGATE and BHAT, 1965; FRANK et al., 1965; GOTO and FUKUNAGA, 1956; ARUNGA, 1982; AMORIM and AMORIM, 1977; EVANGELISTA et al., 2014 a,b; PEREIRA et al., 2014). Failure in fermentation can result in the development of microorganisms that adversely affect coffee character and flavor. Coffee beans resulting from such fermentations are often referred to as “stinkers” (FRANK et al., 1965; ARUNGA, 1982).

Unsatisfactory demucilaging can result from insufficient as well as excessive fermentation (AGATE and BHAT, 1965). Under-fermentation interferes with the drying process, because the mucilage layer is not completely removed. Beans that are under-fermented contain residual mucilage and sugars that impede the drying process and encourage the growth of spoilage bacteria and fungi. However, it can be determined whether or not the beans are ready to drying by periodically testing small

samples from the fermentation tank. The coffee fermentation is adjudged "finished" when the parchment on touch "feels hard" and is free from slippery mucilage. If not, fermentation is continued until demucilaging is completed. Fermented parchment should be washed immediately after fermentation is completed to avoid these problems (AGATE and BHAT, 1965).

If possible, cherries should be processed soon after harvest to avoid overfermentation in the unpulped cherries (GOTO and FUKUNAGA, 1956). Overfermentation frequently results in adverse changes that affect the flavor and odor of the coffee produced (BECKLEY, 1930; GOTO and FUKUNAGA, 1956; SILVA, 2014). Most frequently, acidogenic fermentations by Enterobacteriaceae and acetic acid bacteria occur, and these may possibly be the major cause for cherry spoilage problems arising during processing (SILVA et al., 2008). For exemple, when fermentation is deficient or too slow, butyric or propionic acids can develop, both of which have an undesirable impact on coffee quality (AMORIM and AMORIM, 1977). Species of the *Bacillus*, especially *B. megaterium*, might be responsible for the propionic acid found in coffees processed via dry method (SILVA et al., 2008). The overfermentation can best be avoided by testing small samples during processing to detect complete demucilaging at its earliest stage, so that washing can be instituted before undesirable changes occur. Because of microbial buildup in machinery and equipment as the season progresses, thorough cleaning of the fermentation vats should be carried out frequently (AGATE and BHAT, 1965).

In addition, coffee fermentation and drying must be managed to control the growth of filamentous fungi that can produce off-flavors and mycotoxins (TANIWAKI et al., 2003; SUÁREZ-QUIROZ et al., 2004; BATISTA et al., 2009;). Studies have demonstrated that the high incidence of *Aspergillus, Penicillium* and *Fusarium* is associated with the loss of sensory quality in the final beverage (DAIVASIKAMANI and KANNAN, 1986; PASIN et al., 2002; CHALFOUN et al., 2005). This is probably due to the production of long-chain carboxylic acids (DAIVASIKAMANI and KANNAN, 1986; ALVES and CASTRO, 1998; PASIN et al., 2002; CHALFOUN et al., 2005).

1.10. STARTER CULTURE TECHNOLOGY FOR COFFEE BEAN FERMENTATION

Starter cultures are defined as a preparation or material containing large numbers of variable microorganisms, which may be added to accelerate and improve a fermentation process (HOLZAPFEL, 2002). Microorganisms selected to be used as starter cultures are expected to have some characteristics such as adapting easily to the raw material and process, developing sensory quality, extending shelf life, reducing the processing time and energy during the production, inhibiting food-related pathogenic microorganisms, as well as having probiotic, non-pathogenic, and non-toxigenic properties (CORSETTI et al., 2012). The use of functional starter cultures in the food fermentation industry is widely known, such as cheese, yogurt, bread, beer and wine (STEINKRAUS, 2004; SCHWAN et al., 2014). In many cases, the technology has evolved from a traditional, spontaneous fermentation to a controlled industrialized process based on the use of well defined microbial strains as starter cultures to conduct the fermentation (STEINKRAUS, 2004).

A controlled coffee fermentation process by use of starter culture may guarantee a standardized quality and reduce the economic loss for the producer. In the literature, only few studies have been reported to use of starter culture for coffee fermentation, although the attempt to control coffee fermentation has existed for over 40 years. Early studies performed by Calle (1957, 1965) and Butty (1973) reported the use of residual waters from a previous coffee fermentation as starter. These authors demonstrated that the time required to degrade the mucilage was shortened of 24 to 12 hours, without affecting the final quality of the coffee. A study conducted by Agate and Bhat (1965) was the first to effectively introduce a starter culture for coffee fermentation. In particular, they demonstrated that the incorporation of a mixture of three *Saccharomyces* species (viz., *Saccharomyces marxianus*, *S. bayanus*, *S. cerevisiae* var. *ellipsoideus*) aided the process by accelerating the mucilage-layer degradation. These authors also demonstrated that a pooled yeast enzyme preparation from *Saccharomyces* species was indeed very effective in the sense that it brought about complete elimination of pectic substances within 7 to 8 hr.

However, it was only in the 2000s that next studies on the starter cultures for coffee fermentation were performed again. Avallone et al. (2002) studied the microbial and physicochemical parameters of coffee fermentation inoculated by

different pectolytic microorganism strains isolated from a spontaneous process (viz., *Lactobacillus brevis* L166, *Erwinia herbicola* C26, *Bacillus subtilis* C12, *Kluyveromyces fragilis* K211). The authors noted that inoculations with these pectolytic strains do not speed up polysaccharide degradation. It was observed, however, that organoleptic characteristics of the beverages were not modified by addition of starter cultures, thereby demonstrating that the use of starter culture would be possible in order to limit off-flavour development and to standardize the final coffee quality. More specifically, the authors suggested that it would be preferable to use lactic acid bacteria in order to stay as close as possible to the natural fermentation. In study performed by Massawe and Lifa (2010), yeast strains of *Pichia anomala* and *P. kluyveri* and acid lactic bacteria identified as *Leuconostoc*/*Weissella* sp., Homofermentative *Lactobacillus* spp., Heterofermentative *Lactobacillus* spp., and *Enterococcus* strains were used as starter cultures in coffee fermentations against ochratoxin-producing *Aspergillus ochraceus*. The resulted demonstrated that the two yeast species in combination with selected strains of lactic acid bacteria could be used as biocontrol agents against *A. ochraceus*.

Recently, the use of aromatic yeasts in coffee fermentation to promote flavor development in coffee beverages have been investigated (EVANGELISTA et al., 2014a,b; PEREIRA et al., 2014). Pereira et al. (2014) evaluated the potential impact of selected yeast strains to promote flavor development in coffee beverages for inoculating coffee beans during wet fermentation trials at laboratory scale. The coffee beans were inoculated with single culture of *Pichia fermentans* YC5.2 and co-culture of *P. fermentans* YC5.2 and *Saccharomyces* sp. YC9.15, selected through specific characteristics suitable to drive the fermentation process, viz., coffee fermentation-associated stress tolerance, flavor-active ester compound production and pectinolytic activity. The use of these starter cultures in wet processing resulted in coffee beverages with modified flavors, being rated as having the higher sensory scores for fruity, buttery and fermented aroma. Evangelista et al. (2014 a,b) conducted inoculated coffee fermentations in dry and semi-dry processes using one of the following starter cultures: *Saccharomyces cerevisiae* UFLA YCN727, *S. cerevisiae* UFLA YCN724, *Candida parapsilosis* UFLA YCN448 and *Pichia guilliermondii* UFLA YCN731. The study showed variable results with respect to metabolites formed during the fermentation process and starter cultures growth, making it difficult to draw

firm conclusions. However, the authors observed that it was possible to produce a beverage with special aroma of caramel, herbs and fruits using the starter cultures *Candida parapsilosis* UFLA YCN448 and *Saccharomyces cerevisiae* UFLA YCN727 in coffee processed by the dry method (EVANGELSITA et al., 2014a), while a beverage with caramel and bitter flavors was produced in coffee using the starter cultures *Candida parapsilosis* UFLAYCN448 and *Saccharomyces cerevisiae* UFLAYCN727 in semi-dry fermentation method (EVANGELISTA et al., 2014b). Thus, these recent study have revealed that yeasts have a complementary role when associated with coffee quality through the synthesis of yeast-specific volatile constituents. However, it is not clear how such volatiles might impact on beverage flavor because, firstly, they must diffuse into the beans and, secondly, it is expected that they would be mostly lost by evaporation or otherwise transformed during the roasting operation. Further research detailing these kinetics during coffee fermentation process are required.

1.10.1. Criteria for selecting and developing starter cultures for coffee fermentation

Based on literature data of the microbial ecology and metabolism of coffee fermentation, criteria for the selection and development of starter cultures can be outlined. Basically, these criteria can be considered under three common categories for the food fermentation industry: (1) properties that affect the performance and efficiency of the fermentation process, (2) properties that determine coffee quality and character and (3) properties associated with the commercial production of coffee yeasts (FLEET, 2008).

(1) Properties that affect the performance of the fermentation process. Firstly, the ability of the starter culture to dominate the indigenous microbiota is one of the main criteria for its applicability (DAESCHEL and FLEMING, 1984; LEFEBER et al., 2012; CIANI et al., 2010; PERRONE et al., 2013). The selection of best-adapted strains offer the possibility of effectively using their over indigenous microorganims with lower capacity of adaptation within coffee environment conditions, which might help to develop new, stable, controlled coffee starter cultures for fermentation processes.

In the course of coffee fermentation process, microbial cells are affected by a plethora of stress conditions that can affect their viability and fermentation efficiency, such as limitation of nutrient availability, pH and temperature variation and accumulation of toxic metabolites (e.g., ethanol and organic acids) (WRIGLEY, 1988; AVALLONE et al., 2001; AVALLONE et al., 200, 2001; SILVA et al., 2008; MURTHY and NAIDU, 2011; Velmourougane, 2013; Pereira et al., 2014). The ability of yeast cells to detect and respond to the above-described stress conditions is essential for avoiding important losses of viability. Using strains that are not adapted to stress conditions is a mistake that can compromise an entire fermentative process (Querol et al., 2003). Pereira et al. (2014) used a culture agar plate assay to select stress tolerant yeast strains isolated from a spontaneous coffee fermentation process. Among 144 isolates tested, nine yeast strains were selected possessing the following characteristics: (i) growth capacity in a typical pH range of coffee fermentation (pH 2.0 to pH 8.0); (ii) osmotic pressure tolerance (growth detected in the presence of up to 50% glucose and fructose); (iii) heat tolerance (ability to growat temperatures of 37 to 43 °C); and (iv) metabolite accumulation tolerance (growth capacity up to 12 to 15% ethanol, 2% lactic acid and 2% acetic acid). These included strains of *Pichia fermentans, P. kluyveri, P. guilliermondii, H. opuntiae, C. glabrata* and *Sacharomyces* sp.

One of the main purposes of coffee fermentation is to remove the pectineous mucilage adhering to coffee beans. Therefore, the capacity to degrade the coffee bean mucilage (pectinolytic activity) is another important characteristic that should be considered for a coffee starter culture (MASOUD et al., 2005; MASOUD and JESPERSEN, 2006; SILVA et al., 2012; EVANGELISTA et al., 2014; PEREIRA et al., 2014). Masoud and Jespersen (2006) tested the ability of six strains of *Pichia anomala*, four strains of *Pichia kluyveri* and two strains of *Hanseniaspora uvarum* isolated from arabica coffee processing, to produce polygalacturonase (PG), pectin esterase (PE) and pectin lyase (PL). From this study, it can be seen that only PG was secreted by the investigated yeasts with high amounts produced by two strains of *Pichia anomala* and *P. kluyveri* at pH of 5.5 and 5.0 respectively, which is within the range of pH conditions that occur during coffee fermentation. Silva et al. (2012) tested the ability of bacteria and yeast strains isolated from dry and semi-dry coffee processing to produce pectinolytic enzymes. Among 127 yeasts isolates and 189 bacterial isolates tested, 15 showed the ability to produce PL. These isolates were

strains identified as *Bacillus cereus, B. megaterium, B. subtilis, Candida parapsilosis, Pichia caribbica, P. guilliermondii* and *Saccharomyces cerevisiae*. The majority of positive strains were isolated from dry processing, probably because in dry processing the coffee fruit is fermented with the skin, pulp and mucilage present, which favours microbial species that are able to use pectin as a carbon and energy source (SILVA, 2014). It was also observed that the pectinase activity of all bacteria isolates was an average of 1.7 times lower than the enzymes activity of the yeast isolates (SILVA et al., 2012).

The coffee pulp is substrate favorable to the production of ochratoxin A (OTA) especially when there is a microbiota already established which can alter the substrate and favors the production of OTA by *Aspergillus* or *Penicillium* species (Mantle and Chow, 2000). It has been reported that some yeasts can inhibit growth of filamentous fungi (MASIH et al., 2000). Masoud et al. (2005) and Masoud and Kaltoft (2006) evaluated the effects of *Pichia anomala*, *P. kluyveri* and *Hanseniaspora uvarum* on growth of *Aspergillus ochraceus* and production of OTA. The three yeasts were able to inhibit growth of *A. ochraceus*, with the two *Pichia* species showing the strongest effect. From the results of both these studies, it appears that two mechanisms are involved, viz., effect of volatiles produced by yeast metabolism and competition for nutrients. Thus, for the purpose of preventing production of OTA in coffee, the authors suggested the possibility of using *Pichia anomala* and *P. kluyveri* in biological control of OTA-producing fungi during coffee fermentation.

Recently, it was proposed that lactic acid bacteria species with high antifungal ability may have utility in decreasing the numbers of mycotoxin-producing fungi during coffee fermentation. Djossou et al. (2011) observed that, among 44 bacteria strains isolated from fresh coffee cherries, 10 possessed antifungal effect against *Aspergillus carbonarius*, further identified as belonging for *Lactobacillus plantarum* group. Leong et al. (2013) also observed that lactic acid bacteria species isolated from fresh coffee cherries (viz., *Leuconostoc pseudomesenteroides*, *Leuconostoc citreum*, *Lactobacillus plantarum, Weissella confusa* and *Enterococcus faecalis*) showed inhibitory activities against *Aspergillus flavus* ATCC 32592.

(2) *Properties that determine coffee quality and character*. The microbial activity might be detected in the final coffee aroma and flavor if the microbial metabolites diffused from pulp to bean and remained after roasting (SILVA et al., 2012; PEREIRA et al., 2014). The microbial conversion of coffee pulp constituents

into flavour-active components has emerged, in recent years, as an important, additional mechanism whereby yeasts substantially impact on coffee aroma and flavor (SILVA et al., 2012; EVANGELISTA et al., 2014 a,b; PEREIRA et al., 2014). These profiles vary significantly between yeast species and strains (PEREIRA et al., 2014), so extensive strain screening is necessary to select for those with positive attributes (e.g. enhanced ester formation) and reject those with distinct negative impacts (e.g. overproduction of acetic, butyric and propionic acids). On this basis, yeast species within *Saccharomyces*, *Candida* and *Pichia* have been shown with the greatest potential for enhance quality of coffee fermentation (SILVA et al., 2012; EVANGELISTA et al., 2014 a,b; PEREIRA et al., 2014). Silva et al. (2012) studied the volatile aroma production of coffee yeasts in a coffee peel and pulp media. The authors observed that the evaluated yeasts could affect the final product in different ways. The yeast strains *Saccharomyces cerevisiae* UFLACN727 and *Pichia guilliermondii* UFLACN731 were associated with the production of 1,2-propanediol, hexanoic acid, decanoic acid, nonanoic acid and ethyl acetate, while the strains *Candida parapsilosis* UFLACN448 and *Saccharomyces cerevisiae* UFLACN724 were characterized by the production of guaiacol, butyric acid and citronellol. Pereira et al. (2014) evaluated the volatile aroma production of nine yeast strains isolated from a spontaneous coffee fermentation (*Saccharomyces* sp. YC9.15, *Saccharomyces* sp. YC8.10, *Saccharomyces* sp. YC9.13, *Pichia fermentans* YC8.8, *P. fermentans* YC5.2, *Candida glabrata* CG1.5, *H. opuntiae* YC1.4, *P. guilliermondii* YC1.2 and *P. kluyveri* YH7.16). A total of fourteen compounds were quantified in the headspace of the inoculated fermentations in a coffee pulp simulation medium. These included acetaldehyde, benzaldehyde, caprylic acid, ethanol, ethyl acetate, ethyl laurate, isoamyl acetate, 2,3-butanedione, 1-decanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-hexanol, 2-octanol and 1-octanol. The most important volatile compounds were acetaldehyde, ethanol, isoamyl acetate and ethyl acetate, with the yeast strain *P. fermentans* YC5.2 showing the highest production of ethyl acetate (pineapple-like aroma) and isoamyl acetate (banana-like aroma).

(3) *Properties associated with the commercial production of coffee yeasts*. Finally, for commercial purpose, the yeast must be amenable to large-scale cultivation on relatively inexpensive substrates. Subsequently, it needs to be tolerant of the stresses of drying, packaging, storage and, finally, rehydration and reactivation

processes (SOUBEYRAND et al., 2006). These requirements need to be achieved without loss of the essential and desirable fermentation properties (FLEET, 2008).

1.11. CONCLUSIONS AND FUTURE PROSPECTS

It can be concluded that the distribution of the taxa of yeast and bacteria, as well as their load at the start of fermentation and maximum growth, are highly variable from one coffee ecosystem to another. Yeast, filamentous fungi and bacteria populations are more diverse and are present in greater numbers in natural coffee processing than that in pulped ones (wet and semi-dry) due to the longer exposure period in which the fruits are subjected to contamination during fermentation. On the other hand, lactic acid bacteria are isolated in high numbers in pulped coffees due to the anaerobic or low oxygen conditions present which favor their development.

In dry processing, the common species are *Bacillus subtilis*, species of Enterobacteriaceae family, *Debaryomyces hansenii*, *Pichia guilliermondii* and *Aspergilus niger,* while lactic acid bacteria are rarely found. In pulped coffees (wet and semi-dry processing), bacteria and yeasts represent the most frequently occurring microorganisms, with a predominance of yeasts over bacteria. *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, Enterobacteriaceae, *Bacillus cereus*, *Hanseniaspora uvarum* and *Pichia fermentans* are commonly isolated. Thus, the common species in all types of processing are those species belonging to the Enterobacteriaceae family and *Pichia* genus. The role of microorganisms in the degradation of the mucilage is still matter of debated and further studies are needed to investigate the ability of bacteria and yeast species to degrade the mucilage in vivo (i.e., during coffee processing).

Because coffee beans are generally fermented by various naturally occurring microbiota, the quality of the coffee products varies depending on the raw materials even though it is processed under controlled conditions. The use of starter cultures has recently been suggested as an alternative to control the fermentation process and to promote quality development of coffee product (EVANGELISTA et al., 2014 a,b; PEREIRA et al., 2014). However, the potential use of starter cultures for coffee fermentation is not yet well studied. The prospect of applying starter cultures in

coffee fermentation is to reduce fermentation times (pectinolytic activity), improvement of process control, sensory quality (production of metabolites that confer agreeable flavor to the final product) and safety attributes (inhibition of toxigenic fungi growth). The implementation of coffee starter cultures could be a quite laborious process but, once successfully implemented, can transform an inconsistent process into an economically valuable proposition. In order to explore this avenues, it is first important to understand the ecology, physiology, biochemistry and molecular biology of process. This knowledge can be increased by the applications of more recently, molecular methods, such "omics" technologies, which has never been used in coffee studies. This will open up new horizons in the industrial production of coffee with good taste and high quality.

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CHAPTER II (RESEARCH RESULTS) - ISOLATION, SELECTION AND EVALUATION OF YEASTS FOR USE IN FERMENTATION OF COFFEE BEANS BY THE WET PROCESS

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1.1. ABSTRACT

During wet processing of coffee, the ripe cherries are pulped, then fermented and dried. This study reports an experimental approach for target identification and selection of indigenous coffee yeasts and their potential use as starter cultures during the fermentation step of wet processing. A total of 144 yeast isolates originating from spontaneously fermenting coffee beans were identified by molecular approaches and screened for their capacity to grow under coffee-associated stress conditions. According to ITS-rRNA gene sequencing, *Pichia fermentans* and *P. kluyveri* were the most frequent isolates, followed by *Candida glabrata*, *C. quercitrusa*, *Saccharomyces* sp., *P. guilliermondii*, *P. caribbica* and *Hanseniaspora opuntiae*. Nine stress-tolerant yeast strains were evaluated for their ability to produce aromatic compounds in a coffee pulp simulation medium and for their pectinolytic activity. *Pichia fermentans* YC5.2 produced the highest concentrations of flavor-active ester compounds (viz., ethyl acetate and isoamyl acetate), while *Saccharomyces* sp. YC9.15 was the best pectinase-producing strain. The potential impact of these selected yeast strains to promote flavor development in coffee beverages was investigated for inoculating coffee beans during wet fermentation trials at laboratory scale. Inoculation of a single culture of *P. fermentans* YC5.2 and co-culture of *P. fermentans* YC5.2 and *Saccharomyces* sp. YC9.15 enhanced significantly the formation of volatile aroma compounds during the fermentation process compared to un-inoculated control. The sensory analysis indicated that the flavor of coffee beverages was influenced by the

starter cultures, being rated as having the higher sensory scores for fruity, buttery and fermented aroma. This demonstrates a complementary role of yeasts associated with coffee quality through the synthesis of yeast-specific volatile constituents. The yeast strains *P. fermentans* YC5.2 and *Saccharomyces* sp. YC9.15 have a great potential for use as starter cultures in wet processing of coffee and may possibly help to control and standardize the fermentation process and produce coffee beverages with novel and desirable flavor profiles.

Keywords: wet processing, pectinolytic enzymes, aromatized coffee, *Pichia fermentans*, *Saccharomyces* sp.

1.2. INTRODUCTION

Coffee is an important plantation crop grown in more than 50 developing countries and is one of the most popular non-alcoholic beverages consumed throughout the world (SELVAMURUGAN et al., 2010). Its annual production has reached 8.4 million metric tons, with a turnover close to US $10 billion. Brazil is the leading producer of coffee, followed by Vietnam, Colombia, Indonesia and Mexico (FAO, 2013). Post-harvest processing of coffee cherries is carried out in producing countries using two processes, referred to as wet and dry (PANDEY et al., 2000). Wet processing is used mainly for *arabica* coffee: the ripe fruits are depulped and then submitted to 24–48 h of underwater tank fermentation and dried until a final water content of 10–12% (AVALLONE et al., 2001; MURTHY and NAIDU, 2012). The wet method is widely used in some regions, including Colombia, Central America and Hawaii (VILELA et al., 2010). In the dry processing, in contrast, entire coffee fruits are dried (in the sun) on platforms and/or on a floor without prior removal of the pulp (SILVA et al., 2008). Brazil is the largest producer of coffees obtained by dry process; however, the wet process has increasingly been used as a way to improve the coffee quality (BOREM, 2008; GONÇALVES et al., 2008).

During wet processing, the ripe coffee fruits undergo a spontaneous fermentation, carried out by a complex microbiological process that involves the actions of microorganisms like yeasts, bacteria and filamentous fungi (AVALLONE et al., 2001; SILVA et al., 2008). The fermentation is carried out to eliminate any mucilage still stuck to the beans and helps improve beverage flavor by producing microbial metabolites, which are precursors of volatile compounds formed during roasting (MUSSATTO et al., 2011). Yeasts are among the microorganisms most frequently isolated from fermenting coffee beans, but limited information is available regarding their effect on the development of coffee's taste characteristics (EVANGELISTA et al., 2014). Surveys have shown that the most frequently occurring species during coffee processing are *Pichia kluyveri, P. anomala*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Debaryomyces hansenii* and *Torulaspora delbrueckii* (MASOUD et al., 2004; SILVA et al., 2008; VILELA et al., 2010). In addition, bacteria with pectinolytic activity belonging to the genera *Erwinia*, *Klebsiella*, *Aerobacter*, *Escherichia* and *Bacillus*, as well as a variety of filamentous fungi are often isolated (AVALLONE et al., 2001; SILVA et al., 2012; VAUGHN et al., 1958).

The use of selected yeasts is well known for other fermented foods and beverages, such as wines, to which as many as eight strains or more of *Saccharomyces cerevisiae* may contribute to the one wine fermentation (FLEET, 2008). This was possible after many studies to understand the impact of individual yeast strains upon final product quality (CIANI et al., 2006; ERTEN, 2002; FLEET, 2003; FLEET, 2008; GAO and FLEET, 1998; MENDOZA et al., 2007). For coffee processing that reaches this level, further research is needed to increase the understanding of the microbial ecology, physiology and biochemistry of coffee fermentation and how this scientific knowledge contributes to the development of coffee beverage character. The exploration of the biodiversity of indigenous coffee yeast strains can be an important contribution to the understanding and selection of strains with specific phenotypes able to contribute to the final product quality (MASOUD et al., 2004; SILVA et al., 2012).

To the best of our knowledge, no previous studies have investigated the use of aromatic yeasts as starter cultures during the fermentation step of wet coffee processing and what impact such yeasts might have on coffee beverage flavor. Here we report an experimental approach to target identification and selection of indigenous coffee yeasts and their potential use as starter cultures with the aim of

improving the flavor of coffee beverage processed by wet method. The on-farm implementation of these novel starter cultures is part of a patented process developed in our laboratory (SOCCOL et al., 2013).

1.3. MATERIALS AND METHODS

1.3.1. Spontaneous coffee fermentation and yeast isolation

A total of 150 Kg of coffee cherries (*Coffee arabica* var. Mundo Novo) were manually harvested at the mature stage from a farm located in the city of Lavras, Minas Gerais State, Brazil, and mechanically depulped using a BDSV-04 Pinhalense depulper (Pinhalense, Sao Paulo, Brazil). 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. The environmental temperature was 23-30°C (day-time temperature) and 11-15°C (night-time temperature). Every 8 h, liquid fraction samples were withdrawn in triplicate from the middle depth of the tank fermentation, placed aseptically in sterile plastic bags and transferred to the laboratory in ice boxes. Ten ml 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 [1% yeast extract (Merck, São Paulo, Brazil), 2% peptone (Himedia, São Paulo, Brazil), 2% glucose (Merck) and 2.5% agar (Difco, São Paulo, Brazil); pH = 5.6] containing 100 mg/l chloramphenicol (Sigma, São Paulo, Brazil) and 50 mg/l chlortetracycline (Sigma) to inhibit bacterial growth. Plating was performed, in triplicate, with 100 µl of each dilution. Cultures were incubated at 30 °C for 4 days. According to the macroscopic indications (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 on YEPG agar. The purified isolates were stored at −80 °C in YEPG broth containing 20% (v/v) glycerol (Difco).

1.3.2. Identification of yeast isolates

The yeast DNA was extracted from the pure cultures according to the method described by Pereira *et al.* (2013). The 5.8S ITS rRNA gene region of yeast isolates was amplified using the primers ITS1 and ITS4 (MASOUD et al., 2004). The obtained ITS-rRNA gene region of yeast isolates was digested by restriction endonucleases *Hae*III and *Msp*I, according to the manufacturer's instructions (Invitrogen, São Paulo, Brazil). The PCR products and restriction fragments were separated by gel electrophoresis on 0.7% (w/v) agarose gel, and stained with ethidium bromide (Sigma). The bands were then visualized by UV transilluminator and photographed. A size marker (Gene Ruler of 100 bp DNA Ladder Plus, Fermentans) was used as a reference. The patterns of Amplified rRNA gene Restriction Analysis (ARDRA) were clustered using BioNumerics Version 6.50 (Applied Maths, Sint-Martens-Latem, Belgium). Representative isolates were selected on the basis of genotypic groupings, and 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). The nucleotide sequences of representative isolates were deposited in the GenBank database under access numbers KF747750 to KF747757.

1.3.3. Pre-selection of isolates: evaluation of individual stress factors

All yeast species were evaluated for their ability to grow under stress conditions that occur during the wet fermentation of coffee beans according to the procedure described by Pereira *et al.* (2012). The isolates were transferred from YEPG plates to pre-culture 10 ml YEPG broth and incubated at 30°C for 24 h, 120 rpm. Subsequently, 1 ml of the resulting yeast cultures were transferred to 50-ml

Erlenmeyer flasks containing 10 ml YEPG broth and grown for 3 h at 120 rpm (until early exponential phase). At this point, cells were harvested and diluted in sterile water to Abs600 of 0.2. Spots of 3 µL were placed onto stress plates, which were incubated for at least 48 h at 30 °C.

The test medium used was composed of basal medium [0.05% yeast extract (Sigma), 0.3% (w/v) vitamin-free Casamino Acids (Difco) and 2.5% agar (Difco)] and 5% glucose (Merck). The basal medium without an added carbon source was used as a negative control. The heat stress plates were incubated at 25, 30, 37 or 43 °C. Plates with different glucose or fructose concentration were prepared by adjusting the sugar concentration of the basal medium to 15, 30 or 50 % (w/w) hexose-equivalent; the sugar being added by sterile filtration. Ethanol, acetic acid and lactic acid stress plates were composed of basal medium with glucose supplemented with 6, 8 or 10% (v/w) ethanol (Sigma); 1, 2 or 3% (v/w) lactic acid (Sigma); 1, 2 or 3% (v/w) acetic acid (Sigma) (added aseptically). Acidic stress plates were composed of basal medium with glucose in the pH 2.0, 4.0, 6.0 or 8.0; pH adjustments were made with sterile 1M H_2SO_4 or 1M NaOH.

1.3.4. Formulation of coffee pulp simulation medium and micro-fermentation trials with pre-selected yeasts

A coffee pulp simulation medium was formulated containing 50% (v/v) fresh coffee pulp extract plus 2.0 g/l citric pectin (Sigma), 15 g/l fructose (Merck), 15 g/l glucose (Merck), 5.0 g/l yeast extract (Merk) and 5.0 g/l soya peptone (Oxoid); pH=5.5. Citric pectin, which is present in coffee pulp, was added as energy sources for yeast, plus glucose and fructose, while fresh coffee pulp was added to ensure the availability of nitrogen, trace elements and growth factors naturally present during the wet fermentation of coffee beans. For the preparation of coffee pulp extract, 200 g of the coffee pulp and coffee peel from *Coffea arabica* var. Mundo Novo was mixed with 1 L of sterile water in a blender for 5 min. The medium was sterilized for 15 min at 121 °C.

In preparing the inoculation cultures of pre-selected yeasts, cells from YEPG agar plates were transferred to glass tubes containing 10 ml YEPG broth and incubated with agitation (120 rpm) at 30°C for 24 h. Subsequently, these cells were transferred to 200-ml Erlenmeyer flasks containing 90 ml YEPG broth and grown for 30 h and 120 rpm at 30 °C. After centrifuging cells and washing twice with sterile 0.1% peptone water, 250-ml Erlenmeyer flasks with 200 ml of coffee pulp simulation medium were inoculated with log 6 cells/ml of each yeast species. The fermentations were carried out in duplicate.

The growth of yeast at 48 h of fermentation was calculated by colony-forming unit through plating of tenfold serial dilutions of the samples in sterile 0.1% peptone water that was incubated at the appropriate fermentation temperature for 24 h. The volatile compounds produced after 48 h of fermentation in the headspace of the Erlenmeyer flasks were analyzed by gas chromatography as described in Section 2.5. Samples were withdrawn from the headspace with a 10-ml gas-tight syringe and injected into the gas chromatography apparatus.

1.3.5. Headspace analysis by gas chromatography

Headspace analysis of the volatile compounds was conducted by gas chromatography according to the method of Rossi et al. (2009). Aroma compounds were identified by comparing the peak retention times against those of authentic standards purchased from Sigma in a gas chromatograph (Shimadzu model 17A) equipped with a flame ionization detector at 230 °C. The standards used were 11 alcohols (ethanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-decanol, 2-hexanol, 2-octanol, 2-methyl-1-butanol, n-butanol, 3-methyl-1-butanol), 12 esters (ethyl acetate, propyl acetate, ethyl laurate, ethyl propionate, ethyl isobutyrate, ethyl hexanoate, ethyl octonoate, diethyl succinate, isoamyl acetate, isobutyl acetate, n-butyl acetate, hexyl acetate), 5 ketones (2,3-butanedione, 2-pentanone, 2-hexanone, 2-octanone, 2-heptanone), 3 aldehydes (acetaldehyde, benzaldehyde, and 3-methyl-butanal) and 2 organic acids (acetic acid and caprylic acid). 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, injector temperature at 230 °C. Individual volatiles were expressed as µmol/l of headspace, as ethanol equivalent.

1.3.6. Polygalacturonase activity

Erlenmeyer flasks containing 200 ml of synthetic pectin medium [12% citric pectin (Sigma), 0.3% glucose (Merck), 1.0% KH2PO4 (Merck), 0.5% $MgSO_4.7H_2O$ (Merck), 0.68% $CaCl_2$ (Merck) and 1.0% $(NH4)_2SO_4$ (Merck)] were inoculated in triplicate with 4 log cells/ml of each pre-selected yeast strain. Cultures were incubated at 30 °C for 48 h at 120 rpm and sampled every 24 h. Yeast cells were removed by centrifugation (2060 ×*g*, 20 min, 4 °C) and the cell free supernatants used for determination of polygalacturonase activity by measuring the increase in reducing sugars released from pectin dispersion using 3,5-Dinitrosalicyclic acid DNS (MILLER, 1959). A 0.1 ml aliquot of the supernatant was added to 0.9 ml of 0.1% (w/v) of polygalacturonic acid (Sigma) in 1 M sodium acetate buffer (pH 5.0) and incubated in water bath at 50 °C for 15 min. The reaction was stopped by the addition of 1.0 ml DNS reagent. The mixture was boiled for 5 min and then cooled in an ice bath. Absorbance was read at 600 nm, with the optical density (OD600) determined using an appropriate calibration curve. One unit of polygalacturonase activity (U) was expressed as µmol of galacturonic acid released per min and µg total protein under assay conditions. The total protein was determined by the method of Bradford (1976), with bovine serum albumin (BSA) as the standard. The data was analyzed by Analysis of Variance (ANOVA), and the means were compared using Duncan's test.

1.3.7. Culture of selected yeasts in coffee pulp simulation medium

The influence of fermentation temperature on the growth and volatile compounds production of *P. fermentans* YC5.2 was analyzed in pure and mixed cultures with the pectinolytic *Saccharomyces* sp. YC9.15. Triplicate fermentations were performed in Erlenmeyer flasks containing 200 ml of coffee pulp simulation medium at 15, 28 and 37 °C and 120 rpm for 48 h. The initial yeast cell concentration was of log 6 cells/ml with a 1:1 ratio of *P. fermentans* YC5.2 to *Saccharomyces* sp. YC9.15. Yeast growth was determined by surface inoculation on YEPG agar. Differentiation of the two yeast species on YEPG agar was done by colony morphology and testing of isolated colonies by a specie-specific PCR assay. To develop species-specific primers capable of distinguishing between *P. fermentans* YC5.2 and *Saccharomyces* sp. YC9.15, ITS rRNA gene region sequences obtained from these two species were aligned using the multiple-sequence alignment program clustal_x (1.8) (THOMPSON et al., 1997). Based on these alignments, the *P. fermentans* (PFF2 and PFR2)- and *S. cerevisiae* (SCF1 and SCR1)-specific primers were designed. The sequences of the PCR primers were SCF1 (5′ttcgcctagacgctctcttc3′), SCR1 (5′ctggccttttcattggatgt3′), PFF2 (5′gaaggaaacgacgctcagac3′) and PFR2 (5′atctcttggttctcgcatcg3′). The specificity of the primers was verified by searching for homologous nucleotide sequences in the GenBank database using the blast search program (http://www.ncbi.nlm.nih.gov/) and validated via PCR using genomic DNA from pure cultures of *P. fermentans* YC5.2 and *Saccharomyces* sp. YC 9.15. The yeast DNA was extracted according to Pereira *et al.* (2013). The 25 µL PCR mixture contained 12.5 µl of Mix GoTaq® Green Master 2X (Promega, São Paulo, Brazil), 1.0 µl of DNA and 0.3 µM of each primer. Amplification products were separated by electrophoresis on a 0.7% (w/v) agarose gel, detected by ethidium bromide staining and visualized by UV transillumination. A ladder marker (GeneRuler 100 bp DNA Ladder Plus, Fermentans) was used as a size reference. The quantitative analysis of volatile compounds produced after 48 h of fermentation in the headspace of the Erlenmeyer flasks was done through gas chromatography as describe in Section 1.3.5. The data was analyzed by ANOVA and the means were compared using Duncan's test.

1.3.8. Wet fermentation with selected yeast cultures and sensory evaluation of coffee beverages

Freshly harvested coffee cherries (*Coffee arabica* var. Mundo Novo), obtained from a coffee farm located in Lavras, Minas Gerais State, Brazil, were mechanically depulped (BDSV-04 Pinhalense depulper) to obtain beans with mucilage. Laboratory fermentations were conducted in 6-l Erlenmeyer-flasks containing 1.5 Kg of depulped beans and 3 l of fresh water. The coffee was fermented by inoculation of *P. fermentans* YC5.2 in pure or mixed cultures with *Saccharomyces* sp. YC9.15. The initial yeast cell concentration was 6 log cells/ml with a 1:1 ratio of *P. fermentans* YC5.2 to *Saccharomyces* sp. YC9.15. As a control, spontaneous process was allowed to ferment with indigenous microorganisms present in the coffee fruit. The fermentations were carried out in triplicate at 28 °C for 48 h to simulate the natural process performed on farms. At the end of the fermentation processes, the growth of the inoculated microorganisms was measured by counting viable cells and the volatile compounds in the headspace of the Erlenmeyer flasks were analyzed by gas chromatography as described in Section 3.5. The data was analyzed by ANOVA and the means were compared using Duncan's test.

The resulting parchment coffee was dried in a laboratory oven at 35–40 °C until a water content of 12% (wet basis) was achieved, then roasted at 140 °C for 30 min. The roasted coffee was sampled, added to boiling water at the rate of 2% (w/v) and infused for 5 min. The samples were evaluated by a panel of five experienced judges based on the flavor descriptors: fruity, buttery, caramel aroma, chocolate aroma, fermented aroma and acidic. A note, from 0 (low intensity) to 10 (high intensity), was attributed to each criterion. Results from the two inoculated samples were compared to the control using ANOVA and the means were compared using Duncan's test.

1.4. RESULTS AND DISCUSSION

1.4.1. Isolation and identification of yeast isolates

Yeast (2.7 log cfu/ml) was present at the beginning of the fermentation and grew to a maximum population of 7.15 log cfu/ml during the subsequent 40 h, followed by a drop to 5.2 log cfu/ml by 48 h (FIGURE 2a). A total of 144 yeast were isolated throughout the wet fermentation of coffee beans. Based on ARDRA-PCR profiles (FIGURE 1), eight groups were delineated from which representatives were identified to species level by sequencing. The main species found and their evolution throughout the fermentation are shown in FIGURE 2b. *P. fermentans* (Accession No. KF747751) was the most frequently isolated species, followed by *P. kluyveri* (Accession No. KF747755), *C. glabrata* (Accession No. KF747753) and *C. quercitrusa* (Accession No. KF747756). *Saccharomyces* sp. (Accession No. KF747750) was detected at 24 and 32 h, while *P. guilliermondii* (Accession No. KF747752), *P. caribbica* (Accession No. KF747757) and *H. opuntiae* (Accession No. KF747754) were generally isolated at the start of the fermentation. These yeast species are reported to be fermentative and have been found in soil, fruits and trees (KURTZMAN, 1998). While *P. fermentans* was first isolated as the dominant yeast in spontaneously fermented coffee beans, *P. kluyveri* has been reported in other geographical areas and processing methods (MASOUD et al., 2004; SILVA et al., 2008). Species of the genus *Pichia* have been reported to inhibit ochratoxigenic filamentous fungi growth during coffee fermentation, acting as a possible biological control for the prevention of ochratoxin A in coffee (MASOUD et al., 2005). Interestingly, the phylogenetic position of some isolates belonging to the genus *Saccharomyces* (e.g., the selected *Saccharomyces* sp. YC9.15) had less than 97% similarity to known species, which suggests the isolation of a new candidate species of the genus *Saccharomyces*.

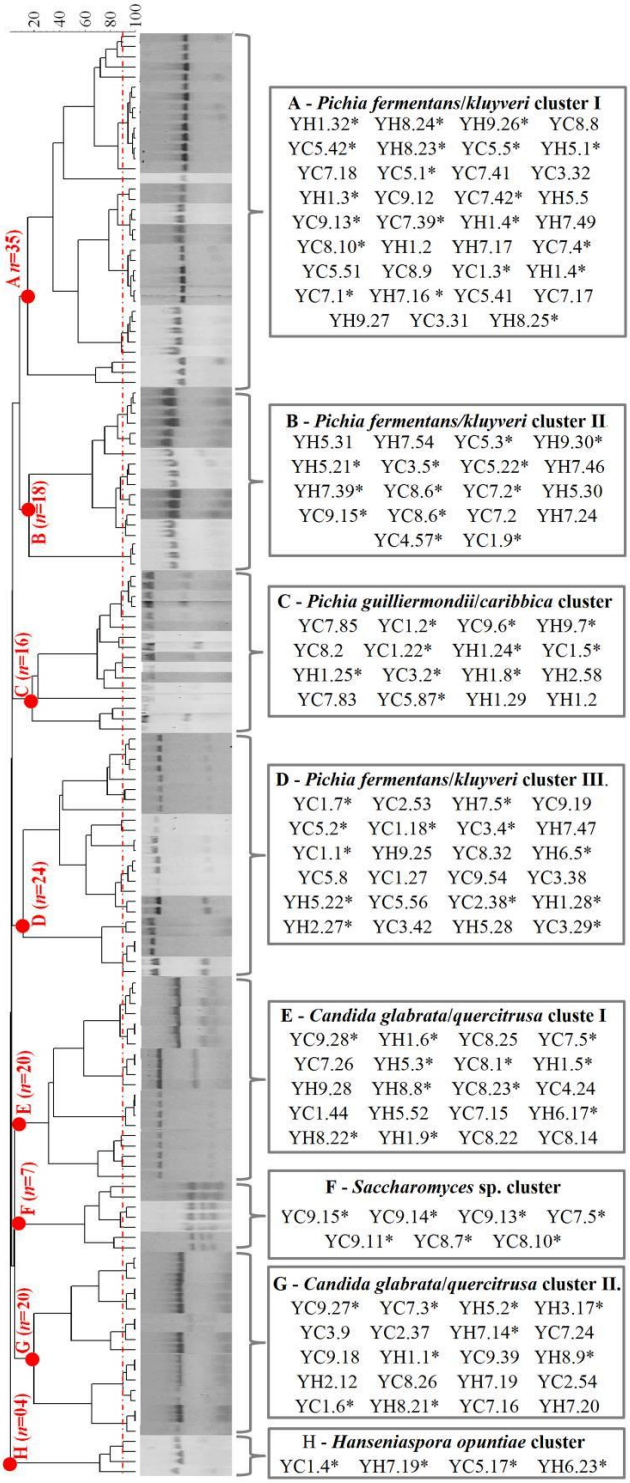

FIGURE 1 - DENDROGRAM BUILT BY CLUSTER ANALYSIS OF (ARDRA)-PROFILES OF YEASTS ISOLATED FROM THE SPONTANEOUS COFFEE FERMENTATION PROCESS. THE DENDROGRAM IS BASED ON DICE'S COEFFICIENT OF SIMILARITY, USING THE UNWEIGHTED PAIR GROUP METHOD WITH THE ARITHMETIC AVERAGES CLUSTERING ALGORITHM (UPGMA). ISOLATES LABELED WITH AN ASTERISK WERE SELECTED FOR ITS-rRNA GENE SEQUENCING

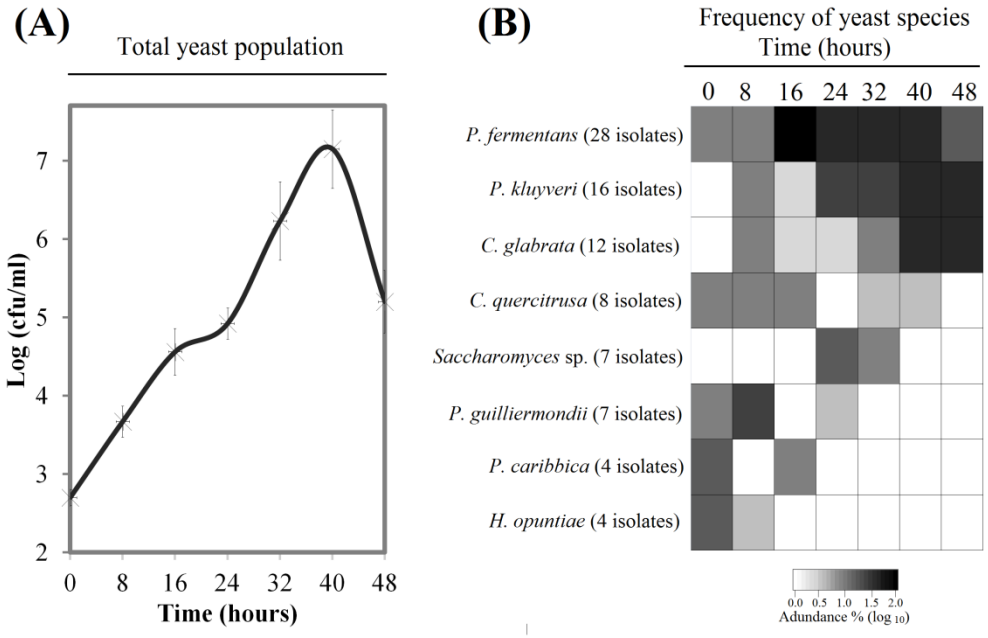

FIGURE 2 - ASSESSMENT OF YEAST DIVERSITY DURING THE SPONTANEOUS COFFEE FERMENTATION PROCESS. (A) DYNAMIC OF TOTAL YEAST POPULATION. BARS REPRESENT STANDARD DEVIATION. (B) HEAT MAP SHOWING THE FREQUENCY AND ABUNDANCE OF INDIVIDUAL YEAST SPECIES. VALUES IN BRACKETS ARE THE NUMBER OF ISOLATES IDENTIFIED BY ITS-RRNA GENE SEQUENCING. THE HEAT MAP WAS GENERATED BY USING VISUAL BASIC FOR APPLICATIONS (VBA) IN CONJUNCTION WITH MICROSOFT® OFFICE EXCEL VERSION 7.0

1.4.2. Pre-selection of yeasts: growth/survival under stress conditions

A preliminary screening was carried out with all 144 isolates to test their capacity to grow under coffee-associated stress conditions. TABLE 1 summarizes the results for growth/survival of yeast isolates under tested conditions. The criteria used were based on the physical and chemical changes that yeast cells face through the fermentation process. Coffee pulp and mucilage is a high density substrate that consists of 27–30% fermentable sugars, in particular glucose and fructose (WRIGLEY, 1988; AVALLONE et al., 2001; MURTHY and NAIDU, 2011). After inoculation, the yeast can experiences hypertonic conditions, which leads to an efflux of water from the cell, diminished turgor pressure and reduced water availability (BAUER and PRETORIUS, 2000). Throughout coffee fermentation process, approximately 60% of the sugars are utilized as substrate for microbial growth which produces significant amounts of ethanol and acetic and lactic acids, resulting in

lowered pH (from 5.5-6.0 to 3.5-4.0) (AVALLONE et al., 2001; JACKELS et al., 2006). Thus, when the cell has adapted to the new environment and the fermentation begins, other stressors become relevant as organic acids and alcohols accumulate, the temperature changes and the environment acidifies (AVALLONE et al., 2000; Silva et al., 2008; VELMOUROUGANE, 2013). Following a successful inoculation of coffee beans, the ability of the yeast to adapt and to cope with the hostile environment and stress conditions prevailing in coffee fermentation matrix are of vital importance to fermentation performance. Based on this, nine stress-tolerant strains, possessing the following characteristics, were pre-selected for further investigation: (i) growth capacity in a typical pH range of coffee fermentation (pH 2.0 to pH 8.0); (ii) osmotic pressure tolerance (growth detected in the presence of up to 50% glucose and fructose); (iii) heat tolerance (ability to grow at temperatures of 37 to 43 °C); and (iv) metabolite accumulation tolerance (growth capacity up to 12 to 15% ethanol, 2% lactic acid and 2% acetic acid). These included YC9.15, YC9.13 and YC.8.10 (classified as *Sacharomyces* sp.); YC5.2 and YC8.8 (classified as *P. fermentans*); YH7.16 (classified as *P. kluyveri*); YC1.2 (classified as *P. guilliermondii*); YC1.4 (classified as *H. opuntiae*); and YH1.5 (classified as *C. glabrata*).

1.4.3. Polygalacturonase activity screening

Secretion of polygalacturonase by the nine pre-selected yeast strains was investigated in a synthetic pectin medium. The results showed that *Saccharomyces* sp. YC9.15*, Saccharomyces* sp. YC8.10 and *P. fermentans* YC8.8 were the strains with higher polygalacturonase activity under the assay conditions ($p < 0.05$), which produced 2.03, 1.90 and 1.72 U/ml polygalacturonase after 48 h of fermentation. The other yeast strains produced less than 1 U/ml (data not shown).

TABLE 1 - COFFEE FERMENTATION-RELATED STRESS CONDITIONS CHARACTERIZATION OF YEAST ISOLATES

Growth at**	ARDRA cluster*							
	PFK cluster I	PFK cluster II	PFK cluster III	PGC Cluster	CGQ cluster I	CGQ cluster II	SAC cluster	HOP cluster
25 ºC	+	+	+	+	+	+	+	+
30 ºC	+	+	+	+	+	+	+	+
37 ºC	+	+	+	+	+	+	+	+
43 ºC	25/45	14/18	-	-	-	10/18	+	2/4
pH 2.0	33/35	9/18	2/24	2/16	-	-	+	-
pH 4.0	+	+	+	+	+	+	+	+
pH 6.0	+	+	+	+	+	+	+	+
pH 8.0	+	+	+	+	+	+	+	+
1% lactic acid	23/35	9/18	5/24	2/16	4/20	7/18	3/7	3/4
2% lactic acid	08/35	5/18	-	1/16	-	-	-	-
3% lactic acid	-	-	-	-	-	-	-	-
1% acetic acid	20/35	11/18	5/24	2/16	3/20	8/18	+	2/4
2% acetic acid	10/35	3/18	-	-	-	-	3/7	-
3% acetic acid	-	-	-	-	-	-	+	-
6% ethanol	+	+	+	+	+	+	+	+
8% ethanol	+	+	+	+	+	+	+	+
10% ethanol	+	17/18	+	+	+	+	+	2/4
15% glucose	+	+	+	+	+	+	+	+
30% glucose	+	+	+	+	+	+	+	+
50% glucose	28/35	17/18	+	+	18/20	15/18	+	2/4
15% fructose	+	+	+	+	+	+	+	+
30% fructose	+	+	+	+	+	+	+	+
50% fructose	28/35	17/18	+	+	19/20	16/18	+	2/4

* The results are presented according to the ARDRA clustering shown in Fig. 2. PFK cluster I = *P. fermentans* and *P kluyveri* cluster I; PFK cluster II = *P. fermentans* and *P kluyveri* cluster II; PFK cluster III = *P. fermentans* and *P kluyveri* cluster III; PGC cluster = *P. guilliermondii* and *P. caribbica* cluster; CGQ cluster I = *C. glabrata* and C*. quercitrusa* cluster I; CGQ cluster II = *C. glabrata* and *C. quercitrusa* cluster II; SAC cluster = *Saccharomyces* sp. cluster; HOP cluster = *H. opuntiae* cluster.
**Characters are scored as: (+) all strains positive; (−) all strains negative; number of strains positive/number tested.

Previous studies also reported the pectinolytic activity of indigenous coffee yeasts, such as species of *Kluyveromyces*, *Saccharomyces*, *Pichia*, and *Candida* (MASOUD and JESPERSEN 2006; SILVA et al., 2012). These pectinolytic strains appeared to have potential to be used as starter cultures for mucilage degradation during coffee fermentation; nevertheless, further studies are needed to investigate the ability of these yeasts to degrade the mucilage *in vivo* (i.e., during coffee processing). The removal of the mucilage by microorganisms facilitates bean drying and produces metabolites that diffuse into the interior of the coffee beans and react with substances responsible for the flavor of the final beverage (SILVA et al., 2012).

1.4.4. Production of volatile aroma compounds by pre-selected yeast strains

The nine pre-selected yeast strains were inoculated into coffee pulp simulation medium and the volatile aroma compound production was quantified after 48 h of fermentation. The composition of the coffee pulp simulation medium supported good growth of the investigated yeasts because all isolates grew about 3 log cfu/ml within 48 h, relative to the initial population inoculated (data not shown). Fourteen compounds were quantified in the headspace of the inoculated fermentations. These included acetaldehyde, benzaldehyde, caprylic acid, ethanol, ethyl acetate, ethyl laurate, isoamyl acetate, 2,3-butanedione, 1-decanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-hexanol, 2-octanol and 1-octanol. The most important volatile compounds (i.e., acetaldehyde, ethanol, isoamyl acetate and ethyl acetate) of the different fermentations are listed in TABLE 2.

TABLE 2 - CONCENTRATIONS OF MAJOR VOLATILE COMPOUNDS PRODUCED BY PRE-SELECTED YEAST STRAINS AFTER 48 H OF FERMENTATION IN COFFEE PULP SIMULATION MEDIUM

	Yeast strain[a]								
Compounds (µmol/l)[b]	SC9.15*	SC8.10	SC9.13	PF8.8	PF5.2*	CG1.5	HO1.4	PG1.2	PK7.16
Acetaldehyde	22.04	0.47	0.86	ND	0.53	0.87	0.73	11.5	ND
Ethanol	121.8	126.9	125.0	40.5	60.7	49.1	47.4	72.8	30.4
Ethyl acetate	0.52	0.37	ND	ND	79.4	ND	ND	0.25	0.72
Isoamyl acetate	ND	ND	ND	ND	23.4	ND	ND	ND	4.48

[a]Abbreviations: SS9.15.: *Saccharomyces* sp. YC9.15; SC8.10.: *Saccharomyces* sp. YC8.10; SC9.13.: *Saccharomyces* sp. YC9.13; PF8.8.: *P. fermentans* YC8.8; PF5.2.: *P. fermentans* YC5.2; CG1.5.: *C. glabrata*; HO1.4.: *H. opuntiae* YC1.4; PG1.2.: *P. guilliermondii* YC1.2; PK7.16.: *P. kluyveri* YH7.16.
[b] Means of triplicate fermentations expressed in µmol/l of ethanol equivalent. Minor volatile compounds, such as benzaldehyde, caprylic acid, ethyl laurate, 2,3-butanedione, 1-decanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-hexanol, 2-octanol and 1-octanol were produced in amounts below 1 µmol/l. *Strains labeled with an asterisk were selected for further investigation.

As expected, *Saccharomyces* strains produced higher amounts of ethanol compared to non-*Saccharomyces* species; *Saccharomyces* sp. YC8.10 produced the highest ethanol levels (126.9 µmol/l), followed by *Saccharomyces* sp. YC9.13 and *Saccharomyces* sp. YC9.15, which produced 125.0 and 121.8 µmol/l, respectively. Isoamyl acetate and ethyl acetate were produced in significant amounts only by *P. fermentans* YC5.2 and *P. kluyveri* YC7.16. *P. fermentans* YC5.2 produced the highest concentrations of ethyl acetate (pineapple-like aroma) and isoamyl acetate (banana-like aroma), reaching concentrations of 79.04 and 23.04 µmol/l, respectively. As these compounds are related to fruity aroma, which participates in

aromatic complexity, this makes *P. fermentans* YC5.2 an attractive yeast to enhance the aromatic value of coffee beans and was chosen to evaluate its behavior in co-culture with the pectinolytic *Saccharomyces* sp. YC9.15 at different temperatures. In addition to pectinolytic production, the *Saccharomyces* sp. YC9.15 was selected also for its high production of acetaldehyde in relation to other pectinolytic strains. Acetaldehyde has been correlated with sensory attributes as it gives a floral and fruity note to the final beverage (SANZ et al., 2002).

1.4.5. Culture of selected yeasts in coffee pulp simulation medium

The growth and volatile compounds production of *P. fermentans* YC5.2 in pure and mixed cultures with pectinolytic *Saccharomyces* sp. YC9.15 are shown in TABLES 3 and 4, respectively. The viable count of *P. fermentans*'s single-culture fermentations peaked at a maximum population exceeding 7 log cfu/ml. On the other hand, in the mixed-culture fermentations, the viable counts of *P. fermentans* from ~5.8 log cfu/ml increased to 6.4 and 6.3 log cfu/ml at 15 and 28 °C, respectively, and declined to 4.5 log cfu/ml at 37 °C, while the viable counts of *Saccharomyces* sp. were over 7 log cfu/ml (7.3, 8.4 and 7.7 log cfu/ml at 15, 28 and 37 °C, respectively) (TABLE 3). The highest isoamyl acetate and ethyl acetate concentrations (59.5 and 171.8 µmol/l, respectively) were determined in pure culture of *P. fermentans* at 28 °C, whereas the production of acetaldehyde and ethanol was greater in mixed fermentations at 28 and 37 °C, respectively (TABLE 4). Thus, it can be see that growth and volatile compounds production of *P. fermentans* were reduced by the presence of *Saccharomyces* sp. in mixed-culture fermentations at all tested temperatures. It should be noted, however, that the metabolism of *P. fermentans* was encouraged when fermentations were carried out at low temperature, and even when in co-culture with *Saccharomyces* sp., a high production of isoamyl acetate was observed (TABLE 4). Several studies also have indicated a higher persistence of some non-*Saccharomyces* yeasts and different fermentation behaviors at low temperatures (CIANI et al., 2006; ERTEN, 2002; GAO and FLEET, 1988; HEARD and FLEET, 1988). This can provide light in future studies aiming to balance the

metabolisms of both these species in an attempt to optimize the production of pectinolytic enzymes while maintaining high production of aromatic compounds.

1.4.6. Inoculation of selected yeasts in wet fermentation of coffee beans and sensory evaluation of coffee beverages

To respond to the new challenges of consumer demands for coffees with high complexity of flavors and stylistic distinction, the time-temperature profile used during roasting is the most used (WANG and LIM, 2013). However, no method of imparting yeast-derived volatile aroma compounds to coffee during wet processing has been established. In order to evaluate the impact of the starter cultures developed in this study on the final beverage flavor, wet fermentations trials at laboratory scale were carried out by inoculating coffee beans. The results presented in TABLE 5 show the yeast counts and volatile aroma compounds produced after 48 h of fermentation, as well as the sensory flavor profiles of coffee beverages produced thereof. Except for *P. fermentans* in mixed fermentation, which had its growth strongly reduced to 3.26 log cfu/ml, the starter cultures were able to grow under wet processing conditions, as observed in the increasing log cfu/ml after 48 h of fermentation compared to their original numbers at time 0.

Ethanol, acetaldehyde, ethyl acetate, isoamyl acetate, 2,3-butanedione and hexanal were the major volatile compounds released in the headspace of spontaneous and inoculated coffee fermentations (TABLE 5). These volatile compounds are proposed to derive from two main sources—namely, those resulted from yeast metabolism (i.e., ethanol, acetaldehyde, ethyl acetate, and isoamyl acetate) and those that derived from thermal reactions during fermentation (i.e., hexanal and 2,3-butanedione) (GONZALEZ-RIOS et al., 2007). Some of these compounds are known to play a role in aroma development during coffee fermentation (e.g., ethanol, ethyl acetate, isoamyl acetate and acetaldehyde) (CZERNY and GROSCH, 2000; EVANGELISTA et al., 2014; GONZALEZ-RIOS et al., 2007). The use of the culture starters developed in this study significantly

increased ($p < 0.05$) the production of these compounds during the fermentation process (TABLE 5).

There were no statistically significant differences among any treatments for the descriptors caramel aroma, chocolate aroma and acidic (TABLE 5). On the other hand, the inoculated fermentations produced beverages with higher sensory scores ($p < 0.05$) for fruity, buttery and fermented aroma compared to the un-inoculated control. Coffee beverage produced from beans inoculated with *P. fermentans* single-culture was rated as having the highest intensity of fruit, which is usually associated with esters produced in the fermentation process (e.g., ethyl acetate and isoamyl acetate). In addition, the significantly more intense buttery and fermented aroma in inoculated coffees could be linked to the total volatile concentration produced in the fermentation processes, such as 2,3-butanedione (buttery flavor), acetaldehyde (fruity flavor), hexanal (green beans flavor), ethanol (alcoholic flavor) and esters (fruity flavor).

TABLE 3 - GROWTH (LOG cfu/ml) OF *P. FERMENTANS* YC5.2 IN PURE OR MIXED CULTURE WITH *SACCHAROMYCES* SP. YC9.15 AT 15, 28 AND 37 °C AFTER 48 H OF FERMENTATION IN COFFEE PULP SIMULATION MEDIUM

		Temperature incubation					
		15 °C (h)		28 °C (h)		37 °C (h)	
		0	48	0	48	0	48
Pure Culture	*P. fermentans* YC5.2	5.6^{a}±0.2	$7.8^{b,c}$±0.2	5.7^{a}±0.1	8.3^{c}±0.2	5.8^{a}±0.3	7.0^{b}±0.1
Mixed Culture*	*P. fermentans* YC5.2	5.8^{a}±0.1	6.4^{b}±0.2	5.7^{a}±0.1	6.3^{b}±0.2	5.8^{a}±0.2	4.5^{c}±0.3
	Saccharomyces sp. YC9.15	5.7^{a}±0.1	7.3^{b}±0.4	5.6^{a}±0.2	8.4^{c}±0.3	5.9^{a}±0.1	$7.7^{b,c}$±0.4

*In mixed culture, the dominance was verified by DNA approaches, as described in the supplementary material. Means of triplicate fermentations in each row bearing the same letters are not significantly different (p > 0.05) from one another, using Duncan's test. (mean±standard deviation)

TABLE 4 - MEAN CONCENTRATIONS* OF VOLATILE COMPOUNDS PRODUCED BY *P. FERMENTANS* YC5.2 IN PURE OR MIXED CULTURE WITH *SACCHAROMYCES* SP. YC9.15 AT 15, 28 AND 37 °C AFTER 48 H OF FERMENTATION IN COFFEE PULP SIMULATION MEDIUM

	Pure culture			Mixed culture		
Coumpounds	15 °C	28 °C	37 °C	15 °C	28 °C	37 °C
Ethanol	114.0^{a}±12.4	$158.6^{a,b}$±1.7	53.0^{c}±2.0	$145.4^{a,b}$± 2.6	163.8^{b}±15.4	$151.5^{a,b}$±24.0
Isoamyl acetate	25.8^{a}±1.8	59.5^{b}±4.5	22.5^{a} ±3.1	26.5^{a}±0.2	24.2^{a}±1.4	2.4^{c}±0,4
Ethyl acetate	14.0^{a}±1.9	171.8^{b}± 27.5	93.6^{c}±4.2	35.8^{d}± 4.0	$22.1^{a,d}$±2.3	0.3^{e}±0.04
Acetaldehyde	$1.6^{a,b}$±0.2	0.2^{b}±0.1	ND	11.32^{c}±0.8	21.8^{d}±0.8	4.2^{a}±0.03
N-Butyl acetate	0.8^{a}±0.1	3.8^{b} ±0.7	3.4^{b}±0.5	ND	ND	ND
Isobutyl acetate	ND	ND	0.9^{a}±0.2	ND	ND	ND
Ethyl isobutyrate	ND	ND	0.7±0.1	ND	ND	ND
3-methyl-1-butanol	0.6^{a}±0.2	1.2^{b} ±0	ND	ND	ND	ND
1-pentanol	0.4^{a}±0.1	ND	0.3^{a} ± 0.1	ND	ND	ND
1-octanol	ND	0.3^{a}±00.07	ND	0.2^{a}± 0.1	0.4^{a}±0.3	ND
2-hexanol	ND	2.0±0.3	ND	ND	ND	ND
1-decanol	ND	ND	0.4^{b} ±0.1	ND	ND	ND
Caprylic acid	5.9^{a}±0.1	0.3^{b}±0.04	0.4^{b}±0.1	ND	0.5^{b}± 0.3	ND
2,3-butanedione	6.0^{a}±1.7	ND	1.3^{b} ±3.4	ND	ND	ND
2-hexanone	ND	ND	ND	0.4 ±0.05	ND	ND
Diethyl succinate	ND	ND	ND	0.32±0.02	ND	ND
Ethyl octonoate	ND	ND	ND	0.20±0.08	ND	ND
2-methyl-1-butanol	ND	ND	ND	ND	1.31±0.41	ND

*Values expressed in µmol/l of ethanol equivalent as means of triplicate fermentations (mean±standard deviation). ND.: not detected. Means in each row bearing the same letters are not significantly different (p > 0.05) from one another, using Duncan's test. (mean±standard deviation)

TABLE 5 - VOLATILE AROMA COMPOUNDS AND YEAST COUNTS AFTER 48 H OF WET FERMENTATION OF COFFEE BEANS AND SENSORY ANALYSIS OF COFFEE BEVERAGES

	Fermentation assay*		
Parameters	Single-culture	Mixed-culture	Un-inoculated control
Aroma compounds (µmol/l)			
Ethanol	43.08^{a} ± 23.81	88.50^{b} ± 61.72	1.29^{c} ± 0.19
Acetaldehyde	0.48^{a} ± 0.01	12.85^{b} ± 0.57	0.49^{a} ± 0,07
Ethyl acetate	42.42^{a} ± 8.38	5.37^{b} ± 2.16	ND
Isoamyl acetate	10.18^{a} ± 1.59	0.32^{b} ± 0.11	ND
Hexanal	21.59^{a} ± 9.30	24.91^{a} ± 6,17	12.59^{b} ± 2.38
2,3-butanediona	17.86^{a} ± 0.74	17.30^{a} ± 3.01	8.15^{b} ± 1.45
Cell growth (log cfu/ml)			
Pichia fermentans YC5.2	7.74^{a}± 0.61	3.26^{b} ± 0.32	ND
Saccharomyces sp. YC9.15	ND	7.40 ± 0.54	ND
Sensory flavor profiles			
Fruity	7.83^{a}±0.14	6.16^{b}±0.28	5.83^{b}±0.30
Buttery	6.25^{a}±0.25	6.30^{a}±0.14	5.40^{b}±0.53
Caramel aroma	6.66^{a}±0.14	6.58^{a} ±0.14	6.75^{a} ±0.25
Chocolate aroma	5.83^{a} ±0.14	5.91^{a}±0.14	5.75^{a}±0.25
Fermented aroma	6.40^{a}±0.38	6.87^{a}±0.17	5.16^{b}±0.14
Acidic	5.83^{a}±0.14	5.91^{a}±0.28	5.8^{a}±0.14

* Single-culture = coffee fermentation conducted with a pure culture of *Pichia fermentans* YC5.2; mixed-culture = coffee fermentation conducted with a mixed culture of *Pichia fermentans* YC5.2 and *Saccharomyces* sp. YC9.15; un-inoculated control = spontaneous fermentation process carried out by indigenous microorganisms from the coffee fruit. In mixed culture, the dominance was verified by DNA approaches. Means of triplicate fermentations in each row bearing the same letters are not significantly different (p > 0.05) from one another, using Duncan's test. (mean±standard deviation)

1.5. CONCLUSION

This study demonstrated a useful approach for target selection of aromatic coffee yeasts and their use as starter cultures during the fermentation step of wet processing. The strong flavor producing *P. fermentans* YC5.2 and the pectinolytic *Saccharomyces* sp. YC9.15 (acetaldehyde producing) were selected as starter cultures for coffee fermentation. The use of these starter cultures in wet processing resulted in coffee beverages with modified flavors, which reveals that yeasts have a complementary role when associated with coffee quality through the synthesis of yeast-specific volatile constituents. A coffee with a distinctive aroma of fruits could be

produced using the starter cultures in coffee processed by the wet method. The selected yeast strains *P. fermentans* YC5.2 and *Saccharomyces* sp. YC9.15 have a great potential for use as starter cultures in wet processing of coffee and may possibly help to control and standardize the fermentation process and produce coffee beverages with novel and desirable flavor profiles. Further studies should be directed toward the implementation of these yeast strains under on-farm coffee processing conditions and their interaction with other microorganisms, such as lactic and acetic acid bacteria and Gram-negative bacteria present during fermentation.

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CHAPTER III (RESEARCH RESULTS) - YEAST STARTER CULTURE IMPLEMENTATION IN COFFEE FERMENTATION DURING WET PROCESSING: GROWTH, METABOLIC ANALYSES AND SENSORIAL EFFECTS

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1.1. ABSTRACT

In this study, we investigated the potential use of *Pichia fermentans* YC5.2 as a starter culture in coffee fermentation during on-farm wet processing. Inoculated fermentations were conducted with or without the addition of 2% (w/v) sucrose, and the resultant microbial growth and metabolism, bean chemistry, and beverage quality were compared with spontaneous (control) fermentation. In both inoculated treatments, *P. fermentans* prevailed over indigenous microbiota, and a restricted microbial composition was observed at the end of fermentation process. The inoculation also increased the production of specific volatile aroma compounds (e.g., ethanol, acetaldehyde, ethyl acetate, and isoamyl acetate) and decreased the production of lactic acid during the fermentation process. Sucrose supplementation did not significantly interfere with the growth and frequency of *P. fermentans* YC5.2 inoculum but maintained high levels of wild bacteria population and lactic acid production similar to the spontaneous process. In roasted beans, the content of sugars and organic acids were statistically ($p<0.05$) similar for all the treatments. However, the inoculated fermentations were shown to influence the volatile fraction of roasted coffee beans by increasing the concentration of yeast-derived metabolites compared to control. Sensory analysis of coffee beverages demonstrated that the use of the YC5.2 strain was favorable for the production of coffee with distinctive characteristics, viz., intense perception of ‘vanilla’ taste and ‘floral’ aromas. In

conclusion, the use of *P. fermentans* YC5.5 in coffee processing shown to be a viable alternative to control the fermentation process and to obtain beverages of distinctive flavor and high quality.

Keywords: coffee beverage, starter culture, *Pichia fermentans*, wet method, aromatized coffee

1.2. INTRODUCTION

The quality of a coffee beverage is strongly related to the chemical composition of the roasted beans but is also dependent on the postharvest processing (ILLY and VIANI, 2005). Three different methods are employed in producing countries to process coffee fruit, referred to as dry, wet, and semi-dry (PANDEY et al., 2000). Wet processing is used mainly for arabica coffee: the ripe fruits are de-pulped and then submitted to 24–48 h of underwater tank fermentation and dried until a final water content of 10–12% is achieved (AVALLONE et al., 2001; MURTHY and NAIDU, 2012). In dry processing, in contrast, entire coffee fruits are dried (in the sun) on platforms and/or on a floor without prior removal of the pulp (SILVA et al., 2008). Semi-dry processing is a combination of both methods, in which coffee fruits are de-pulped, but the fermentation process occurs directly under the sun on a platform (VILELA et al., 2010).

During on-farm wet processing, the fermentation step is carried out to eliminate any mucilage still stuck to the beans and helps improve beverage flavor by producing microbial metabolites, which are precursors of volatile compounds formed during roasting (MUSSATTO et al., 2011). The microorganisms responsible for the fermentation are indigenous species that originate as natural contaminants of the process, including yeasts, bacteria, and filamentous fungi. Surveys have shown that the most frequently occurring species during coffee fermentation are *Pichia kluyveri*, *P. anomala*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Debaryomyces hansenii* and *Torulaspora delbrueckii* (MASOUD et al., 2004; SILVA et al., 2008;

VILELA et al., 2010). In addition, bacteria with pectinolytic activity belonging to the genera *Erwinia*, *Klebsiella*, *Aerobacter*, *Escherichia,* and *Bacillus*, and a variety of filamentous fungi are often isolated as well (AVALLONE et al., 2000; SILVA et al., 2012). These fermenting organisms utilize the bean pulp as a carbon and nitrogen source and produce significant amounts of ethanol, acetic, and lactic acids 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).

Presently, coffee fermentation is conducted as a simple, traditional process and still relies on the naturally occurring microbiota of the fresh raw materials. However, spontaneous fermentation processes suffer from a lack of control, often resulting in end-products of inconsistent quality. The challenge is to develop this fermentation into a more modern, controlled process that gives greater consistency and predictability and yields a value-added product, as has occurred with many other fermented foods and beverages, such as cheese, yogurt, bread, beer, and wine (STEINKRAUS 2004; SCHWAN et al., 2014). Starter cultures are microbial preparations of a large number of cells of one or more microbial strains, which are added to the raw material to accelerate and control the fermentation process (LEROY and DE VUYST, 2004). Good candidate functional starter cultures are mainly wild-type strains that originate from the natural ecosystem, as these usually exert more elaborate metabolic activities in comparison with industrial bulk starters, often from diverse sources (LEROY and DE VUYST, 2004; PEREIRA et al., 2012b; WOUTERS et al., 2013). In this way, the frequent appearance of *Pichia* yeasts as prevailing in coffee fermentations (MASOUD et al., 2004; SILVA et al., 2008; PEREIRA et al., 2014) makes this common genus a promising candidate for starter culture trials. Furthermore, several *Pichia* strains possess functional properties, such as the capacity to degrade the coffee bean mucilage (pectinolytic activity), to resist stress conditions prevailing in coffee fermentation matrix, to produce flavoring compounds and to inhibit ochratoxigenic filamentous fungi growth during coffee fermentation (MASOUD et al., 2005; MASOUD and JESPERSEN, 2006; SILVA et al., 2012; EVANGELISTA et al., 2014; PEREIRA et al., 2014). All these features make their application as starter cultures even more interesting.

This study was aimed at the implementation of *P. fermentans* YC5.2 as a starter culture for coffee fermentation during wet processing. It is a competitive and aromatic yeast strain isolated from coffee processing that has successfully been

used as a starter culture in coffee fermentation trials on a laboratory scale (PEREIRA et al., 2014).

1.3. MATERIAL AND METHODS

1.3.1. Microorganism and lyophilized inoculum preparation

The yeast strain used in this study*, P. fermentans* YC5.2 accession no. KF747751 (http://www.ncbi.nlm.nih.gov/genbank), was originally isolated from a spontaneous coffee fermentation process and selected as detailed by Pereira et al. (2014). For biomass production, pre-culture was prepared by inoculating 100 mL of yeast extract peptone Glucose (YEPG) broth with 1 mL of the thawed stock culture and incubating for 48 h at 30°C and 120 rpm. This pre-culture was transferred to 3-L Erlenmeyer flasks containing 1 L YEPG broth and grown for 24 h at 30 °C and 120 rpm. Subsequently, 400 mL of the resulting yeast culture was transferred to a 6-L Erlenmeyer flask containing 4 L YEPG broth and grown for 24 h at 30 °C and 150 rpm. The culture was centrifuged for 15 min at 4500 Xg and 4 °C, washed in distilled water, and centrifuged again. Lyophilized culture was prepared by resuspeding the pellet in skimmed UHT milk, rapidly freezing in an ethanol-dry Ice mixture, and freeze-drying in a Modulyod Freeze Dryer 230 (Thermo Electron Corporation, Waltham, USA) under negative pressure of 50 mBar at −45°C. The lyophilized culture was analyzed for total viable cell count by standard dilution method on YEPG agar and conditioned in packs of 10 g.

1.3.2. Fermentation experiments

The fermentation experiments were conducted at the Apucarana coffee farm localized in the Cerrado Mineiro region at Minas Gerais state, Brazil. The Apucarana farm is situated at 1270 m above sea level atop the mineral-rich dome of an unformed volcano and is known for consistently producing high-quality coffees. Freshly harvested coffee cherries (*Coffee arabica* var. Catuí) were depulped using a BDSV-04 Pinhalense depulper (Pinhalense, Sao Paulo, Brazil) to obtain beans with mucilage. Fermentations were conducted in cement tanks with inner dimensions 2.42 × 1.94 × 0.96 m containing 20 Kg of depulped beans and approximately 500 L of fresh water in accordance with the local wet processing method. The lyophilized starter culture was rehydrated by adding water at 37–40 °C (10 g/L) and stirred gently over a period of 5 min. This solution was spread into the fermentation tank to reach a concentration of approximately 10^7 cells/mL. During the fermentation trials, three different batches were performed: (i) inoculated starter culture with fermenting mass being supplemented with 2 % (w/v) sucrose; (ii) inoculated starter culture with no sugar supplementation; and (iii) noninoculated control (i.e., spontaneous fermentation). The fermentations were conducted simultaneously and repeated three time. The depulped coffee beans were fermented for 24 h and then sun-dried until 11–12% moisture was reached. The environmental temperature was 24–32 °C (daytime temperature) and 12–15 °C (nighttime temperature).

1.3.3. Sampling and pH

Samples (liquid fraction plus beans) were withdrawn in triplicate (0 and 24 h) at random to perform microbial counts and metabolite target analysis. At every sampling point, the pH of the fermenting mass was measured with a portable pH metre, model AK90 (AKSO, São Leopoldo, Brasil).

1.3.4. Enumeration of microorganisms

Ten-milliliter samples were homogenized in 90 ml saline-peptone water [(v/v) (0.1% bacteriological peptone (Himedia), 0.8% NaCl (Merck, Whitehouse Station, USA)], in a Stomacher at normal speed for 5 min (10-1 dilution) and diluted serially. Yeast were enumerated by surface inoculation on YEPG agar [1% yeast extract (Merck), 2% peptone (Himedia), 2% glucose (Merck) at pH 5.6] containing 100 mg/L chloramphenicol (Sigma) to inhibit bacterial growth. Nutrient agar containing 0.1% cycloheximide was used as a general medium for count of viable bacteria population. The plates were incubated at 30 °C for 48 h. Following incubation, the number of colony-forming units (cfu) was recorded.

1.3.5. Verification of inoculum dominance

The capability of *P. fermentans* to dominate indigenous yeast flora was verified through DNA approache. All yeast colonies from the YEPG plates at an appropriate dilution (section 3.4.1) were transferred into single wells of a 96-well plate containing 40 µl sterile distilled water and heated to 95 °C for 10 min. 1 µL of each yeast extract was used directly in 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′). Amplification products were separated by electrophoresis on a 0.7% (w/v) agarose gel, detected by ethidium bromide staining and visualized by UV transillumination. A ladder marker (GeneRuler 100 bp DNA Ladder Plus, Fermentans) was used as a size reference. A 136-bp amplification product pointed to the identification of *P. fermentans,* and the estimated average levels (log cfu/ml) were obtained at the sampling time. To confirm the identification of *P. fermentans*, the 5.8S ITS rRNA gene region of representative yeast isolates was amplified using the primers ITS1 and ITS4 (MASOUD *et al.,* 2004) and 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).

1.3.6. Identification of bacterial isolates

Bacterial colonies from the Nutrient agar plates were initially grouped using repetitive extragenic palindromic (rep)-PCR according to according to the protocol of Pereira *et al.* (2012a). Representative isolates were selected and the 16S rRNA gene was partially sequenced (about 850–1,000 bp). Amplification with primers 27f and 1512r was performed as previously described (WANG et al., 2006). The PCR products were sequenced using an ABI3730 XL automatic DNA sequencer. The sequences were aligned using the BioEdit 7.7 sequence alignment editor and compared to the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, Maryland, USA).

1.3.7. Volatile compounds

A carboxen/poly (dimethylsiloxane) (DVB/CAR/PDMS) type 75 µm SPME fiber (Supelco Co., Bellefonte, PA, USA) was used to extract volatile constituents from the headspace of the fermenting coffee pulp–bean mass and roasted coffee beans. The coffee beans were roasted as described in section 1.3.9. Five grams were triturated with 20 mL of ultrapure water in a blender for 30 s. Ten milliliters of this extract or of the fermenting coffee pulp–bean mass were placed in a 20 mL hermetically sealed flask and heated for 10 min at 60 °C, to reach sample headspace equilibrium. Then, volatile compounds were extracted by placing the SPME fiber in the headspace for 30 min at 60 °C. The volatile compounds were identified by comparing the peak retention times against those of authentic standards purchased from Sigma in a gas chromatograph (Shimadzu model 17A) equipped with a flame ionization detector at 230 °C. The standards used were 11 alcohols (ethanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-decanol, 2-hexanol, 2-octanol, 2-methyl-1-butanol, n-butanol, 3-methyl-1-butanol), 12 esters (ethyl acetate, propyl

acetate, ethyl laurate, ethyl propionate, ethyl isobutyrate, ethyl hexanoate, ethyl octonoate, diethyl succinate, isoamyl acetate, isobutyl acetate, n-butyl acetate, hexyl acetate), 5 ketones (2,3-butanedione, 2-pentanone, 2-hexanone, 2-octanone, 2-heptanone), 3 aldehydes (acetaldehyde, benzaldehyde, and 3-methyl-butanal), and 2 organic acids (acetic acid and caprylic acid). 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, injector temperature at 230 °C. Individual volatiles were expressed as µmol/l of headspace, as ethanol equivalent.

1.3.8. Sugars and organic acids

The concentration of sugars (glucose and fructose) and organic acids (lactic, acetic, citric, fumaric, succinic, and malic acids) of the fermenting coffee pulp–bean mass and roasted coffee bean extract were determined by high-performance liquid chromatograph (HPLC) apparatus (Shimadzu LC 10AD VP; Shimadzu Corp., Kyoto, Japan) equipped with an Aminex HPX 87 H column (300 by 7.8 mm; Bio-Rad Laboratories, California) connected to a refractive index (RI) detector (HPG1362A; Hewlett-Packard Company). The column was eluted with a degassed mobile phase containing 5 mM H_2SO_4 at 60 °C at a flow rate of 0.6 ml/min.

1.3.9. Coffee cup quality evaluation

Green coffee samples were roasted in a semi-industrial roaster (Probatino, Leogap model, Brazil) with capacity of 1,300 g. The roasting cycle was defined through the sensory markers technique. This procedure consists of defining the thermal load during the roasting using the perception of specific aromatic notes, which are evaluated by a highly-trained coffee roaster. The roasted coffee was ground in a G3 Bulk Coffee Grinder (Bunn Corporation, USA) to an average particle

size between 360 and 420 microns. The coffee samples for cupping were prepared using 105 g of roasted and ground coffee in 1,500 ml of filtered water (Everpure Water Filter System, USA) using a VP17-3 BLK Coffee Brewer (Bunn Corporation, USA) with paper filter method (Melitta orginal 1 × 4). The water was treated to avoid the influence of different solutes and contained 90 ppm of total dissolved solid with a balance relation of 1:4 sodium and calcium, and had a pH of 6.50.

Cup quality was assessed by a panel of three expert coffee tasters with Q-Grader Coffee Certificate, coordinated by Ensei Neto, titular member of the Technical Standards Committee SCAA (2004–2010). The preparations (80 mL) were served in 240-mL snifters of 8 cm diameter to allow dispersion of volatile compounds to olfactory perception. Assessments started when the beverage temperature reached 65 °C for the olfactory step and 43 °C for the gustatory step. A first sensory evaluation was conducted assessing the following attributes: aroma, taste, acidity, body, balance, aftertaste, and overall quality. A note from 0 to 10, with 0.25 increments, was attributed to each criterion. After that, a descriptive analysis and a total score for each sample were assigned. At the beginning of each analysis, the panelists were familiarized with the two basic tastes, sweet and acid, with known concentrations of glucose, fructose, and sucrose (0.5, 1.0, 2.0, and 3 g/L) and citric acid (0.24, 0.48, and 0.90 g/L), respectively. Furthermore, a combination of citric acid and sugars (glucose, fructose, and sucrose) was used to determine the quality of acidity because the absence of sugar propitiates the acidity with an astringent perception. Thus, solutions were prepared containing a fixed concentration of citric acid (0.9 g/L), varying concentrations of each sugar into 1.0% w/v (‘sweet acidity’), 2.0% w/v (‘bright acidity’), and 3.0% w/v (‘liquorous acidity’). A solution with no sugar was prepared as a reference (‘sour acidity’). The solutions were prepared the same day of the session and tested at room temperature.

1.3.10. Statistical analyses

Means and standard deviations of metabolite target analysis and sensory evaluation data were calculated and subjected to ANOVA followed by post-hoc

comparison of means by Tukey'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.

1.4. RESULTS

1.4.1. pH and microbiological analysis

The pH and microbiological analysis (total microbial growth and inoculum persistence) of the starter culture-added fermentations (with and without sucrose supplementation) and spontaneous control are shown in TABLE 1. All fermentations were characterized by an initial pH value of ~5.4. The final pH values were correlated closely with bacterial growth; i.e., spontaneous and inoculated supplemented fermentations showed the lowest pH values (4.0 and 4.1, respectively) and higher bacterial growth (6.22 and 6.45 log cfu/mL, respectively), compared to the inoculated unsupplemented treatment, which had higher pH value (4.4) and lower bacterial population (5.68 log cfu/mL). Initial yeast counts in both inoculated fermentations were 2 log larger than in the spontaneous process and increased to significantly ($p<0.05$) higher numbers after 24 h of fermentation (6.45, 6.22, and 5.68 log CFU/ml in inoculated supplemented, inoculated unsupplemented, and spontaneous fermentations, respectively) (TABLE 1).

The frequency of *P. fermentans* yeast in the different fermentations was assessed with a DNA approach (TABLE 1).

TABLE 1 - PH, VIABLE COUNTS OF YEAST AND BACTERIA POPULATIONS, AND FREQUENCY OF *P. fermentans* YC5.2 IN FERMENTING COFFEE PULP–BEAN MASS OF THE TWO INOCULATED FERMENTATIONS (WITH OR WITHOUT SUCROSE SUPPLEMENTATION) AND SPONTANEOUS (CONTROL) FERMENTATION.

	pH		Total bacterial count log (cfu/ml)		Total yeast count log (cfu/ml)		*P. fermentans* frequency (%)	
Fermentation assay	T0	T24	T0	T24	T0	T24	T0	T24
Spontaneous	5.3±0.03[a]	4.0±0.10[a]	5.42±0.59[a]	6.22±0.12[a]	5.32±0.17[a]	5.78±0.21[a]	78.26	14.63
Inoculated	5.3±0.07[a]	4.4±0.10[b]	4.04±0.05[ab]	5.68±0.43[b]	7.46±0.14[b]	8.77±0.35[b]	81.82	83.93
Inoculated sup.	5.4±0.05[a]	4.2±0.05[ab]	4.75±0.13[bc]	6.45±0.10[a]	7.22±0.18[b]	8.62±0.42 [b]	78.57	84.62

* Means of triplicate fermentations in each row bearing the same letters are not significantly different ($p > 0.05$) from one another, using Duncan's test. (mean±standard deviation). Dominance was verified by PCR analysis, as described in the materials and methods section.

After the specific PCR analysis of the DNA extracted from the pooled colonies on the plates and confirmation, the dominance of *P. fermentans* was observed at the beginning of all fermentation processes, representing over 75% of the total yeast population. However, after 24 h of fermentation, the number of *P. fermentans* in the spontaneous process decreased drastically to 14.65% while maintaining over 80% in both inoculated fermentations.

One hundred and ninety-six bacterial isolates were recovered from different fermentation processes and grouped by $(GTG)^5$-PCR fingerprinting (data not show). Of this total, 78 isolates were selected for sequence-based identification (TABLE 2). *Bacillus subtilis* dominated both inoculated fermentations while *Microbacterium* sp. was prevalent during spontaneous process. A wide bacterial diversity was revealed at the beginning of all fermentation processes, encompassing species of *Microbacterium*, *Streptomyces*, *Nocardia*, *Rhodococcus*, *Arthrobacter*, *Pseudomonas*, *Cellulosimicrobium,* and *Curtobacterium.* However, at the end of both inoculated treatments, this diversity decreases dramatically, while remained high in spontaneous process.

TABLE 2 - DISTRIBUTION OF BACTERIAL ISOLATES FROM INOCULATED FERMENTATIONS (WITH OR WITHOUT SUCROSE SUPPLEMENTATION) AND SPONTANEOUS (CONTROL) FERMENTATION.

Isolate identification*	Spontaneous		Inoculated		Inoculated sup.	
	0 (h)	24 (h)	0 (h)	24 (h)	0 (h)	24 (h)
Bacillus subitilis	1**	2	2	5	1	4
Microbacterium testaceum	2	1	1	0	1	2
Microbacterium radiodurans	1	0	0	0	0	0
Microbacterium oxydans	0	1	1	0	0	1
Microbacterium flavescens	0	0	0	0	1	0
Microbacterium sp	2	4	0	2	1	3
Streptomyces misionensis	1	3	1	0	0	0
Streptomyces sp.	0	2	1	1	1	2
Nocardia niigatensis	1	0	0	0	0	0
Nocardia transvalensis	2	1	1	0	1	0
Nocardia sp.	1	1	0	0	1	0
Rhodococcus wratislaviensis	0	1	0	0	0	0
Arthrobacter scleromae	1	0	2	1	1	0
Pseudomonas sp.	0	1	0	0	2	1
Cellulosimicrobium sp.	0	2	1	0	1	0
Curtobacterium sp.	1	1	0	0	3	0

. *The BLAST search was based on sequences of type and cultured strains at GenBank (National Center for Biotechnology Information). The isolates were assumed to belong to a given species if the similarity between the query rDNA sequence and the sequences in the databases was higher than 97%. **Numbers following species names indicate the number of isolates per species from the same time point.

1.4.2. HPLC analysis

To assess the overall metabolic activity occurring in each fermentation process, the consumption of sugars (i.e., glucose and fructose) and formation of organic acids (i.e., lactic, acetic, citric, fumaric, succinic, and malic acids) were assayed (TABLE 3). The coffee pulp at the start of fermentation consisted of approximately 0.23 g/L citric acid, 0.13 g/L succinic acid, and nearly identical concentrations of glucose and fructose (4.0 g/L). Sugars consumption was higher ($p<0.05$) in inoculated fermentations, which showed the lower levels of residual glucose (in the case of inoculated unsupplemented fermentations) and fructose (in both inoculated fermentations). The inoculated supplemented fermentation ended up with high residual glucose content (4.07 g/L) compared to the inoculated unsupplemented (2.08 g/L) and control (2.86 g/L).

Lactic acid was a major end-metabolite of carbohydrate metabolism during all fermentations quantified by HPLC (TABLE 3). However, final lactic acid concentration of the spontaneous fermentation was approximately twice that of fermentations with added yeast. Citric acid was present in similar concentrations at the start of all the fermentation processes; after 24 h of fermentation, however, citric acid reached higher levels ($p<0.05$) in both inoculated fermentations compared to the control. Fumaric acid was formed only at the end of inoculated fermentations while the content of acetic and succinic acids was statistically similar ($p<0.05$) for all the fermentations.

In roasted coffee beans, citric and malic acids were predominant (TABLE 3). No differences ($p<0.05$) in the concentrations of sugars (glucose and fructose) and organic acids (lactic, acetic, citric, fumaric, and malic acids) were observed in roasted beans from any treatment.

TABLE 3 - CONCENTRATIONS OF SUGARS AND ORGANIC ACIDS IN FERMENTING COFFEE PULP-BEAN MASS AND ROASTED BEAN OF THE TWO INOCULATED FERMENTATIONS (WITH OR WITHOUT SUCROSE SUPPLEMENTATION) AND SPONTANEOUS (CONTROL) FERMENTATION.

Compounds	Fermentation assay (g/L fermenting coffee pulp-bean mass)						Roasted bean (mg/g coffee bean)		
	Spontaneous		Inoculated		Inoculated sup.				
	T0	T24	T0	T24	T0	T24	Spontaneous	Inoculated	Inoculated sup.
Glucose	4.11±0.19[a]	2.86±0.56[b]	4.17±0.17[a]	2.08±0.12[c]	4.28±0.23[a]	4.07±0.13[a]	0.62±0.08[a]	0.59±0.13[a]	0.55±0.17[a]
Fructose	4.24±0.21[a]	3.54±0.17[b]	4.56±0.13[a]	2.91±0.82[c]	3.42±1.14[a]	2.47±0.17[c]	0.24±0.12[a]	0.19±0.09[a]	0.23±0.10[a]
Citric acid	0.23±0.02[a]	0.25±0.04[a]	0.28±0.10[ab]	0.32±0.09[b]	0.29±0.15[ab]	0.73±0.1[d]	3.50±0.75[a]	3.45±0.24[a]	3.05±0.05[a]
Malic acid	ND	ND	ND	ND	ND	ND	2.50±0.70[a]	2.15±0.22 [a]	2.9±0.57[a]
Succinic acid	0.13±0.02[a]	0.15±0.01[a]	0.13±0.05[a]	0.16±0.09[a]	0.14±0.08[a]	0.17±0.02[a]	ND	ND	ND
Lactic acid	ND[a]	0.74±0.02[b]	ND[a]	0.26±0.09[c]	ND[a]	0.33±0.01[c]	0.97±0.11[a]	0.89±0.06[a]	0.92±0.10[a]
Fumaric acid	ND[a]	ND[a]	ND[a]	0.11±0.01[b]	ND[a]	0.14±0.05[b]	0.85±0.20[a]	0.65±0.15[a]	0.65±0.08[a]
Acetic acid	ND[a]	0.18±0.10[b]	ND[a]	0.24±0.08[b]	ND[a]	0.25±0.06[b]	1.80±0.12[a]	1.85±0.19[a]	1.55±0.27[a]

* Means of triplicate fermentations in each row bearing the same letters are not significantly different (p > 0.05) from one another, using Duncan's test. (mean±standard deviation).

1.4.3. GC–SPME analysis

The volatile compounds detected in the fermentation processes are shown in TABLE 4. Ethanol, acetaldehyde, and ethyl acetate were the most important compounds quantified in the fermentations. The next highest volatile compound concentrations corresponded to isomyl acetate and 2-heptanone. The levels of these compounds in fermentations with added yeast were comparable to and higher than the control ($p<0.05$). Propyl acetate, ethyl hexanoate, n-butyl acetate, and n-butanol were detected only in the control fermentation, while hexyl acetate and isobutyl acetate were detected in both inoculated treatments.

Among the volatile compounds identified in roasted beans, benzaldehyde was present in the largest amount, followed by isobutyl acetate, 2-pentanone, and 2-octanone (TABLE 4). The concentrations of acetaldehyde, ethyl acetate, and isoamyl acetate had a significant increase ($p<0.05$) in roasted beans from inoculated treatments compared to the control. Interestingly, these compounds were produced in higher concentrations in inoculated fermentations as mentioned above. In the same way, propyl acetate, ethyl hexanoate, and n-butyl acetate were detected only in control roasted beans and their respective fermentation process. A range of other compounds were identified in roasted beans but not detected in any fermentation process, such as alcohols (3-methyl-1-butanol, 1-pentanol, 1-hexanol, 2-hexanol, 1-octanol, and 1-decanol), esters (ethyl laurate, ethyl isobutyrate, hexyl acetate, isobutyl acetate, and ethyl propionate) and ketones (2-pentanone, 2-hexanone, and 2-octanone).

TABLE 4. CONCENTRATIONS OF VOLATILE FLAVOR COMPOUNDS IN FERMENTING COFFEE PULP-BEAN MASS AND ROASTED BEANS OF THE TWO INOCULATED FERMENTATIONS (WITH OR WITHOUT SUCROSE SUPPLEMENTATION) AND SPONTANEOUS (CONTROL) FERMENTATION.

Compounds	Sensory descriptor	Fermentation assay (µl/L fermenting coffee pulp-bean mass)						Roasted bean (µl/L headspace)		
		Spontaneous		Inoculated		Inoculated sup.				
		T0	T24	T0	T24	T0	T24	Spontaneous	Inoculated	Inoculated sup.
Acetaldehyde	Pungent; Sweet	0.005±0.002[a]	0.004±0.001[a]	0.014±0.015[a]	0.350±0.011[b]	0.011±0.027[a]	0.121±0.075[c]	0.006±0.001[a]	0.019±0.003[b]	0.045±0.001[b]
Benzaldehyde	Bitter; Almond	ND	ND	ND	ND	ND	ND	0.754±0.011[a]	0.471±0.018[b]	0.694±0.032[a]
Ethanol	Alcohol	0.024±0.011[a]	0.078±0.023[a]	0.068±0.006[a]	0.137±0.026[b]	0.089±0.055[a]	0.631±0.155[c]	ND	ND	ND
n-butanol	Banana; Vanila	ND[a]	0.005±0.001[b]	ND[a]	ND[a]	ND[a]	ND[a]	ND	ND	ND
3-methyl-1-butanol	Bitter; Chocolate	ND	ND	ND	ND	ND	ND	0.058±0.025[a]	0.053±0.019[a]	0.032±0.017[a]
1-pentanol	Fruity; Balsamic	ND	ND	ND	ND	ND	ND	0.004±0.001[a]	0.003±0.001[a]	ND[b]
1-hexanol	Green; Grass	ND	ND	ND	ND	ND	ND	0.018±0.005[a]	0.009±0.004[a]	0.019±0.004[a]
2-hexanol	Wine-like	ND	ND	ND	ND	ND	ND	0.017±0.003[a]	0.014±0.005[a]	0.019±0.008[a]
1-octanol	Intense citrus; Roses	ND	ND	ND	ND	ND	ND	0.022±0.012[a]	0.009±0.002[a]	0.013±0.011[a]
2-octanol	Earthy; Fatty	ND	ND	ND	ND	ND	ND	ND[a]	0.097±0.015[b]	ND[a]
1-decanol	Orange; Flowery	ND	ND	ND	ND	ND	ND	0.003±0.001[a]	0.005±0.001[a]	ND[b]
Ethyl acetate	Anise; Pineapple	0.005±0.002[a]	0.0173±0.049[a]	0.006±0.002[a]	0.567±0.114[b]	0.008±0.002[a]	0.435±0.085[c]	ND[a]	0.053±0.003[b]	0.011±0.005[c]
Isobutyl acetate	Apple; Banana	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	0.006±0.001[b]	0.12±0.07[a]	0.32±0.20[a]	0.51±0.09[b]
Isoamyl acetate	Banana; Pear	ND[a]	0.029±0.013[b]	ND[a]	0.007±0.001[c]	ND[a]	0.032±0.007[b]	0.008±0.001[a]	0.015±0.002[b]	0.017±0.003[b]
Hexyl acetate	Pleasant fruity; Pear	ND[a]	ND[a]	ND[a]	0.014±0.019[b]	ND[a]	0.004±0.002[a]	0.116±0.017[a]	ND[b]	0.104±0.009[a]
Ethyl laurate	Fruity; Floral	ND[a]	0.015±0.006[b]	ND[a]	ND[a]	ND[a]	0.001±0.002[b]	0.012±0.002[a]	0.020±0.006[a]	0.015±0.003[a]
Ethyl isobutyrate	Citrus; Strawberry	ND	ND	ND	ND	ND	ND	0.008±0.003[a]	0.003±0.003[a]	0.008±0.001[a]
Propyl acetate	Pear	ND[a]	0.027±0. 10[b]	ND[a]	ND[a]	ND[a]	ND[a]	0.009±0.001[a]	ND[b]	ND[b]
Ethyl propionate	Fruit, strong	ND	ND	ND	ND	ND	ND	0.014±0.004[a]	0.008±0.012[a]	0.012±0.002[a]
Ethyl hexanoate	Green apple	ND[a]	0.047±0.004[b]	ND[a]	ND[a]	ND[a]	ND[a]	0.099±0.023[a]	ND[b]	NDb
n-Butyl acetate	Fruity	ND[a]	0.023±0.011[b]	ND[a]	ND[a]	ND[a]	ND[a]	0.141±0.020[a]	ND[b]	ND[b]
2,3-butanedione	Buttery; Caramel-like	ND	ND	ND	ND	ND	ND	0.094±0.012[a]	0.052±0.003[b]	0.055±0.013[b]

To be continued...

Continued...

2-heptanone	Fruity; Flowery	ND[a]	0.036±0.0153[b]	ND[a]	0.014±0.005[c]	ND[a]	0.002±0.001[d]	ND [a]	0.043±0.004[b]	ND [a]
2-pentanone	Potato; Apple	ND	ND	ND	ND	ND	ND	0.146±0.058[a]	0.149±0.022[a]	0.206±0.030[b]
2-hexanone	Fruity; Spicy	ND	ND	ND	ND	ND	ND	0.034±0.013[a]	0.022±0.003[a]	0.033±0.004[a]
2-octanone	Mould; Green	ND	ND	ND	ND	ND	ND	0.562±0.103[a]	ND[b]	0.301±0.030[c]

ND. not detecte

1.4.4. Coffee cup quality evaluation

Sensory analysis of beverages produced with roasted coffee beans of the two inoculated fermentations and control received different scores for several important sensory attributes (FIGURE 1).

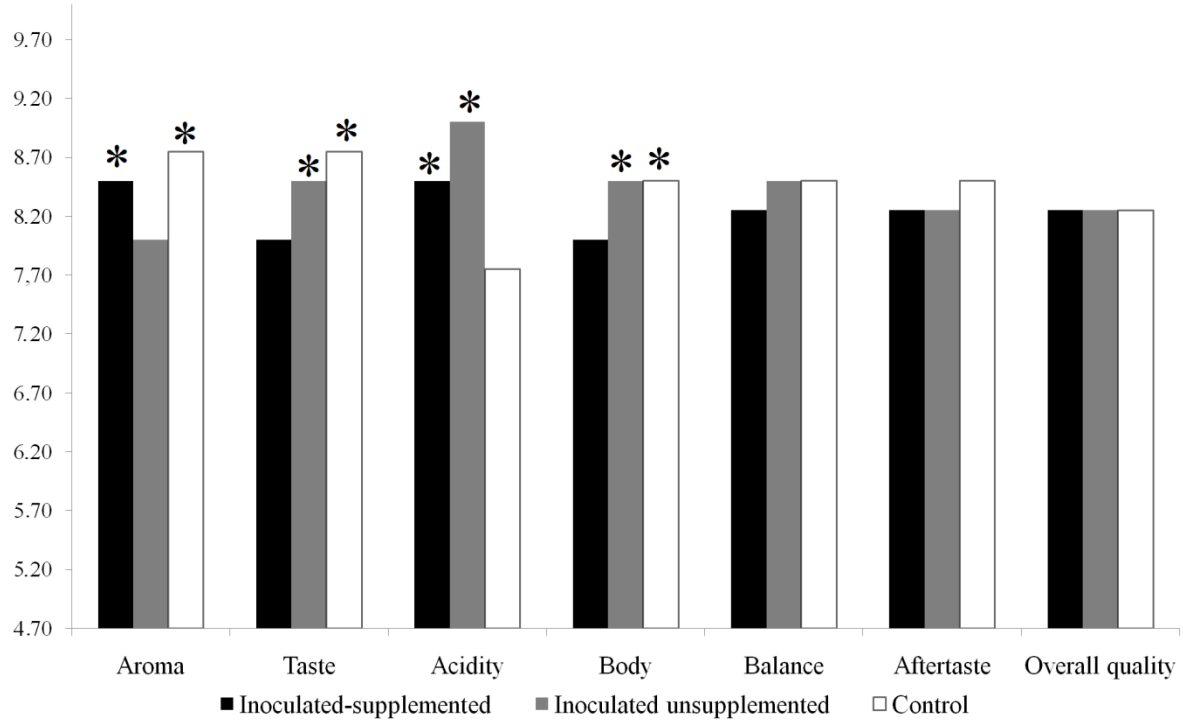

FIGURE 1 - SENSORY DIFFERENCES IN COFFEE BEVERAGES PRODUCED WITH FERMENTED, ROASTED COFFEE BEANS OF THE TWO INOCULATED FERMENTATIONS (WITH OR WITHOUT SUCROSE SUPPLEMENTATION) AND SPONTANEOUS (CONTROL) FERMENTATION. COMPARISONS WERE MADE BY A PANEL OF THREE EXPERIENCED COFFEE TASTERS. SIGNIFICANTLY DIFFERENT IN A TWO-SIDED P-VALUE < 0.05.

Body and taste attributes reached higher scores ($p<0.05$) in coffee beverages produced from spontaneous and inoculated unsupplemented processes, while aroma was scored significantly higher in beverages from inoculated supplemented and spontaneous processes and acidity in beverages from both inoculated treatments. These sensory variations resulted in coffee beverages with close similarity ($p<0.05$) in their overall quality, aftertaste, and balance attributes.

The sensory descriptive terms selected for the characterization of the different coffee beverages are listed in Table 5.

Table 5. Sensory descriptive terms and total score of coffee beverages produced with fermented, roasted coffee beans of the two inoculated fermentations (with or without sucrose supplementation) and spontaneous (control) fermentation.

Sample	Sensory descriptive terms	Total score
Control	Caramel, banana raisin, lactic, apricot	89
Inoculated	Citric, lactic, phosphoric, velvety, toffee	89
Inoculated sup.	Floral, Sicilian lemon, vanilla, elegant	87.5

All beverages showed a positive final evaluation (scored over 87 points) and desirable sensory characteristics. However, the results indicated that each treatment resulted in a beverage with distinctive sensory characteristics. The coffee beverage produced from an inoculated unsupplemented process presented velvet-like body perception, caramel-like taste, and intense perception of ‘lactic,’ ‘citric,’ and ‘phosphoric’ acids. The coffee beverage produced from the inoculated supplemented process presented characteristics such as ‘vanilla’ taste and intense perception of ‘Sicilian lemon’ and ‘floral’ aromas, being considered an extremely ‘elegant’ beverage by the panel of expert coffee tasters. Finally, the uninoculated control beverage presented with intense ‘caramel’ and ‘lactic’ tastes and exotic sensory notes of ‘apricot’ and ‘banana raisin.’

1.5. DISCUSSION

So far, the coffee industry has only paid attention to the aroma compounds formed during the roasting process and has ignored the possibility of controlling coffee fermentation during on-farm processing. This study reports for the first time the use of a yeast starter culture to conduct coffee fermentation during the wet process. Commercially available yeast starter cultures are currently not directly designed for fermenting coffee beans, mainly due to the physical and chemical peculiarities of this

process (PEREIRA et al., 2014). We instead used a selected yeast starter culture*, P. fermentans* YC5.2, originally isolated from wet processing and selected through specific characteristics suitable to drive the fermentation process, viz., coffee fermentation-associated stress tolerance and flavor-active ester compound production (PEREIRA et al., 2014). For its general evaluation, on-farm coffee fermentations (with or without sucrose supplementation) were inoculated with this starter culture strain and compared with spontaneous (control) fermentation. Sucrose supplementation of fermenting coffee pulp is a practice usually adopted by some coffee farmers from the Cerrado Mineiro region as an empirical way to optimize the fermentation process.

The inoculation of *P. fermentans* YC5.2 affected the fermentation courses in different ways. For example, its addition in high numbers ensured the persistence and dominance of this strain over wild yeast and bacteria populations in the culture-added fermentations. Even though a similar initial bacterial diversity was found for all fermentation processes, the inoculation affected growth and death of these microorganisms, and formed a restricted microbial composition at the end of the inoculated treatments. Some bacteria isolated in this study are considered to be clinically important species (e.g., *Nocardia niigatensis* and *Nocardia transvalensis*) which indicated human contact with the beans, while others species may be associated with fruit surfaces, water, soil, and the material used (e.g., *Bacillus subitilis*, *Microbacterium oxydans*, *Microbacterium flavescens*, *Arthrobacter scleromae*, *Curtobacterium* sp., *Microbacterium* sp., and *Cellulosimicrobium* sp.) (MCNEIL et al., 1992; WEN et al., 2004; SILVA et al., 2008). Thus, *P. fermentans* YC5.2 inoculum can be used to ensure the hygiene control of fermentation process and to prevent that microbial contamination spoils the beans. As was proposed in our previous work (PEREIRA et al., 2014), the ability of *P. fermentans* YC5.2 to withstand stress conditions imposed by the coffee fermentation was assumed to contribute to the efficient growth of the strain, though other factors such as efficient consumption of coffee pulp nutrient, spatial interference mechanisms, killer toxin, and quorum sensing, are likely play a role (FLEET, 2003). Such prevalence of the added starter culture strain is one of the main criteria for its applicability, which has been demonstrated previously in the use of yeast starter cultures in the production of, among others, cocoa and wine (CIANI et al,. 2010; LEFEBER et al., 2012; PERRONE et al., 2013).

Pulp sugars provide the substrates that drive microbial fermentation and create the environmental conditions that stimulate the production of important microbial metabolites (HO et al., 2014). In this study, although a residual content of pulp fructose and glucose was observed at the end of all the fermentations, its consumption was more efficient in inoculated processes. However, the inoculated supplemented fermentation ended up with high residual glucose content, probably resulting from hydrolysis of extra added sucrose by yeast metabolism. Some actions that may be adopted in order to improve the sugar pulp consumption by the added starter culture include cleaning and sanitizing of the fermentation equipment prior to fermentation, mixing and aeration of the bean mass, temperature control, and/or nitrogen supplementation (SCHWAN, 1998). Regarding organic acid production, one of the main influences of the application of the YC5.2 starter culture strain was the low presence of pulp lactic acid, and, consequently, higher final pH values. It was expected that, in the presence of low total bacteria population, there would be a weaker fermentation of pulp sugars by lactic acid bacteria and production of lactic acid. However, the concentrations of lactic acid and all other organic acids analyzed in this study in roasted beans were similar for all treatments. Thus, it is assumed that organic acids in the roasted beans originate by mechanisms that might occur within the seeds and not by diffusion from the pulp environment.

Directly, yeast fermentation of pulp sugars produces a vast array of volatile metabolites that are well known for their aromatic and flavorant properties (SWIEGERS et al., 2005). In the case of coffee bean fermentation, it is not clear how such volatiles might impact beverage flavor because, firstly, they must diffuse into the beans and, secondly, it is expected that they would be mostly lost by evaporation or otherwise transformed during the roasting operation. In this study, data from SPME/GC showed that the fermentations involving the yeast starter culture contained compounds typically reported in the literature as attributable to metabolic active yeasts, i.e., ethanol, acetaldehyde, ethyl acetate, and isoamyl acetate (STAM et al., 1998). Significantly, roasted beans from inoculated treatments gave more of these yeast-derived metabolites (in the case of acetaldehyde, ethyl acetate, and isoamyl acetate), which suggests their diffusion into the seed during the fermentation. We are not aware of any other studies on these kinetics during the coffee fermentation process. Although no experimental evidence has been given, it is often mentioned in the literature that such metabolites might contribute unique fruity, floral,

sweet, and other notes to food products' character (TABLE 4). In addition, some specific metabolites (e.g., propyl acetate, ethyl hexanoate, and n-butyl acetate) were detected only in fermentation and roasted beans from control treatment, thereby demonstrating that wild micloflora are responsible for their production and can impact coffee beverage flavor. It may be desirable to include other yeast and/or bacteria species in a mixed starter culture with the YC5.2 strain in order to obtain coffee beverages with increased chemical complexity. Currently, the use of mixed fermentation has also been proposed as a practical way to improve the complexity and to enhance the particular characteristics of a wine (CIANI et al., 2010). However, the knowledge regarding the impact of bacteria and yeast upon final coffee quality and process efficiency is limited, and more studies are needed to fill these important gaps. Finally, a range of other compounds that were identified in roasted beans were not detected in any fermentation process, leading to the conclusion that these volatiles are mostly generated during the course of fermentation by biochemical reactions within the bean or even by reactions that occur during bean roasting.

The quality of coffee beverages is largely determined by sensory perception. In this study, all prepared coffee beverages were scored over 87 points, which indicates very high coffee quality according to Specialty Coffee Association of America Cupping Protocols (SCAA, 2010). However, some sensory differences in body, taste, aroma, and acidity of the coffee beverages were detected by the expert coffee tasters. These sensory variations may be the result of the different compositions of the final beverages but had no influence on their overall quality, aftertaste, and balance.

In the sensory descriptive analysis, positive attributes detected in this study, such as fruity, floral, acid, citric, and caramel, are always mentioned in coffee beverage evaluations (LELOUP et al., 2004; NEBESNY and BUDRYN, 2006). The perception of exotic sensory notes of 'apricot' and 'banana raisin' in beverages from uninoculated treatment can be associated with specific compounds detected in this process and strongly suggests an important role for wild microflora in the development of these flavors as mentioned above. On the other hand, the use of the yeast culture was demonstrated to be favorable for the production of coffee with distinctive sensory profiles. The 'floral,' 'Sicilian lemon,' and 'vanilla' perceptions detected in the inoculated supplemented treatment and 'citric,' 'lactic,' 'phosphoric,' and velvet-like body perception in the inoculated unsupplemented treatment are

desirable sensations. In addition, both inoculated processes produced coffee beverages with high acidity, which is greatly appreciated in specific consuming regions. These coffee beverages can be used in blends to achieve desirable acidity and distinctive flavor.

In summary, the result from this study showed that *P. fermentans* YC5.2 was successfully implemented as a starter culture in coffee fermentation during wet processing, with the ability to dominate this process. Its metabolic activity during the fermentation process was shown to influence the final volatile fraction of roasted beans. Coffee beverages with distinctive flavor and high sensory quality were produced from inoculated beans and can be used to acquire different coffee market segments. These results suggest that yeasts have a greater impact on the chemical qualities of coffee than previously assumed. Our findings also point out the need for more research to better understand how the “other” wild bacteria and yeast species might impact the fermentation process and bean and beverage quality. Future research should be focused on the investigation of the possibility of creating multi-purpose mixed cultures by combining the aromatic and functional properties of various yeast and/or bacteria species.

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