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Faculdade de Engenharia Química  
Programa de Pós-graduação em Engenharia de Alimentos

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**LUDMILLA JANNE CARVALHO FERREIRA**

**CONTROLLED FERMENTATION OF ARARA COFFEE  
FROM CERRADO MINEIRO**

**Patos de Minas - MG**  
**2023**

**LUDMILLA JANNE CARVALHO FERREIRA**

**CONTROLLED FERMENTATION OF ARARA COFFEE  
FROM CERRADO MINEIRO**

Dissertação de mestrado apresentada ao Curso de Mestrado em Engenharia de Alimentos, da Universidade Federal de Uberlândia, comorequisito parcial para obtenção do título de mestre em Engenharia de Alimentos.  
Orientador(a): Líbia Diniz Santos  
Coorientador(a): Liliane Maciel de Oliveira

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## RESUMO

O café é um dos produtos mais relevantes para a economia brasileira. Consumidores tem buscado por cafés de qualidade e características sensoriais únicas, o que tem levado a mudanças nas tecnologias de processamento pós-colheita. A fermentação é uma ferramenta potencial para produção de cafés especiais. Entretanto, ainda acontece em pequenos volumes e possui baixa reprodutibilidade. O objetivo deste trabalho foi estudar a influência de parâmetros físicos (adição de água e influência de frutos imaturos) e microbiológicos (uso de microrganismos inoculantes) durante o processo fermentativo na qualidade sensorial e composição química do café. Foi realizado uma revisão bibliográfica de artigos de fermentação de café dos últimos 10 anos. A parte experimental foi realizada utilizando frutos inteiros de café arábica, cultivar Arara, da Fazenda Chuá do Grupo DB, no município de Patos de Minas, submetidos a fermentação em anaerobiose auto-induzida em estado sólido e com adição de água, submerso. Foram estudadas duas porcentagens de frutos imaturos (0.3 e 11%) durante a fermentação natural. E ainda, o uso de *Saccharomyces cerevisiae* e *Bacillus amyloliquefaciens* como inoculantes. Os experimentos foram avaliados quanto aos efeitos na composição físico-química dos cafés (açúcares, ácidos orgânicos, etanol, glicerol, umidade, proteínas, cinzas, acidez titulável e lipídeos) e também na qualidade sensorial da bebida avaliada segundo o protocolo SCA. De acordo com a revisão verificou-se que o processamento pós-colheita do café não é mais limitado a úmido, seco e semi-seco, e a etapa de fermentação pode ser determinada pelo tratamento do café, disponibilidade de oxigênio, adição de água e uso de inóculo. Existe uma migração da utilização de processos em ambientes abertos para ambientes fechados com anaerobiose induzida. Contudo, ainda não é possível definir um único processo fermentativo capaz de aumentar a qualidade do café ou desenvolver um padrão sensorial específico em qualquer condição ambiental. De acordo com os experimentos realizados, a porcentagem de frutos imaturos alterou significativamente o conteúdo inicial de açúcares (sacarose, glucose e frutose), cinzas e acidez titulável. A adição de água durante o processo fermentativo não influenciou significativamente a umidade, proteínas e as concentrações de ácidos cítrico e propiônico. O café fermentado com 0.3% de frutos imaturos apresentou grande produção de ácido láctico no processo submerso (67.44 mg/g), enquanto no processo em estado sólido, foram maiores para etanol (42.84 mg/g) e glicerol (1.68 mg/g). Em comparação com o estado sólido, o processo submerso deu origem a cafés com concentrações mais baixas de etanol, glicerol, cinzas, lipídios, ácidos succínico e acético. Frutos imaturos presentes durante a fermentação em porcentagem menor ou igual a 11% podem não influenciar negativamente a qualidade sensorial da bebida do café. A água foi capaz de modificar a atuação do inóculo. Os cafés coinoculados obtiveram as maiores pontuações sensoriais no processo submerso, acima de 86 pontos. O tipo de processo mostrou mais influência nas análises químicas e sensoriais que o tipo de inóculo utilizado. Os cafés do processo submerso mostraram descrições sensoriais de nozes, cacau, e doce, enquanto o processo de estado sólido produziu bebidas de café com atributos ácido, azedo e alcoólico.

Palavras-chave: Pós-colheita; fermentação de café; análise sensorial; análise química; qualidade do café.

## ABSTRACT

Coffee is one of the most relevant products in Brazilian economy. Consumers have been looking for quality coffee with unique sensory characteristics, which has led to changes in post-harvest processing technologies. Fermentation is a potential tool for specialty coffees production. However, it still occurs in small quantities and has low reproducibility. The objective of this work was to study the influence of physical parameters (water addition and immature coffee fruit effect) and microbiological parameters (microorganisms' inoculation) during the fermentation process on the sensorial quality and chemical composition of coffee. A literature review of coffee fermentation articles from the last 10 years was performed. The experimental section was carried out using whole fruits of Arabica coffee, cultivar Arara, from Fazenda Chuá of Grupo DB, in the city of Patos de Minas, submitted to fermentation in Self-Induced Anaerobic Fermentation (SIAF) in solid-state and submerged process. Two percentages of immature coffee fruits (0.3 and 11%) were studied during spontaneous fermentation. Also, the use of *Saccharomyces cerevisiae* and *Bacillus amyloliquefaciens* as inoculants were investigated. The experiments were evaluated regarding to effects on the physical-chemical composition of the coffee fruits (sugars, organic acids, ethanol, glycerol, moisture, protein, ash, titratable acidity, and lipids) and about the beverage sensorial quality evaluated according to the SCA protocol. According to the review it was found that post-harvest processing of coffee is no longer limited to wet, dry, and semi-dry, and the fermentation step can be determined by coffee fruit treatment, oxygen availability, water addition, and inoculum utilization. There is a migration from using processes in open environments to closed environments with induced anaerobiosis. However, it is not yet possible to define a single fermentative process capable to increase coffee quality or developing a specific sensory standard under any environmental condition. According to the experiments performed, the percentage of immature coffee fruits significantly altered the initial content of sugars (sucrose, glucose, and fructose), ash, and titratable acidity. The water addition during the fermentation process did not significantly influence moisture, protein, and citric, and propionic acid concentrations. The coffee fermented with 0.3% immature fruit showed high lactic acid production in the submerged process (67.44 mg/g), while in the solid-state process, they were higher for ethanol (42.84 mg/g) and glycerol (1.68 mg/g). Compared to the solid-state, the submerged process yielded coffees with lower concentrations of ethanol, glycerol, ash, lipids, succinic and acetic acids. Immature fruits present during fermentation in a percentage less than or equivalent to 11% may not negatively influence the sensorial quality of the coffee beverage. Water was able to modify the performance of the inoculum. Co-inoculated coffees obtained the highest sensorial scores in the submerged process, above 86 points. The type of process showed more influence in the chemical and sensorial analyses than the type of inoculum used. Coffees from the submerged process showed sensory descriptions of nutty, cocoa, and sweet, while the solid-state process produced coffee beverages with acidic, sour, and alcoholic characteristics.

**Keywords:** Post-harvest; coffee fermentation; sensory analysis; chemical analysis; coffee quality.

## 1 INTRODUÇÃO

O café é uma bebida consumida em todo o mundo, muito conhecida pelo seu sabor e aroma característicos. Apesar da ampla variedade de espécies, as mais comercializadas são *Coffea arabica* (café arábica) e a *Coffea canephora* (café robusta). O café arábica se destaca por apresentar bebidas de maior qualidade, mais suave, com maior diferenciação em sabor e aromas (BATISTA et al., 2016). Historicamente o café é um produto de grande importância econômica para o Brasil. O país é o maior produtor e exportador mundial do grão, sendo que em 2022 a produção chegou a 32.721 mil sacas de café arábica (ABIC, 2022). Juntamente com o aumento da produção, a busca de cafés com melhor qualidade cresce exponencialmente. O país exportou 38 milhões sacas de 60 kg de cafés diferenciados de janeiro a dezembro de 2021 com receita cambial de US\$ 5.803.514 (ABIC, 2021a). Além de ser uma cultura importante para exportação, o café tem sido valorizado também pelos próprios brasileiros, visto que o consumo nacional chegou a 21,5 milhões de sacas em 2021 registrando um crescimento de 1,71% em relação ao mesmo período analisado no ano anterior (ABIC, 2021b).

A qualidade do café é uma característica multifatorial e diretamente influenciada pelos processos de pós-colheita. Esta deve ser construída ao longo de todo o processo produtivo pois, depende de atributos relacionados ao grão, como cor, aparência, número de defeitos, e de atributos como o aroma e sabor da bebida (HAMEED et al., 2018; PEREIRA et al., 2017). Após a colheita, os frutos de café são transformados em grãos de café verde, que têm vários precursores aromáticos e não aromáticos. Estes precursores são transformados durante a torrefação para determinar o aroma e sabor final do café (LEE et al., 2015; MONTAVON; MAURON; DURUZ, 2003; PEREIRA et al., 2019). No mercado do café, o termo "café especial" refere-se a uma bebida que tem uma qualidade superior bem como um sabor e características únicas (MARTINEZ et al., 2021). A Specialty Coffee Association (SCA) é responsável pelo protocolo de classificação de cafés especiais aceito mundialmente (CARDOSO et al., 2021). O protocolo consiste numa análise sensorial desenvolvida por Q-graders certificados, utilizando amostras de café torrado. No teste, as amostras são avaliadas em relação a dez atributos primários de café: fragrância, sabor, gosto residual, acidez, corpo, uniformidade, equilíbrio, doçura, limpeza, e pontuação (SCA, 2018). Para o SCA, um café é considerado especial quando alcança pelo menos 80 pontos na escala de classificação e possui elevado potencial de expressão de aroma e sabor (CARDOSO et al., 2021).

Procurando ir ao encontro do mercado que procura cafés com diferentes perfis sensoriais, os produtores têm tentado desenvolver novas práticas pós-colheita, visando aumentar a qualidade dos seus cafés e a diferenciação sensorial. A fermentação é uma etapa espontânea e natural do processo produtivo do café, que ocorre devido à presença de substrato presente na mucilagem e microrganismos endógenos vindos da planta e do solo (HAMEED et al., 2018; PEREIRA et al., 2017). Estudos mostram que compostos desejáveis podem ser produzidos durante a fermentação e a migração desses compostos para o grão tem sido relacionada com a modulação de aroma e sabores finais do café, contribuindo assim para o aumento da qualidade (ELHALIS; COX; ZHAO, 2023; LEE et al., 2015; PEREIRA et al., 2021). Nos últimos anos os processos fermentativos de café têm avançado e muitos métodos de fermentação associados à pós-colheita tem surgido. Processos comumente aplicados como seco, semi-seco e úmido tem dado lugar para processos anaeróbicos, com uso de biorreatores. Além disso, muitos estudos apontam para os benefícios da utilização de microrganismos selecionados durante o processo fermentativo (BRESSANI et al., 2021; DA SILVA VALE et al., 2023; ELHALIS; COX;

ZHAO, 2023; HUCH; FRANZ, 2015; LEE et al., 2015; MARTINEZ et al., 2021; PEREIRA et al., 2021).

A Fermentação Anaeróbica Autoinduzida (SIAF) é uma tecnologia emergente baseada no acondicionamento de frutos de café em recipientes fechados, com ou sem adição de água. A anaerobiose é causada pelo consumo de  $O_2$  e a produção de  $CO_2$  pelo metabolismo dos microrganismos fermentadores. O método SIAF tem sido capaz de melhorar a qualidade do café, resultando em pontuações mais elevadas de acordo com o método SCA. Este é o método anaeróbico mais acessível aos produtores e está relacionado com alterações no comportamento microbiano, maior concentração de ácido láctico, modificações na concentração de compostos voláteis, e diferenciação nos perfis sensoriais resultando em cafés com atributos frutados mais intensos. (BRESSANI et al., 2021; CASSIMIRO et al., 2022; DA MOTA et al., 2020, 2022; MARTINEZ et al., 2021; PEREIRA et al., 2022). Entretanto, os parâmetros do processo ainda não estão completamente definidos. Atualmente o maior problema envolvendo a fermentação é a falta de controle do processo, que impede a reprodutibilidade dos resultados obtidos de um ano para outro.

Visto a importância do café para o mercado nacional e internacional, o objetivo deste trabalho foi realizar uma revisão bibliográfica para descrever detalhadamente os diferentes protocolos existentes para a condução da etapa de fermentação do café. Além disso, propomos neste trabalho uma nova forma de identificação do processo centrada em considerar a fermentação como uma etapa influenciada por quatro variáveis de controle: tratamento do café, disponibilidade de oxigênio, adição de água, e utilização de culturas iniciais. Além disso, a etapa experimental foi realizada para avaliar e identificar possíveis parâmetros de controle capazes de melhorar o processo fermentativo do café e possibilitar reprodutibilidade e maior produtividade. Parâmetros de processo como a disponibilidade de oxigênio, influência de frutos imaturos e influência da adição de água no processo fermentativo de café foram abordados pela primeira vez neste estudo. Tais parâmetros foram avaliados para fermentações espontâneas e com uso de microrganismos inoculantes. Os cafés produzidos tiveram a qualidade sensorial da bebida avaliada e relacionada com as características químicas de compostos identificados nos frutos após a fermentação.

## 1.1 OBJETIVOS

### Objetivo geral:

Estudar, implementar e avaliar técnicas de fermentação como ferramenta para produção de cafés especiais na região do Cerrado Mineiro, Brasil.

### Objetivos específicos:

- Estudar a influência da porcentagem de frutos de café verdes durante a fermentação em biorreator;
- Estudar a fermentação de micro lotes do café cereja natural através do método de fermentação SIAF (Self-Induced Anaerobic Fermentation), utilizando processamento submerso e em estado sólido.

- Estudar a influência da utilização de *Saccharomyces cerevisiae* e *Bacillus amyloliquefaciens* como culturas iniciadoras durante o processo fermentativo do café.
- Realizar um planejamento fatorial para identificar os efeitos das variáveis no processo fermentativo.
- Avaliar a influência da composição química dos frutos de café após a fermentação nas características sensoriais da bebida de café.

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## CAPÍTULO 1 - Coffee fermentation process: A review

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### ABSTRACT

In recent years, the importance of controlling coffee fermentation in the final quality of the beverage has been recognized. Several studies have been developed to evaluate and propose fermentation conditions that result in sensory improvements in coffee. So, this review aims to describe detailed the different protocols for conducting the coffee fermentation step and how they could influence the sensory quality of coffee based on the Specialty Coffee Association protocol. We propose a new way to identify coffee post-harvest processing not based on the already known wet, dry and semi-dry processing. The new identification is focused on considering fermentation as a step influenced by the coffee fruit treatment, availability of oxygen, water addition, and starter culture utilization. The findings of this survey showed that each type of coffee fermentation protocol can influence the microbiota development and consequently the coffee beverage. There is a migration from the use of processes in open environments to closed environments with controlled anaerobic conditions. However, it is not possible yet to define a single process capable of increasing coffee quality or developing a specific sensory pattern in any environmental condition. The use of starter cultures plays an important role in the sensory differentiation of coffee and can be influenced by the fermentation protocol applied. The application of fermentation protocols well defined is essential in order to have a good product also in terms of food safety. More research is needed to develop and implement environmental control conditions, such as temperature and aeration, to guarantee the reproducibility of the results.

**Key words:** Post-harvest; Specialty coffee; Flavor development; Natural Coffee; Pulped Coffee; Solid-state fermentation; Submerged fermentation, Sensory quality.

## 1. Introduction

Coffee is one of the most important crops, and it is of enormous economic relevance (Bastian et al., 2021). According to the International Coffee Organization (2022), the Arabica coffee world production in 2020 was 105.262 thousand of 60 kg bags, and this showed an increase of 13.7% compared to 2019 year. In the same period, the Brazilian Arabica coffee production was 60.292 thousand of 60 kg bags, and increase 26%. From February/2021 to January/2022, 124.608.939 of 60 kg bags of coffee were exported to all destinations. Brazil has than one-third of global production and exportation being the biggest coffee producer and exporter, followed by Vietnam, Indonesia, and Colombia (Hall et al., 2022). Arabica coffee stands for 70% global market and produces a beverage with a typical sweet aromatic (Batista et al., 2016; Hall et al., 2022).

In the coffee market, the term “specialty coffee” refers to a beverage that has a higher quality than the ones available for commodity prices, as well as a unique flavor and characteristics (Martinez et al., 2021). This quality of the coffee beverage is considered a consolidated criterion for reaching the markets that best pay for the product's added value. The Specialty Coffee Association (SCA) defined standardized methodologies to assist buyers and producers in evaluating the sensory quality of their products, especially for fair and more attractive trade (Cardoso et al., 2021). For the international market and coffee fermentation researchers, the method developed by SCA is the only one to define specialty coffee. The method consists of a sensory analysis released by certificated Q-graders, using roast coffee samples. In the cupping test, the samples are evaluated in relation to ten primary attributes of coffee: fragrance, flavor, aftertaste, acidity, body, uniformity, balance, sweetness, cleanliness, and score (SCA, 2018). For the SCA, the definition of beverage quality comes from specialty coffees characterized for not having any defect in the beverage, achieving at least 80 points in the rating scale for specialty coffees, and presenting differentiated quality and a high potential for aroma and taste expression (Cardoso et al., 2021). The specialty coffee production can be advantageous as it promotes a greater appreciation of coffee since the price of coffee is influenced by this classification – it is currently the main form of evaluation in the world market. According to Magalhães Júnior et al. (2021) specialty coffees can be commercialized for US\$ 20/kg, while commodity coffee beans only US\$ 2.5/kg. The growing demand for specialty coffee follows the increasing consumer interest in how coffee is brewed, as well as where, how and by whom the coffee was grown (European Market Potential for Specialty Coffee, 2020).

Seeking to meet the market that is looking for coffees with different sensory profiles, producers have been trying to develop new post-harvest practices, aiming to increase the grade of their coffees and sensory differentiation. Increases in final scores, even with small values, are reported as an improvement in quality. Furthermore, the final score is complementary to the flavor descriptive analysis, so it is not considered in isolation. Post-harvest processes have evolved over the years and the need to carry out the fermentation step in a controlled manner, mainly linked to the use of starter cultures, has already been pointed out as promising for flavor development, creation of volatile compounds, and product uniformity (Bressani, Martinez, Batista, et al., 2021; Brioschi Junior et al., 2021; Carvalho Neto et al., 2018; Elhalis, Cox, Frank, et al., 2020a; Evangelista, Silva, et al., 2014; Martinez et al., 2021; G. V. de M. Pereira et al., 2015,

2020; L. L. Pereira et al., 2020; Ribeiro, Ribeiro, et al., 2017). The fermentation has been carried out to develop new sensory profiles and increase the final score in coffees processed by different methods, aiming to achieve a better position in the market and a greater appreciation of the final product. In the literature there are already published patents on coffee fermentation. Most of them propose the development of equipment for use in induced fermentation of coffee fruits under different conditions of oxygen availability, temperature, agitation, and use of inoculating microorganisms (Cardoso et al., 2017; de Bortoli & Wirth, 2022; Palini & Alves, 2022; Socol et al., 2016, 2018, 2022).

Given this, the different ways of post-harvest processing including fermentative process application, show that the processing is no longer linked only to the old terms already known such as dry, semi-dry, and wet. This review aims to describe in detail the different protocols for conducting the coffee fermentation step. The new identification is focused on considering fermentation as a step influenced by four control variables: coffee treatment, oxygen availability, water addition, and starter cultures utilization. In addition, it is critical to discuss how those variables applied in an induced fermentation can influence microbial development and sensory specialty quality coffee based on SCA protocol. A better understanding and study of the influence of these variables allows us to improve the nomenclature of the coffee fermentation process and to promote better process control conditions and, consequently, greater reproducibility of the results.

## **2. Methodology for literature review**

The literature review included scientific articles that presented case studies published in the last ten years (2012-2022). The literature review was conducted in the Science Direct and Springer databases, and patents in INPI (Brazilian National Institute of Industrial Property) and ESPACENET, with the structured set of keywords: "coffee fermentation". After this primary search, 237 articles and 34 patents were compiled. In this review, only articles and patents which investigated the fermentation phase of Arabica coffee fruits in post-harvest processing were included. In addition, three older articles with important concepts about the fermentative process were included. Finally, 74 references were selected.

## **3. Postharvest coffee processing**

Coffee processing begins just after the harvesting of coffee cherries and the goal is to convert coffee cherries into green beans (Hameed et al., 2018; Wu et al., 2022). This process reduces fruit moisture and removes the protective outer coverings (husk, pulp, mucilage, and skin) around the pair of beans to make them more stable and suitable for storage, transportation, and roast process (Bastian et al., 2021; Hameed et al., 2018). The type of process employed depends on the area of production, infrastructure on farm, climate conditions, soil, and the genetic characteristics of the different varieties cultivated in each property (L. L. Pereira et al., 2021). After harvest, caution with the processing method is necessary. It is known that the coffee processing method influences the endogenous metabolic factors in coffee fruit and exogenous factors such as microbial

ecology and fermentation conditions and consequently causes changes in the biochemical composition of coffee (Lee et al., 2015; L. L. Pereira et al., 2021). Therefore, the type of process has a strong influence on coffee quality, from changes in coffee flavors and aromas to the dominance rate of these attributes in the final beverage (Hameed et al., 2018; T. S. Pereira et al., 2022; Zhang et al., 2019).

In general, three types of coffee processing procedures are used to obtain green coffee beans: wet, dry, and semi-dry methods, based on the fruit's initial condition. Wet process refers to pulped coffee submitted to underwater tank fermentation. Dry process refers to drying of entire coffee fruits on platforms. Semi-dry processing refers to drying of pulped coffee (Bastian et al., 2021; Lee et al., 2015; G. V. de M. Pereira, Soccol, et al., 2016). However, coffee processing includes several other steps, which may vary according to the farm infrastructure and the producer strategy. Currently, the fermentation step could be considered an independent step: after coffee fruit treatment and before drying (Figure 1). Because of that, these old definitions are limited, and are not able to cover all processing parameters clearly and objectively.

The on farm steps that involve the coffee processing include harvest, cleaning and separation, coffee treatment, fermentation, and drying. The first step is the harvest, which can be carried out manually or mechanically. After harvest, the coffee fruits can be washed and separated from impurities (sticks, stones, and leaves). As there are differences in the level of maturity of coffee fruits from the same tree, the washing process is used to separate them according to their maturation stage based on the density of fruit (immatures, cherries, and raisin/overripe). After that, each coffee type is processed separately because the selection of the appropriate cherry maturity level is important to the quality the coffee manufacturing process (Bastian et al., 2021; G. V. de M. Pereira et al., 2017; Vale et al., 2019). In the next step, coffee may be used in three different forms. One of them is the whole fruits, which are called natural coffee. The other forms are pulped coffee and demucilaged coffee, which are obtained after two different treatments. Mechanical removal of the skin and part of the mucilage give rise to the pulped coffee, and removal of all the mucilage by washing the pulped coffee gives rise to the demucilaged coffee (Figure 1). In the past, fermentation was considered only in wet processing (Lee et al., 2015). As coffee fruits are rich in moisture and sugars, mainly found in mucilage, it is possible to affirm that all coffee fruits will undergo some fermentation process, given the action of epiphytic microorganisms along with the processing (L. L. Pereira et al., 2021). Because of the high availability of the substrate the fermentation can occur simultaneously with drying or in induced fermentation, done before the drying in a separated step. The induced fermentation application is not obligatory. The aim of conducting the fermentation separated from drying is to provide a controlled environment and promote favorable biochemical reactions in coffee due to endogenous fruit metabolism and microorganism development. The induced fermentation can be carried out using natural or pulped coffee, on bioreactors with or without water, with or without starter culture adding, and in different conditions of oxygen availability. Finally, drying is performed on open terraces (cement, asphalt, or suspended) and/or on mechanical dryers until fruits reach 10-12% of moisture (Hameed et al., 2018; G. V. de M. Pereira et al., 2019).

Figure 1 - Post-harvest coffee processing flowchart.

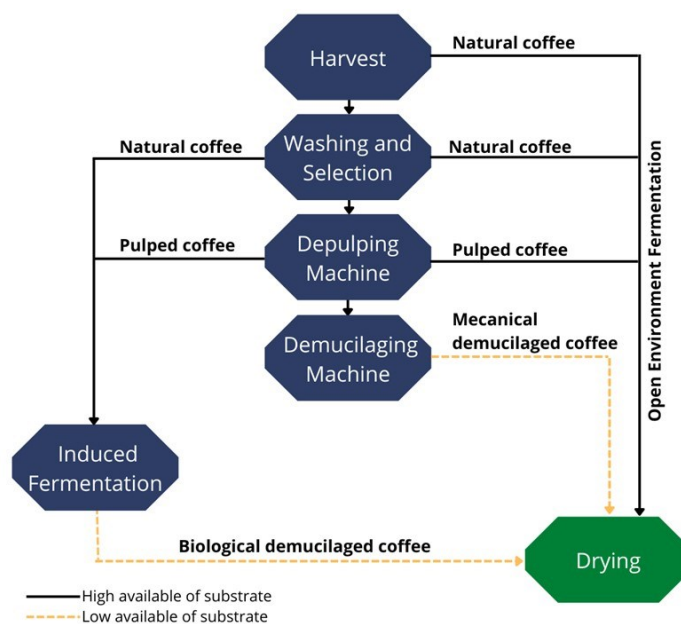


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### 3.1.Coffee fermentation step

Fermentation is the catabolic process of oxidation of organic substances, especially sugars, which are transformed into energy and simpler compounds such as ethanol, acetic acid, lactic acid, and butyric acid, by bacteria and yeast (L. L. Pereira et al., 2021). In coffee processing, the word “fermentation” has been generalized to every microbial metabolism that occurs during processing using the mesocarp as an energy source. The fermentation of coffee fruits occurs naturally regardless of the processing method and it is a complex process that involves the development of different microorganisms that can either improve or reduce the coffee quality (Cardoso et al., 2021; C. F. Silva et al., 2013). The first objective of fermentation is to remove the mucilaginous layer, which is rich in polysaccharides (pectin) (Hall et al., 2022; G. V. de M. Pereira et al., 2019; C. F. Silva et al., 2013). The Arabica coffee mucilage constitutes 2–5% of the fruit in dry weight, with 94% moisture, 4% sugar, 0.7% protein, and 1–3% pectin (B et al., 2019). The fermentation is also used to modify the coffee beans composition, and to develop flavor precursors (Lee et al., 2015).

The natural microorganisms in coffee are called epiphytic and they are mainly mesophilic, as the process is conducted at room temperature, mostly in tropical countries (Bastian et al., 2021). The diversity of microbial communities found in coffee during postharvest processing is influenced by the processing method applied (Hamdouche et al., 2016; G. V. de M. Pereira, Soccol, et al., 2016). Microorganisms’ development occurs from the harvest until drying (Evangelista, Miguel, et al., 2014; Evangelista, Silva, et al., 2014; T. S. Pereira et al., 2022; Ribeiro, Miguel, et al., 2017). Coffee fruits provide substrates for the development of bacteria, yeast, and filamentous fungi, supplying them with carbon and nitrogen sources, as they have cellulose, hemicellulose, pectin, reducing sugars, sucrose, starch, oils, proteins, acids, and caffeine (L. L. Pereira et al., 2021). During fermentation, the microorganisms can produce different enzymes and use different compounds to survive: polysaccharides are hydrolyzed into simple sugars and

used to produce alcohol, mannitol, glycerol, and acids; proteins are hydrolyzed into amino acids and can be metabolized into fusel alcohols, aldehydes, sulphurous compounds; lipids are hydrolyzed into fatty acids and can form secondary metabolites like esters, methyl ketones, alcohols and lactones; chlorogenic acids can be hydrolyzed into phenolic acids (Cardoso et al., 2021). Changes in the composition of the pulp and mucilage caused by the endogenous metabolic activity of beans and the microbial metabolic activity cause a decrease in pH from 5.5–6.0 to 3.5–4.0 and water activity from 0.9 to 0.7–0.8, allowing microbial growth (Bastian et al., 2021; Cardoso et al., 2021).

The microorganism growth and metabolites production are dependent on the cellular metabolism type which could be aerobic or anaerobic, according to the availability of oxygen (L. L. Pereira et al., 2021). Briefly, cellular respiration is a series of coupled oxidation and reduction reactions that result in the transfer of electrons from an appropriate electron donor (such as a reduced coenzyme) to an appropriate acceptor of electrons. Whatever the electron acceptor, respiration provides the ability to conserve energy in the form of adenosine triphosphate (ATP) or perform energy-demanding processes. Aerobic respiration produces more ATP, CO<sub>2</sub>, and water, promoting a higher growth. In terms of energy conservation, respiration-coupled oxidative phosphorylation via the proton motive force is substantially more efficient than fermentation, allowing faster growth and attainment of higher yields of biomass per mole of energy substrate used (Crocomo & Gutierrez, 2013; Nicholls et al., 1992). On the other hand, the anaerobic metabolism produces less ATP compared to aerobic respiration, however, it produces more organic acid by-products and volatile compounds that are relevant for changing sensory profiles. Coffee has great microbiological diversity, with the presence of microorganisms classified as facultative, in other words, they can choose one of these two routes depending on the environmental conditions. The yeast *Sacharomyces cerevisiae*, which is widely used industrially for different purposes and found naturally during coffee processing, is known to produce mainly ethanol under anaerobic conditions, but under semi-anaerobic and aerobic conditions it is capable to produce compounds such as succinic acid, lactic acid, gluconate, 1,2-propanediol, isobutanol, 2,3-butanediol (Gambacorta et al., 2020).

There are different discussions regarding the origin of the aromas and flavors in coffees subjected to different forms of processing that include direct drying or induced fermentation (L. L. Pereira et al., 2021). The formation of coffee flavor depends on several reactions that occur during roasting such as Maillard reactions, caramelization, and other thermal reactions involving aroma precursors (Lee et al., 2015). Some aroma and flavor precursors are formed during fermentation because of biochemical processes such as alcoholic fermentation, lactic and heterolactic fermentation, lipid degradations, acetification, and enzymatic hydrolysis. These precursors may confer additional flavor notes due to their potential to migrate into the seed (Cardoso et al., 2021). Specific non-aromatic compounds like sugars, acids, phenolic compounds (chlorogenic acid and trigonelline), caffeine, lipids, proteins, and amino acids are also important compounds present in green coffee beans which play an essential role in coffee aroma formation (Lee et al., 2015).

In the past, fermentation of coffee beans emerged to promote a faster degradation of the mucilage (G. V. de M. Pereira, Soccol, et al., 2016). Nowadays, it is known that coffee quality is closely related to several physicochemical constituents associated with flavor precursors produced during the fermentation step and related to sensory analysis (Bressani, Martinez, Batista, et al., 2021; Elhalis, Cox, Frank, et al., 2020b; Evangelista, Silva, et al., 2014; Lee et al., 2015; L. L. Pereira et al., 2021; Ribeiro, Ribeiro, et al., 2017). Hadj Salem et al. (2020) proved that occurs mass transfer of volatile compounds

from liquid phase to coffee beans during a submerged process. The authors showed that butanal, 2-phenylethanol and isoamyl acetate had different mass transfer rates and their transfer were influenced by several factors, such as the parchment resistance, the interaction with the yeast cell wall and the degradation reactions into the coffee beans. Therefore, the fermentation can affect the coffee aroma by degrading undesirable compounds or depositing favorable metabolites onto the beans (Pothakos et al., 2020).

The fermentation can be important to develop new flavors in coffee. However, the lack of control of the coffee fermentation process is harmful because it results in the loss of predictability of final coffee beans quality. Undesired products might be formed because of an uncontrolled fermentation, such as butyric or propionic acids, or even mycotoxins (Carvalho Neto et al., 2018; Evangelista, Silva, et al., 2014). It can still represent a problem related to food security, as well as difficulties in meeting the requirements of the global market (L. L. Pereira et al., 2021).

#### **4. Coffee fermentation processing methods**

It is known that coffee post-harvest processing is not developed in a sterile environment. In a lot of steps of the productive chain, several microorganisms are incorporated into coffee fruit, coming from the geographic region and manipulation (soil, air, equipment, people, and anything else that make physical contact with the fruits). The microbiota associated with the environmental conditions and the process control at the farm might or might not produce coffee with high-quality characteristics. The use of suitable processes associated with food safety and hygiene at all stages of processing can result in quality improvement (L. L. Pereira et al., 2021).

The coffee fermentation process is a multivariate system that is influenced by the environmental conditions such as temperature, pH, water presence, and epiphytic microbiota, and other than that, characteristics related to coffee fruit such as variety and coffee treatment (natural or pulped) (Bressani, Martinez, Batista, et al., 2021; Elhalis, Cox, Frank, et al., 2020b; Evangelista, Miguel, et al., 2014; G. V. de M. Pereira et al., 2017; Ribeiro, Miguel, et al., 2017). The fermentation method used influences the oxygen concentration and consequently the microbial metabolism. The oxygen concentration during coffee fermentation can promote alterations in the sensory profiles and the development of different flavors in coffee. The methods are based on the utilization of an open or closed environment, with or without the use of starter cultures inoculation, in solid-state or submerged fermentation (T. S. Pereira et al., 2022). The variables of coffee processing can be identified by four terms/stages: type of fruit treatment (natural or pulped), type of environment condition (oxygen availability), water adding (solid-state or submerged), and starter culture utilization. Based on the variations of the coffee fermentation process, Figure 2 shows the possible methods of conduction for fruit treatment, oxygen availability, water addition, and starter culture addition.

**Figure 2** – Conduction possibilities of coffee fermentation processes, regarding to coffee treatment, oxygen availability, water adding, and culture starter adding.

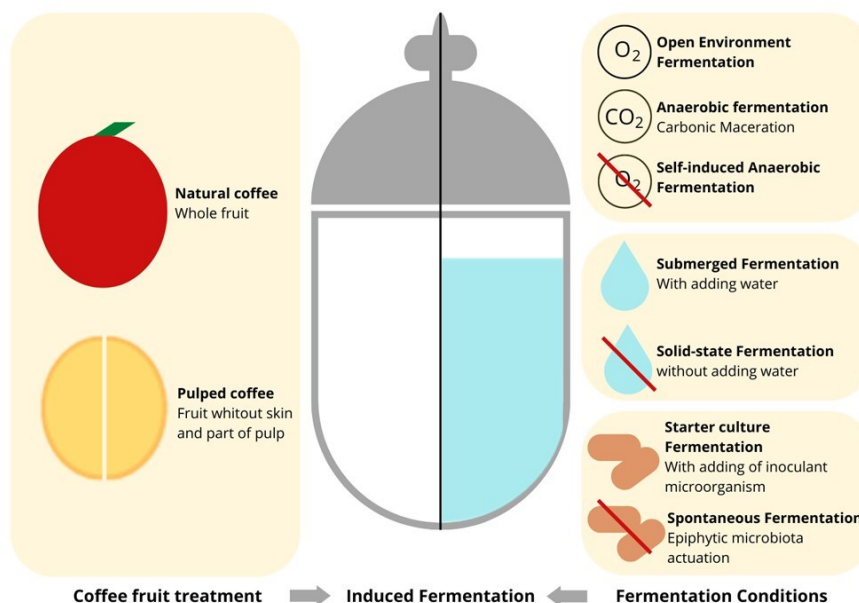


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## 4.1. Oxygen availability in fermentative process

### 4.1.1. Open environment fermentation

The open environment process is the oldest and simplest method of coffee processing. This method is characterized by fermentation and drying occurring simultaneously. After harvest, the fruits are spread on platforms or on the floor without prior removal of the pulp. The coffee is turned up and put on in layers that are approximately 5 - 10 cm thick, heaped at night, and respread each day until moisture decreases to 12–11%. It may last up to 10 - 25 days, depending on environmental temperature and humidity (Evangelista, Silva, et al., 2014; G. V. de M. Pereira et al., 2017; L. L. Pereira et al., 2021). When this process occurs with natural coffee cherries, it is called dry process, unwashed or natural. However, if the coffee is pulped the process is called semi-dry (G. V. de M. Pereira et al., 2017; G. V. de M. Pereira, Soccol, et al., 2016). In this process, the fermentation is associated with sun drying and it is the most common in tropical countries like Brazil and Ethiopia which expect extended periods of sunshine (Lee et al., 2015). During natural coffee fermentation, the microorganism access to the mucilage occurs through enzymatic actions as pectinolytic and cellulolytic enzymes produced by epiphytic microorganisms, opening the micropores on the skin (Evangelista, Silva, et al., 2014; Lee et al., 2015). During the dry process, the metabolism of the coffee beans continues, but the germination is inhibited (Bastian et al., 2021).

The process is generally a spontaneous fermentation conducted on the floor or in suspended terraces, and the bacteria diversity present is generally greater than the yeast diversity (Evangelista, Miguel, et al., 2014; Evangelista, Silva, et al., 2014; Martinez et al., 2017; Ribeiro, Miguel, et al., 2017; Ribeiro, Ribeiro, et al., 2017). However, the microbiota can change if the coffee treatment includes a washing process used to remove impurities in coffee cherries. Evangelista, Silva, et al., (2014), demonstrated that the microbial population was higher in non-washed coffee, and the water promoted a physical removal of microorganisms, mainly yeasts. It is possible to use selected microorganisms in dry and semi-dry processes and they are mainly inoculated by spraying microorganism solution while the natural or pulped coffee is placed on the drying platforms (Evangelista, Miguel, et al., 2014; Evangelista, Silva, et al., 2014; Martinez et al., 2017; Ribeiro, Miguel, et al., 2017; Ribeiro, Ribeiro, et al., 2017). However, the inoculation can also be carried on in a step before the drying, as a kind of pre-fermentation. Martinez et al. (2017) and Bressani et al. (2018) inoculated yeast solution on pulped and natural coffee cherries placed in open polystyrene buckets for 16 h and only after that, the fermented beans were transferred to a drying platform. The authors named this procedure bucket inoculation, and they observed that this method favored the permanence of the bacteria and yeast during the coffee process, especially in the treatment inoculated with *S. cerevisiae*. The natural coffee inoculated with yeast *S. cerevisiae* CCMA 0543 presented a significant difference in the sensory analysis, indicating the best result when inoculated using the bucket method. It resulted in a characteristic banana and cashew flavor and increased 4 points on the SCA scale, compared to coffee without inoculation (Bressani et al., 2018). On other hand, for pulped coffee, the sensorial results showed that no significant difference were observed between treatments (types of yeast) and inoculation methods (Martinez et al., 2017).

The process control and quality are variable, and directly related to the farm practices. In the first hours, the coffee moisture and sugar content are high, and the fruits are turned up (heaped and respread) more frequently. At night, the producers usually gather the coffee and cover them to prevent losing heat and receiving air moisture. In the dry and semi-dry processes, due to the coffee turning and the contact with the atmospheric air, it is possible to say that the fermentation is mostly characterized by aerobic conditions with high concentrations of sucrose, glucose, and fructose substrates (Hameed et al., 2018). It is important to highlight that in this environment the high availability of O<sub>2</sub> ends up being unfavorable for the anaerobic metabolism pathway and the microorganisms can act in a respiratory and non-fermentative way, given that several microorganisms have facultative metabolism and in the presence of O<sub>2</sub> they may breathe and reproduce instead of ferment and metabolize secondary compounds (L. L. Pereira et al., 2021). The availability of oxygen in this process can favor aerobic microorganisms, however, the measurement of oxygen concentration in the coffee mass has not been studied yet. Despite the availability of oxygen, it is not possible to consider that this is a full aerobic process, due to the existence of facultative microorganisms, the existence of coffee packaging regions and the lack of control over the availability and uniformity of oxygen in the coffee mass during solid-state fermentation.

At the beginning of the drying process in open environment fermentation, there are favorable conditions for filamentous fungi to develop because of the high sugar content, moisture, and temperature (L. L. Pereira et al., 2021). The *Aspergillus*, *Fusarium*, and *Penicillium* are predominant fungi genera during coffee fermentation responsible to produce mycotoxins and off-flavor (Bastian et al., 2021). The dry and semi-dry processes presented higher filamentous fungi diversity (Hameed et al., 2018). Fungus development is not totally controllable only with the good farm practices, since the procedure used for

each producer is very different and relies on the environmental conditions, that include the rain possibility. In these processes, the starter culture utilization can have an important role in food security. G. V. de M. Pereira, Beux, et al. (2016) found that in laboratory conditions the use of *P. fermentans* LPBYB13 in coffee cherries artificially contaminated with *Aspergillus westerdijkiae* showed efficacy in reducing ochratoxin A (OTA) content up to 88%.

Studies on dry and semi-dry processes used mainly yeasts as inoculum, and these microorganisms are known to have facultative metabolism. The use of starter culture in an open environment can promote beverages with a distinctive flavor and good sensory quality, but not always results in an increase of the beverage's final score according to the SCA protocol (Evangelista, Miguel, et al., 2014; Evangelista, Silva, et al., 2014; Martinez et al., 2017). Evangelista, Silva, et al., (2014) used the dry process with inoculation and obtained the caramel and fruit flavors, which was not identified in the process without inoculum addition.

In the literature, final quality observed in inoculated coffees were also dependent on plant variety (Ribeiro, Miguel, et al., 2017; Ribeiro, Ribeiro, et al., 2017). Ribeiro et al. (2017) observed a small difference in the attributes of fragrance/aroma and global described by the tasters in the cupping test. All processes evaluated, including spontaneous fermentation, resulted in scores higher than 80 points. The inoculation for Mundo Novo (MN) variety did not show a striking effect, because the major score observed, 84.25, was obtained by spontaneous fermentation. On the other hand, the inoculation was able to increase by 1.87 points in the Ouro Amarelo (OA) final score. Ribeiro et al. (2017) observed chocolate, lemon, nuts, and caramel flavor for OA without inoculation, while the inoculum *S. cerevisiae* 0543 provided a delicate acidity of lemon, caramel, and sweet flavor and the highest scores for flavor, acidity, body, aftertaste, balance, overall impression and final score (82.17). In general, OA showed better sensory characteristics than MN after inoculation.

The pre-fermentation was used by Bressani et al. (2020) and Bressani, Martinez, Sarmento, et al. (2021) with stainless-steel containers (open bioreactors) for natural and pulped coffee fermentation for 40 and 27 hours, respectively. The use of stainless-steel containers improved the hygiene during the fermentation of coffee and Bressani, Martinez, Sarmento, et al. (2021) showed that samples fermented with *S. cerevisiae* (CCMA 0543) presented the highest sensory scores in both processing (84.75 and 84.92). Processes in an open bioreactor were more efficient in pulped natural coffee because this treatment increased the score by 1.67 points compared to fermentation without starter addition. These results suggest that pre-fermentation can be benefit open environment processes. The major aseptis in the first hours of fermentation was benefic for inoculum development and promoted more evident diversification of sensory attributes. Further information about process variables, description, and sensory evaluation are described in the Supplementary Data Table 1. **(CITE: Supplementary Data Table 1 - Effect of processing variables in sensory results during open environment coffee fermentation)**

The studies presented in this review demonstrated that it is possible to obtain specialty coffees using the process conducted in an open environment, but some care should be taken. The main advantage of this method is its low implementation cost. However, the difficulty of control during the process represents the biggest disadvantage. Because it is a slow drying process, it can cause problems to be controlled, especially along a simultaneous fermentation process. It can be difficult to maintain large-scale production of a specialty coffee using this method due to the high possibility of contamination in the initial period. As it is a simpler process, the dry method can often be

performed with less care by the producer, ignoring the steps of coffee cleaning and preparation to achieve uniformity in maturation. However, the processing can be benefited by use of only ripe fruits and the pre-fermentation process. The application of pre-fermentation using starter culture can favor the sanitary quality of the coffee and allow the development of different sensory notes in the beverage.

#### 4.1.2. Anaerobic fermentation

In the condition of full anaerobiosis, the microorganisms follow the fermentative pathway, transforming the raw material, mainly sugars, into energy and other metabolic products, such as ethanol, CO<sub>2</sub>, glycerol, organic acids (pyruvic and succinic), volatiles alcohols, and ester (Crocomo & Gutierrez, 2013; Mauricio et al., 1997). Fermentation always occurs in the cytoplasm of the cell with the aid of enzymes, which act as catalysts (L. L. Pereira et al., 2021). Compared with respiration, fermentation processes consume much more hexose molecules generating the same number of ATP molecules (Cardoso et al., 2021).

Anaerobic fermentation has been used in Brazil and Thailand to modify sensory profiles and creating specialty coffees with a consistent level of quality in every season on an industrial level. Carbonic maceration or semi-carbonic maceration is a process inspired by wine processing. It is a solid-state fermentation based on the fermentation of whole coffee cherries (unruptured cherries) in an anaerobic (oxygen-free) environment of carbon dioxide (CO<sub>2</sub>) in a closed container (Jitjajoen, 2021; L. L. Pereira et al., 2021). Firstly, the coffee cherries absorb CO<sub>2</sub>, and an oxygen-free fermentation breaks down the sugars and increases acidity. This causes a modification of pulp color, which turns into a pink/purplish color because of the migration of anthocyanins and tannin from the skin (L. L. Pereira et al., 2021).

The process can be performed with or without inoculation. The use of the inoculum can accelerate the degradation of mucilage, which helps decrease the stickiness and increase simple sugars, adding more sweetness to the coffee (Jitjajoen, 2021). The temperature control is crucial to provide the ideal conditions for microorganisms to act in fermentation. At higher temperatures, it is possible to reduce the fermentation time, which lasts around 48 h, while in low-temperature fermentation, it is necessary to extend the fermentation step by 5–8 days. It is recommended that the carbonic maceration process should be performed with strategies of at least 4 days of fermentation at a constant temperature of 36 °C or 6–8 days with temperatures of 26 °C (L. L. Pereira et al., 2021).

Brioschi Junior et al. (2021) used sterile plastic bags injected with CO<sub>2</sub> to spontaneous fermentation of Catuaí Vermelho variety. They evaluated different fermentation times and temperatures. The microbiota of the coffee varied with the time and temperature of fermentation. The fungal community had low variation over time and was smaller in 120 h compared to the initial population. On the other hand, the bacterial diversity increased over time and was correlated with the increase in aftertaste and fragrance. In the cupping test, there was an increase in the global note of coffees with the increase in time and temperature of the fermentation. The process using 38°C and 120 hours had the best score (85.15). The organic compounds like trigonelline, formic acid, lipids, and  $\gamma$ -butyrolactone contributed to the score and sensory quality of the coffee beverage. Further information about process variables, description, and sensory evaluation are described in the Supplementary Data Table 2. **(CITE: Supplementary**

**Data Table 2 - Effect of processing variables in sensory results during anaerobic coffee fermentation.)**

Many producers are applying O<sub>2</sub> restriction processes to promote anaerobic fermentation with or without added microorganisms. Under anaerobic conditions, the cell produces less energy, which can consequently affect the growth of microorganisms and the production of enzymes (L. L. Pereira et al., 2021). According to Elhalis, Cox, Frank, et al. (2020a) by-products of lower molecular weight are interesting for flavor development in coffee and ethanol is important for beverage viscosity and can act as a solvent for volatiles.

There are still few studies related to the application of anaerobic fermentation in coffee, and we suggest that the anaerobic process with pulped coffee should be studied. The advantage of this method is that it provides an environment with more asepsis and the possibility of reproducing the environmental conditions of anaerobiosis, inhibiting the growth of pathogenic aerobic microorganisms. On the other hand, the disadvantage is that it is a method that requires bioreactors with pressure control and CO<sub>2</sub>. Since this process requires greater investment for application this method should be better studied, as it may be a promising way to produce specialty coffees as seen above.

#### **4.1.3. Self-Induced anaerobic fermentation**

Self-induced anaerobic fermentation (SIAF) is a new technology to ferment coffee that was first described by da Mota et al. (2020) using closed cylindrical polyethylene bioreactors. This method is based on coffee fermentation in a closed environment without adding water, that is, in solid-state fermentation. SIAF process has an aerobic and an anaerobic stage. The anaerobic condition is achieved slowly due to the metabolism of the microorganisms which consume O<sub>2</sub> and produce CO<sub>2</sub>. Commonly, it has been carried out in polyethylene bioreactors with small dimensions, ranging between 20 and 200L. In these cases, the anaerobic environment is achieved by using an airlock valve, that makes possible the escape of part of the CO<sub>2</sub> produced inside the reactor, but it does not allow the entry of oxygen. This is a method that can be carried on with natural or pulped coffee cherries and normally bioreactor homogenization and feeding are done manually (Martinez et al., 2021). This technology enhances the fermentative performance of lactic acid bacteria (LAB) and yeast, increasing metabolite production and beverage sensory quality (T. S. Pereira et al., 2022). The SIAF positively impacts microbial behavior, resulting in coffees with a more intensified fruity attribute. It is noticeable the presence of metabolites like chlorogenic acid, sucrose, lactic acid, and trigonelline in coffees produced by this process (T. S. Pereira et al., 2022).

The temperature inside a bioreactor is dependent on environmental temperature and increases during the time of fermentation (Martins et al., 2020). In SIAF method, the measurement of temperature is very important because the finish time is defined by temperature stabilization. Martinez et al. (2021) implemented and evaluated the performance of 300 L stainless steel bioreactors that were built to increase specialty coffee production under monitored fermentation conditions. The installation of thermometers made continuous temperature monitoring possible until the end of fermentation. The authors observed that the temperature variation inside the reactor was different depending on the coffee treatment, and consequently, the time of fermentation: natural coffee fermentation lasted longer than pulped coffee fermentation (16 and 8 hours respectively).

The use of closed bioreactors ensures the best performance of the yeast starter cultures. However, the coffee treatment (natural or pulped), the region (altitude and temperature), and the strain of the microorganisms used as starter cultures influenced on results obtained using SIAF (Bressani, Martinez, Batista, et al., 2021; da Mota et al., 2020; Martinez et al., 2021). For Martinez et al. (2021) the SIAF method produced coffee beverages with 82.6 points using spontaneous fermentation (natural and pulped). However, the inoculation of *S. cerevisiae* CCMA 0535 increased 1.73 points in pulped coffee and the coffee beverage showed yellow fruit, chocolate, caramel and floral flavors, while in natural treatment the inoculation increased 1.40 points and showed chocolate, fruits, floral, citric, prune and peach flavors. Da Mota et al. (2020) showed that *Torulaspora delbrueckii* inoculation had more influence on natural coffee and the best results were achieved in Southern Minas Gerais and Cerrado. This inoculum was capable to increase the final score by 5.75 points (SCA scale). According to Bressani, Batista, et al. (2021) all coffees produced using SIAF obtained a final score greater than 85 points. The chocolate and caramel were common in all treatments, including fermentations without inoculum. Despite that, it was possible to observe that the treatments resulted in different perceptions depending on the inoculum combination. The best coffee score was 86.9 for fermentation released with co-inoculation with *S. cerevisiae* CCMA 0543, *Candida parapsilosis* CCMA 0544, and *T. delbrueckii* CCMA 0684, which showed chocolate, caramel, molasses, fruity, red fruit, citric and mint flavors. The authors also showed that the total phenolic content has a strong and positive correlation with the fermentation time and a moderate and positive correlation with the beverage antioxidant activity. Further information about process variables, description, and sensory evaluation are described in the Supplementary Data Table 3. **(CITE: Supplementary Data Table 3 - Effect of processing variables in sensory results during self-induced anaerobic coffee fermentation.)**

The use of SIAF method is capable to produce specialty coffees in spontaneous fermentation (Bressani, Batista, et al., 2021; da Mota et al., 2020; Martinez et al., 2021). Furthermore, depending on the coffee treatment and strain used as a starter culture, an increase of more than 5 points on the SCA scale may be achieved (da Mota et al., 2020). The best result found in this review was a score of 87.25 points, obtained by a natural coffee fermentation of Bourbon Amarelo variety, from high altitude (1661 m), and fermented for 70 hours using *T. delbrueckii* inoculation (da Mota et al., 2020). The main advantage of this method is that it allows a low-cost anaerobiosis environment, making it affordable for small producers. However, the disadvantages are mainly related to the absence of process control tools, especially regarding the use of polypropylene drums. In these cases, normally there are no pH or temperature sensors, or any device to promote coffee agitation. Therefore, it is necessary to open the bioreactor to carry out homogenization, pH, and temperature checking, allowing the oxygen to enter. No data were found regarding the measurement of oxygen concentration inside the reactor. Therefore, this could bring more clarity about the oxygen variation within the reactor and how the metabolism of microorganisms could be affected. It is also important to emphasize the importance of defining homogenization standards during the fermentation time, since in solid-state fermentation, heat transfer is difficult. Ideally, producers that opt for this method would invest in a bioreactor like the one proposed by Martinez et al. (2021).

## 4.2. Water addition in fermentative process

Arabica coffee submerged fermentation, using water and pulped coffees, is known as the wet process. However, this process can also use natural coffee (Cassimiro et al., 2022). The spontaneous fermentation applied to the wet process is the oldest known fermentative process, mostly used in regions of high relative humidity like Colombia, Central America, Hawaii, and South America (Hameed et al., 2018; Lee et al., 2015; L. L. Pereira et al., 2021). This method is most used on Arabica coffees, which the coffee cherries are harvest, and the peel and pulp are removed mechanically, leaving the mucilage adhered to the beans. Subsequently, these pulped coffees, rich in simple sugars and pectin, are transferred to water tanks where they are allowed to ferment for 6 to 72 h, depending on the environmental temperature, being 24 to 48h the most common. During fermentation, the tank can be homogenized (from 4 to 4 hours) but this is not a standard. The process ends when a complete degradation and solubilization of the mucilage layer is achieved, so that the coffee can be removed from the tanks and sun dried (Evangelista et al., 2015; G. V. de M. Pereira, Soccol, et al., 2016; Ribeiro et al., 2018). The advantage of this process is the low infrastructure investment required because it is possible to use a tank normally made of cement (Lee et al., 2015; L. L. Pereira et al., 2021). The volume of water added to the tank for fermentation is not totally standardized. The ratio of pulped coffee and water can be done in 1:1 (m/v) (Carvalho Neto et al., 2018; Evangelista et al., 2015), 1:5 (Bodner et al., 2019), 1:25 (G. V. de M. Pereira, Carvalho Neto, et al., 2016), 1:0,7 (Ribeiro et al., 2020). It is also common to carry out this type of fermentation without specifying the volume of water, using the amount needed to cover the fruits (Carvalho Neto et al., 2017; Elhalis, Cox, Frank, et al., 2020a; Ribeiro et al., 2018).

In a submerged process, the fermentation occurs simultaneously with the germination process, increasing the number of amino acids (Bastian et al., 2021). The use of the submerged process increases water activity and gradually decreases oxygen availability. It has an advantage in use as it facilitates homogenization and can increase the speed of mucilage removal, as the components are mostly soluble. Submerged fermentation is an alternative to enable greater control of the fermentation process, since it is possible to control the aeration rate, thus controlling the time of aerobic and anaerobic phases. Carvalho Neto et al. (2018) were the first to set up coffee fermentation in a stirred-tank bioreactor (STR), according to the literature consulted in this review. Conditions of sanitization, aeration (12 hours aerobic and 12 hours anaerobic), and temperature were controlled to achieve an improved process. These authors found that the application of controlled conditions without inoculation increased the coffee quality from 82.25 to 85.5 points.

The end of the fermentation can be evaluated by the reduction in pH levels below 4.5 or by touch because the parchment that surrounds the grains loses its viscous texture and gives a feeling of greater harshness (friction between the coffee beans) (G. V. de M. Pereira, Carvalho Neto, et al., 2016; L. L. Pereira et al., 2021; Ribeiro et al., 2018). However, these methods are not enough to contemplate the diverse biochemical reactions that can take place in the bioreactor after mucilage solubilization, and the consequent migration of bioproducts to the seed. The fermentation time leads to a great change in the total volatile organic compounds. Although the fermentation time may vary with coffee variety due to differences in mucilage composition, fermentation times longer than 48 hours are not recommended due to reduced activity of the enzyme isocitrate lyase. This enzyme is directly associated with volatile organic compounds formation (Bodner et al., 2019; Ribeiro et al., 2018). Bodner et al. (2019) showed that the total volatile organic

compounds decrease with the increase of the fermentation time independent of the roasting degree. Furthermore, according to the same authors, the coffee beans fermented for a prolonged period contain less acidic notes, less low molecular weight sugars, and more phenolic compounds, which does not benefit coffee quality. Similarly, Peñuela-Martínez et al. (2018) showed that the best coffee quality was obtained from the submerged process that used short process times (24 hours) and low temperature (17 °C).

The submerged process produces coffee with different composition and sensory characteristics when compared to coffee processed by the solid-state methods, which are alternatives used by the producer to suit different markets. This process promotes a microaerobic environment because oxygen gets into water by diffusion from the air, by aeration promoted with agitation, or as a waste product of photosynthesis. The microaerophilic environment and nutritional selectivity of coffee pulp lead to the development of a diverse range of microbial groups, including lactic acid bacteria (LAB), acetic acid bacteria (AAB), *Bacillus*, *Enterobacteriaceae*, yeasts, and filamentous fungi (Elhalis, Cox, Frank, et al., 2020a; G. V. de M. Pereira et al., 2020; L. L. Pereira et al., 2021). Most of the microbiota present throughout the submerged processing is also detected in coffee fruit. The submerged processing favored bacteria development, observing a high increase in the population into the first 36 hours of fermentation. An important characteristic of submerged processing is that it does not favor the growth of filamentous fungi that produce mycotoxins (Hameed et al., 2018).

The submerged fermentation application can result in changes of the final score and sensory profile. Nonetheless, those results are dependent on the coffee variety (Ribeiro et al., 2018) and altitude (region) (Bodner et al., 2019; Evangelista et al., 2015; L. L. Pereira et al., 2020). The spontaneous submerged fermentation results in the accumulation of major groups of volatiles including alcohols, esters, organic acids, ketones, and aldehydes, and impact the sensory quality of coffee beans and the beverage (Elhalis, Cox, Frank, et al., 2020b). According to L. L. Pereira et al. (2020), the results using the submerged fermentation may be more favorable for areas above 893 m, which can be confirmed by the significant linear relationship with altitude for all attributes of the SCA protocol. The highest sensory score of 85.39 was observed at 1033 m. On the other hand, in the study carried out by Bodner et al. (2019), coffee from a smaller altitude (900 m) got the best results regarding the production of volatile organic compounds when compared to coffee processed at 1500 m. The total volatile organic compounds were higher at both altitudes for a shorter fermentation time (24h). However, this study did not perform any sensory analysis. Therefore, the influence of altitude can be highlighted as an essential factor in submerged fermentation. From the studies found, it is not possible to define the ideal altitude.

There are interesting results using coffee submerged fermentation with starter cultures inoculation. The use of yeast culture *S. cerevisiae* improved coffee quality in lower altitude, indicating potential for quality improvement through induced fermentation (L. L. Pereira et al., 2020). G. V. de M. Pereira et al. (2015) used the *Pichia fermentans* YC5.2 and found that lactic acid was a considerable end-metabolite of carbohydrate metabolism during submerged fermentation. Other important compounds quantified were ethanol, acetaldehyde and ethyl acetate. The inoculated coffee produced a beverage with velvet-like body perception, caramel-like taste, and intense perception of 'lactic', 'citric' and 'phosphoric' acids. Although, beverage from the uninoculated fermentation presented intense 'caramel' and 'lactic' taste and exotic sensory notes of 'apricot' and 'banana raisin'. Despite having different sensory characteristics, the inoculated and uninoculated coffee achieved the same final score of 89 points in the cupping test.

Studies have shown that bacteria can also be used as inoculum, and they can also increase quality and develop new sensory profiles in the submerged process. Carvalho Neto et al. (2018) obtained the best results in the submerged process using the inoculation of acid lactic bacteria *Lactobacillus plantarum*. The bacteria strain also significantly increased the formation of lactic acid (twice as high in the inoculated process compared to spontaneous treatment) and volatile aroma compounds during the fermentation process, such as butyl acetate, ethyl acetate, 1-octanol, isoamyl acetate, ethyl hexanoate, ethyl succinate, and ethyl octanoate. Aroma, flavor, acidity, body, and balance reached higher scores in the inoculated treatment compared to the spontaneous one, while sweetness, clean cup, and uniformity were statistically similar for both treatments. Nonetheless, both beverages scored over 80 points (91.5 and 85.5 for inoculated and spontaneous processes, respectively). One of the limitations of introducing starter cultures in submerged processing is that coffee fermentations are conducted in open cement tanks that facilitate contamination by natural microbiota (Carvalho Neto et al., 2018). Further information about process variables, description, and sensory evaluation are described in the Supplementary Data Table 4. **(CITE: Supplementary Data Table 4 - Effect of processing variables in sensory results during submerged coffee fermentation.)**

Closed tank made of stain steel would be more interesting for the process and would facilitate cleaning, sanitation, and temperature control. The use of water during coffee fermentation can be advantageous because it facilitates homogenization and heat transfer processes. While the main disadvantage may be associated with increased water consumption during post-harvest and the need for higher investments in wastewater treatment. In this review, no data were found which highlighted the action of water during coffee fermentation. More studies are necessary to evaluate the physicochemical and sensory differences that may occur when choosing a submerged or solid-state process.

#### 4.3.Processing conditions and microbiota relationship

The microorganisms found in coffee comes from multiple ecosystems, such as water, native soil, air, and plant. Their presence in coffee fruits is dense and diverse and includes yeasts, filamentous fungi, and bacteria (Lee et al., 2015; G. V. de M. Pereira et al., 2020; C. F. Silva et al., 2013). Coffee processing is not sterile, which makes fermentation a complex process that exhibits a succession of microbial populations. The metabolism of these microorganisms plays a specific role in the biochemical reactions within the bean and influences coffee sensory properties (Bressani, Martinez, Batista, et al., 2021; G. V. de M. Pereira, Soccol, et al., 2016).

Total microbial population and prevailing species during the fermentation process depends on several factors such as coffee species/varieties, the processing method employed, geographical origin, altitude, and climatic factors (Donfrancesco et al., 2019; Hameed et al., 2018; Martins et al., 2020; L. L. Pereira et al., 2021). Initial and final population for each group may vary depending on the region and type of processing (G. V. de M. Pereira et al., 2017). The fermentation can be conducted as spontaneous fermentation when it is promoted to epiphytic microbiota. Although, the use of starter cultures for quality improvement and as a process control tool have been widely studied.

### 4.3.1. Spontaneous fermentation

During spontaneous coffee fermentation, the majority of the microbiota present throughout the process is naturally present on the plant and coffee fruit surface (Cardoso et al., 2021; Evangelista et al., 2015). These microorganisms are namely endophytic or epiphytic, and their diversity and dominance during fermentation are variable (Elhalis, Cox, & Zhao, 2020; Ribeiro et al., 2018). The epiphytic microorganisms and the dominant microbiota can be affected by several processing conditions such as temperature, pH, aeration, homogenization, coffee variety (Ribeiro et al., 2018), region, altitude (Elhalis, Cox, & Zhao, 2020; Evangelista et al., 2015), and coffee treatment (natural or pulped) (Martins et al., 2020). For this reason, some strains are specifically found in certain countries or regions. In Brazil, the *Saccharomyces* spp. is a yeast frequently found and well-adapted to the coffee fermentative process (Carvalho Neto et al., 2017; G. V. de M. Pereira et al., 2014), while it was not found by Elhalis, Cox, & Zhao, (2020) in Australia, where they identified for the first time the yeast *Pichia kudriavzevii*. In the Sierra Nevada of Santa Marta in Colombia, LAB of the genus *Leuconostoc* and yeasts of the genus *Kazachstania*, which includes *K. gamospora* and *K. humilis*, were the predominant microorganisms during the submerged coffee fermentation (Cruz-O'Byrne et al., 2021). Finally, according to Evangelista et al. (2015), it can be observed that the diversity of species of prokaryotic and eukaryotic microorganisms differs among different regions in Brazil, and this may be due to differences in environmental temperature, humidity, the composition of the remaining pulp, mucilage surrounding the coffee beans, and the altitude.

The microbial population can grow significantly during the fermentation using mainly sugars from mucilage as substrate, and produce a wide array of metabolic compounds, as discussed before (Elhalis, Cox, & Zhao, 2020; Elhalis, Cox, Frank, et al., 2020b; T. S. Pereira et al., 2022). The production of metabolites, mainly organic acids, causes the acidification of the environment and decreases the pH along the time of fermentation and, these variations restrict microbial diversity present in the bioreactor (Elhalis, Cox, & Zhao, 2020; Evangelista et al., 2015; Pothakos et al., 2020). The microbial population during the coffee fermentation is complex and affected by the coffee fermentation method and environmental conditions (Martins et al., 2020; T. S. Pereira et al., 2022). The main groups monitored during fermentation are mesophilic bacteria, LAB and yeast. In an open environment Silva et al. (2008) and Vilela et al. (2010) showed that bacteria had the largest population during the first days of fermentation and the decrease in the bacterial population was simultaneously related to the significant increase in yeasts. Similarly, in SIAF process during natural and pulped coffee fermentations, microbial successions are initiated by bacteria followed by yeasts (T. S. Pereira et al., 2022). In submerged fermentation Pothakos et al. (2020) carried out the pulped submerged process in Ecuador and identified three successive microbial phases with the following dominant groups: in the first 8 hours of fermentation, enterobacteria, acetic acid bacteria, and some yeasts (*Hanseniaspora*, *Candida*, *Pichia*, and *Wickerhamomyces*) prevailing at the start; from 8 to 24 h were predominant the LAB group, and from 24 to 64 h acid-tolerant LAB group. Table 1 shows the dominant microbiota described to their approximate population in different spontaneous fermentation processes.

**Table 1** - Dominant microbiota and variation of population (initial - final) during the spontaneous fermentation in different process.

| Process Fermentation                | Mesophilic Bacteria                                                                                                                                                                                                                                                                                                                                                           | Lactic Acid Bacteria (LAB)                                                                                                                                                                                                                              | Yeast                                                                                                                                                                                                                                                                                                                                                                                                                                                     | References                                                                                                        |
|-------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|
| Submerged Fermentation              | <i>Acinetobacter lwoffii</i> ,<br><i>Enterobacter</i> ,<br><i>Enterobacter asburiae</i> ,<br><i>Pantoea dispersa</i> , <i>Pantoea vagans</i> , <i>Erwinia persicina</i> ,<br>3.8 to 5 -<br><i>Staphylococcus warneri</i> ,<br>2.5 to 8.5<br><i>Bacillus subtilis</i> <i>Bacillus megaterium</i> ,<br><i>Bacillus cereus</i> group, and<br><i>Cellulosimicrobium cellulans</i> | <i>Leuconostoc mesenteroides</i> ,<br><i>Lactococcus lactis</i> ,<br><i>Lactobacillus plantarum</i> ,<br><i>Enterococcus hirae</i> , and<br><i>Weissella</i>                                                                                            | <i>Hanseniaspora uvarum</i> ,<br><i>Hanseniaspora opuntiae</i> , <i>Pichia fermentans</i> , <i>Pichia kluyveri</i> ,<br><i>Pichia guilliermondii</i> , <i>Pichia caribbica</i> , <i>Pichia kudriavzevii</i> ,<br><i>Candida railenensis</i> ,<br><i>Cystofilobasidium</i> , <i>Candida glabrata</i> , <i>Candida quercitrusa</i> ,<br><i>Rhodotorula</i> , <i>Meyerozyma caribbica</i> ,<br><i>Torulaspora delbrueckii</i> , and <i>Saccharomyces</i> sp. | (Evangelista et al., 2015)<br>(Ribeiro et al., 2018)<br>(Elhalis et al., 2020) (G. V. de M. Pereira et al., 2014) |
| Self-Induced Anaerobic Fermentation | <i>Arthrobacter koreensis</i> ,<br><i>Acinetobacter johnsonii</i> ,<br><i>Acinetobacter oryzae</i> ,<br><i>Bacillus cereus</i> ,<br><i>Cellulosimicrobium</i> sp,<br><i>Enterobacter cloacae</i> ,<br><i>Enterobacter hormaechei</i> ,<br><i>Zymomonas mobilis</i> , and<br><i>Pantoea agglomerans</i>                                                                        | <i>Levilactobacillus brevis</i> ,<br><i>Lactiplantibacillus plantarum</i> ,<br><i>Leuconostoc mesenteroides</i> ,<br><i>Pediococcus pentosaceus</i> ,<br><i>Leuconostoc lactis</i> , <i>Weissella cibraria</i> , and <i>Weissella paramesenteroides</i> | <i>Torulaspora delbrueckii</i> ,<br><i>Hanseniaspora opuntiae</i> , <i>Pichia kluyveri</i> , <i>Hanseniaspora uvarum</i> ,<br><i>Saccharomyces cerevisiae</i> ,<br><i>Cystofilobasidium infirmominiatum</i> ,<br><i>Cystofilobasidium ferigula</i> ,<br><i>Rhodotorula mucilaginosa</i> , and<br><i>Meyerozyma caribbica</i>                                                                                                                              | <2 to 4.76<br>- 3.6 to<br>6.5 log<br>CFU/g<br>(Martins et al., 2020) (T. S. Pereira et al., 2022)                 |

|                                     |                                                                                                                                                                                                                                                                                                                                  |                                       |                                                                                                    |                                     |                                                                                                                                                                                                                                                                                                                                                    |                                     |                                                  |
|-------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|----------------------------------------------------------------------------------------------------|-------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|--------------------------------------------------|
| Open<br>Environment<br>Fermentation | <i>Bacillus cereus</i> , <i>Bacillus subtilis</i> , <i>Bacillus macerans</i> ,<br><i>Bacillus polymyxa</i> ,<br><i>Bacillus megaterium</i> ,<br><i>Bacillus polymyxa</i> ,<br><i>Acinetobacter</i> sp.,<br><i>Arthrobacter</i> sp.,<br><i>Enterobacter agglomerans</i> ,<br><i>Escherichia coli</i> , and<br><i>Yersinia</i> sp. | <1 to 3.5 -<br><1 to 5.7<br>log CFU/g | <i>Lactobacillus plantarum</i> ,<br><i>Lactobacillus brevis</i> , and<br><i>Lactococcus lactis</i> | <1 to 2.8 -<br><1 to 5<br>log CFU/g | <i>Candida saitoana</i> , <i>Candida fermentati</i> ,<br><i>Stephanoascus smithiae</i> ,<br><i>Debaryomyces polymorphus</i> ,<br><i>Pichia guilliermondii</i> ,<br><i>C. membranifaciens</i> , <i>D. hansenii</i> , <i>P. burtonii</i> , <i>P. anomala</i> , <i>D. polymorphus</i> , <i>P. guilliermondii</i> ,<br>and <i>Arxula adeninivorans</i> | <1 to 3.6 -<br><1 to 6<br>log CFU/g | (Vilela et al.,<br>2010) (Silva<br>et al., 2008) |
|                                     |                                                                                                                                                                                                                                                                                                                                  |                                       |                                                                                                    |                                     |                                                                                                                                                                                                                                                                                                                                                    |                                     |                                                  |

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Table was created by authors.

Mesophilic bacteria can be important through the fermentative process because some *Bacillus* species produce cellulases and pectinases that degrade the pulp and mucilage (Vilela et al., 2010). The mesophilic bacteria were identified at the beginning of coffee fermentation in a high diversity of species and the total population started from 4.10 to 4.84 (log CFU/g) in submerged fermentations carried out by Ribeiro et al. (2018) and from 4,80 to 6,09 (log CFU/g) in SIAF performed by T. S. Pereira et al. (2022). The highest bacterial population was normally detected at the beginning or in the middle of the fermentation process, when they achieved 9,17 (log CFU/g) and then decreased until values of 2.48 to 2.78 (log CFU/g) (Ribeiro et al., 2018) and 3,82 to 6,39 log CFU/g (T. S. Pereira et al., 2022). Vilela et al. (2010) found in the semi-dry process that *Bacillus* species were predominant in the initial samples and during the first 48 h of fermentation with a population size of 2.7 to 5.7 log CFU/g. The maximum population is variable, and each microorganism can achieve it at a different time in the same process. In the study of Evangelista et al. (2015) in the submerged process, the most abundant mesophilic bacteria identified in coffee fruit and during fermentation were *Staphylococcus warneri* (8.5 log CFU/g at 36 h), *Erwinia persicina* (5.5 CFU/g at 12 h) and *Enterobacter asburiae* (7.4 log CFU/g at 36 h) (Table 1).

The lactic acid bacteria (LAB) are found in less diversity than mesophilic bacteria, and the type of fermentative process and altitude are capable to influence the LAB population. In an open environment fermentation performed by Vilela et al. (2010), the *Lactobacillus plantarum* varied from 2.8 to 4.9 log CFU/g in the first 48 hours of processing. The low oxygen concentration normally leads to an increase in the LAB population (G. V. de M. Pereira et al., 2017). In submerged fermentation carried out by Elhalis, Cox, & Zhao, (2020) *Leuconostoc mesenteroides* and *Lactococcus lactis* increased in the first 24h, varying from 3.1 and 2.6 log CFU/g to reach 5.5 and 5 log CFU/g, respectively. The same authors found a dropped to 5 and 4.7 log CFU/g, respectively, at 36 h of fermentation. In SIAF, the LAB population during the fermentative process is dependent on the region and coffee treatment. In a study released by T. S. Pereira et al. (2022), the initial LAB population in natural coffee varied from 3.45 to 4.97 log CFU/g, while in pulped coffee was from 4.55 to 6.72 log CFU/g. The maximum population identified was 7.14 log CFU/g, however, the LAB behavior during the SIAF process and final population were very variable (from 4.5 to 9.5 log CFU/g). According to Martins et al. (2020) in the SIAF process using pulped coffee, the LAB population at low altitudes (800 and 1000 m) was considerable at the beginning of the fermentation (varying from 6,9 to 7,2 log CFU/g) and decreased during the fermentative process. On the other hand, in the same process, the LAB population in high altitudes (1200 and 1400 m) was smaller at the begging (4,6 log CFU/g) and increased during the fermentative process. In natural coffee SIAF fermentation, a similar initial behavior was observed and followed by an increase to 9.5 log CFU/g in all altitudes. LABs are favored in coffee fermentation because, in addition to traditional sources such as mucilage sugars, they are also capable of using phenolic compounds and organic acids, such as citric acid and malic acid, as a source of energy (Pothakos et al., 2020). The LABs are the main producers of lactic acid during coffee fermentation. The lactic acid has a significant influence on the decrease of the pH and on the final coffee quality (Evangelista et al., 2015; Ribeiro et al., 2018). Moreover, the alcohol produced by yeasts is capable to promote the suppression of bacterial growth, especially LAB (Elhalis, Cox, Frank, et al., 2020a) (Table 1).

Most yeast species, although not detected at the beginning of the fermentation, exhibited an increase in population after 24 hours when the water activity was reduced and the pH decreased because of acids production by the bacteria population (Elhalis,

Cox, & Zhao, 2020; Martins et al., 2020; T. S. Pereira et al., 2022; Vilela et al., 2010). The initial yeast population is normally smaller than the bacterial population, independent of the processing applied (Elhalis, Cox, & Zhao, 2020; Elhalis, Cox, Frank, et al., 2020a; Martins et al., 2020; T. S. Pereira et al., 2022; C. F. Silva et al., 2008; Vilela et al., 2010). An open environment fermentation performed by Vilela et al. (2010) showed that the dominant yeast in the semi-dry process achieved the major population from 24 hours of fermentation. In the submerged fermentation developed by Evangelista et al. (2015), *Meyerozyma caribbica* was identified as the most prevalent yeast in the submerged process, achieving the maximum population of 4.9 log CFU/g at 36 h of fermentation. G. V. de M. Pereira et al. (2014) showed that the yeast groups in the wet process grew to a maximum population of 7.15 log CFU/mL during the first 40 h of wet fermentation, followed by a decrease to 5.2 log CFU/mL during the next 8 hours. In the SIAF process, Martins et al. (2020) found that the yeast population increased along the fermentation time in pulped and natural coffee processed by SIAF conditions in all altitudes evaluated. The initial population was <2.0 log CFU/g in all processes, and the final population varied from 3.6 to 4.6 log CFU/g with pulped coffee, and in natural coffee from 5.4 to 6.5 log CFU/g. According to T. S. Pereira et al. (2022) during the SIAF process, the yeast population also varied by type of coffee treatment and region. The initial population varied from < 2.0 to 4.76 log CFU/g and the final population varied from 3.80 to 5.85 log CFU/g (Table 1).

During coffee fermentation, yeasts can be important because of the production of pectinases, which are essential in degradation of mucilage rich in pectin (Evangelista et al., 2015; G. V. de M. Pereira et al., 2014; T. S. Pereira et al., 2022). G. V. de M. Pereira et al. (2014) showed that two strains of *Saccharomyces* sp. and *P. fermentans*, both isolated during submerged coffee fermentation, demonstrated high polygalacturonase activity. However, it is not possible to consider that all yeasts have this same capacity. In a spontaneous submerged process performed by Elhalis, Cox, Frank, et al., (2020a), *H. uvarum*, *Candida* and *Wickerhamomyces* made little or no contribution to the degradation of mucilage, however, they played an important role in the production of metabolites and flavor compounds. The growth and activities of yeasts reduced the growth of undesirable microorganisms such as ochratoxigenic filamentous fungi and the production of undesirable metabolites such as butanoic and acetic acids. They also increased the antioxidant capacity, and the concentration of alcohols, esters, aldehydes, glycerol, and organic acids in the fermented green beans (Elhalis, Cox, Frank, et al., 2020a; G. V. de M. Pereira et al., 2014; T. S. Pereira et al., 2022). Studies have shown that spontaneous fermentation can be used to produce specialty coffees according to the SCA protocol. G. V. de M. Pereira et al. (2015) managed to achieve 89 points with caramel, banana raisin, lactic, and apricot flavors in a submerged process at a farm with a cement tank and spontaneous fermentation for 24 hours. L. L. Pereira et al. (2020) performed a submerged process with 36 hours of fermentation and temperature-controlled at 38°C and achieved coffee with 87.6 points.

The microbiological diversity presented in the regions associated with the type of fermentation process utilized can produce coffees with unique sensory profiles, with scores above 80 points. However, the challenge of performing spontaneous fermentation is related to the reproducibility of these results over the years, since the environmental conditions are constantly changing, and the farm does not have the necessary structure for process control as applied in some studies.

#### 4.3.2. Starter cultures utilization

Starter cultures in coffee fermentation are preselected microorganisms, basically composed of one or multiple microbial cultures with high cell concentration, capable of colonizing the product and dominating the high population of microorganisms naturally present in the medium along the fermentative process (Cardoso et al., 2021; Carvalho Neto et al., 2020; Evangelista, Silva, et al., 2014; G. V. de M. Pereira, Soccol, et al., 2016; C. F. Silva et al., 2013). The most common strategy is to select and use a single yeast or bacteria as a starter culture. Although, it is possible to use multiple strains as a starter culture, being called co-inoculation process (Bressani, Martinez, Batista, et al., 2021; Cassimiro et al., 2022; Vale et al., 2019). The use of previously selected strains results in a greater microbiological uniformity, and consequently, the uniformity of metabolites. The form of inoculum utilization is variable, normally prepared with the lyophilized starter culture rehydrated by adding water. The solution with starter culture can be applied by spreading over the coffee (G. V. de M. Pereira et al., 2015; Ribeiro, Miguel, et al., 2017).

The implementation of starter cultures can reduce the fermentation time and transform an inconsistent process into an economically viable proposition, improving the quality by providing better fermentation control and predictability of the final product (Evangelista, Silva, et al., 2014; G. V. de M. Pereira, Carvalho Neto, et al., 2016; G. V. de M. Pereira et al., 2015; G. V. de M. Pereira, Soccol, et al., 2016). According to Magalhães Júnior et al. (2021), the cost to process the coffee using inoculated starter culture in bioreactors is 2.45\$/Kg, while the spontaneous fermentation is 1.75 and 1.71 \$/Kg in bioreactor and open tanks, respectively. The increase in production cost is the main disadvantage of using inoculants. Although the production costs are higher, a controlled coffee fermentation process using a starter culture may guarantee a standardized quality and reduce the economic loss for the producer (G. V. de M. Pereira et al., 2017). The use of starter cultures is a promising alternative and economically viable approach to improve the sensory quality of the coffee beverage and to increase the final product value (Bressani et al., 2018). The starter cultures selection for coffee fermentation is carried out from strains identified in coffee fruit or spontaneous fermentative process (Elhalis et al., 2021; Junqueira et al., 2022; G. V. de M. Pereira, Beux, et al., 2016; G. V. de M. Pereira et al., 2014). Although a broad microbial diversity is generally observed, only a few numbers of species are usually selected (G. V. de M. Pereira, Soccol, et al., 2016).

Observing the dominant microorganisms during a fermentative process, it is possible to say that most epiphytic microorganisms are probably not necessary to obtain a final product with high quality (G. V. de M. Pereira, Soccol, et al., 2016). During the fermentative process, the environmental conditions may become unfavorable. Therefore, the strains are evaluated for their ability to grow under stress conditions, such as the pH range of coffee fermentation (pH 2.0 to pH 8.0); high osmotic pressure (up to 50% glucose and fructose), high temperature (up to 37 to 43 °C) and metabolite accumulation tolerance (12 to 15% ethanol, 2% lactic acid and 2% acetic acid) (Elhalis et al., 2021; G. V. de M. Pereira et al., 2014).

Enzymatic activities of yeasts and bacteria for degrading macromolecules are also very important. It can be evaluated by the pectinolytic, cellulolytic, proteolytic, and amylase activities. Nevertheless, the most measured is pectinolytic (polygalacturonase, pectin lyase, and pectin methylesterase) production, which are the enzymes able to

promote the degradation of the coffee mucilage (Elhalis et al., 2021; Lee et al., 2015; G. V. de M. Pereira, Soccol, et al., 2016; C. F. Silva et al., 2013). This evaluation is important because not all species of bacteria and yeasts have high pectin lyase activity, and thus, not all species would enhance pectin hydrolysis at the beginning of the fermentation (C. F. Silva et al., 2013). The bacteria strains are also tested for their capacity to increase in population, decrease medium pH, and produce organic acids (lactic, malic, succinic, citric, and propionic acids), and volatile organic compounds in the coffee pulp medium (Ribeiro et al., 2020). It is possible to use starter cultures for the fermentation of any coffee processing, coffee variety, and treatment (Bressani, Batista, et al., 2021; Bressani, Martinez, Sarmento, et al., 2021; Evangelista, Silva, et al., 2014; G. V. de M. Pereira et al., 2015; L. L. Pereira et al., 2020). The formation of volatile compounds and the influence of fermentation and starter addition on coffee flavor were previously reported by the reviews from Lee et al. (2015) and G. V. de M. Pereira et al. (2019). The sugar consumption is higher in inoculated fermentations, which showed the lowest levels of residual glucose and fructose (G. V. de M. Pereira et al., 2015).

The microorganisms most used as starter in coffee are yeasts: *S. cerevisiae* CCMA 0543, CCMA 0200, UFLA YCN727, UFLA YCN724, CCMA 0535, *C. parapsilosis* CCMA 0544, UFLA YCN448, *T. delbrueckii* CCMA 0684, *Meyerozyma caribbica* (CCMA 0198), *Pichia guilliermondii* UFLA YCN731, and *Pichia fermentans* YC5.2. At the beginning of the fermentative process, high monosaccharide concentrations could have stimulated the overproduction of glycerol by yeast (da Mota et al., 2020). In coffees inoculated with yeasts it was found mainly alcohols, aldehydes, and acetic acid (Bressani, Martinez, Batista, et al., 2021; G. V. de M. Pereira et al., 2019). It is also possible to direct the coffee flavor precursors using different strains of bacteria. Ribeiro et al. (2020) highlighted *Leuconostoc mesenteroides* CCMA 1105 and *Lactobacillus plantarum* CCMA 1065 as potential bacteria to be used in coffee fermentation, which is related to the production of compounds that can improve cupping qualities: isovaleric acid; 2,3-butanediol; phenethyl alcohol;  $\beta$ -linalool; ethyl linoleate; and ethyl 2-hydroxypropanoate. *Lactobacillus plantarum* (LPBR01 and CCMA 1065) is the most LAB studied. It was used on farm during a submerged fermentation in cement tank (G. V. de M. Pereira et al., 2020; Ribeiro et al., 2020). In submerged fermentation using pulped coffee G. V. de M. Pereira, Carvalho Neto, et al. (2016) found that the inoculation *L. plantarum* LPBR01 showed the initial population was higher than in the spontaneous process, 7.35 and 5.49 log CFU/mL, respectively. The same LAB increased during the process of fermentation. In the end, the population in the inoculated process was 10.92, while the spontaneous was 6.16 log CFU/mL.

The use of *L. plantarum* LPBR01 also significantly increased the production of volatile aroma compounds during the fermentation process: butyl acetate, ethyl acetate, 1-octanol, isoamyl acetate, ethyl hexanoate, ethyl succinate, and ethyl octanoate. The coffee beverage obtained from inoculated treatment resulted in a complex coffee beverage, with noticeable hints of fresh fruit like banana, orange, and pineapple. It also was full-bodied with a very smooth sensation due to the lactic acid, scoring 88.3 points, while the beverage from the conventional process showed a lower sensory quality and a basic sensory profile: citric, caramel, and banana as dominant notes scored with 80.3 points.

Some studies did not achieve improvements in coffee quality using a single starter. It is possible to use more than one strain of microorganism to form an inoculum. This can be done with yeast only or yeast and bacteria. The use of yeast co-inoculation for Bressani, Batista, et al. (2021) and Bressani, Martinez, Batista, et al. (2021) resulted in a high-quality coffee, considering sensory notes, diversity of descriptors, and the final

concentration of organic acids. The use of inoculum composed of yeast and LAB can be advantageous due to the positive synergic interaction between these two microbial groups, capable of improving fermentation efficiency and positively influencing the chemical composition of coffee beans. Vale et al. (2019) demonstrated the great potential of the combined use of *P. fermentans* and *Pediococcus acidilactici* in ripe coffee fruits to increase pulp sugar consumption and production of lactic acid, ethanol, and ethyl acetate. Cassimiro et al. (Cassimiro et al., 2022) found that the use of *L. plantarum* CCMA1065 and *S. cerevisiae* CCMA0543 co-inoculation produced coffee beverage with citrus fruits and floral flavor, reaching a score above 80, intensifying sweetness, acidity, and body. (B et al., 2019) used the consortia of *S. cerevisiae*, *L. plantarum*, and *Bacillus sphaericus* (1:1:1) at 10% inoculum concentration, and found significant in mucilage removal of coffee and improvement in alcohol, sugar, and pectinase compared to spontaneous fermentation. The inoculum was significant even at 0.1%, with the highest enzyme production and release of secondary metabolites, which also revealed the assertiveness and co-existence of strains.

Thus, starter cultures are good alternatives for obtaining a differentiated product with high quality and distinctive flavor (Evangelista, Miguel, et al., 2014; Evangelista, Silva, et al., 2014; G. V. de M. Pereira, Soccol, et al., 2016). However, it is not possible to affirm that an inoculum will grow in the same way in every coffee variety, coffee treatment, region (altitude), and fermentation process. Even using the same starter culture, the profiles of volatile and nonvolatile compounds, and the sensory perception of fermented coffee may be different. The physicochemical conditions during fermentation, such as oxygen availability and temperature, will influence inoculum growth and metabolite production (da Mota et al., 2020). Each inoculated sample showed a strongly dominant sensation and differences between them (Evangelista, Silva, et al., 2014).

The altitude influences coffee chemical composition, microbial diversity, and coffee sensory profile. According to Oliveira et al. (2021), the predominant compounds responsible for coffee chemical characterization in altitudes below 969 m were lipids, trigonelline, citrate, and malate. On other hand, in the high altitudes (> 1000 m), this discrimination was due to the HMF, quinic acid, caffeine, and formic acid. The high quality in sensory profile can be obtained in high altitudes using the spontaneous fermentative process in pulped or natural coffee (Martins et al., 2020). However, at low altitudes the quality can be improved by starter utilization (Bressani, Martinez, Batista, et al., 2021; L. L. Pereira et al., 2020). L. L. Pereira et al. (2020) evaluated the submerged process fermented spontaneously or with *S. cerevisiae* as a starter culture in different altitudes. The authors suggest that spontaneous fermentation may be more favorable for areas above 893 m due to the natural microbiota of the coffees, which can be confirmed by the significant linear functional relation between final score and altitude obtained in the study. The same authors found that the use of *S. cerevisiae* improved the final score up to 2,9 points in lower altitude (774 and 788 m), indicating potential for quality improvement through induced fermentation. Bressani, Martinez, Batista, et al. (2021) evaluated the single and co-inoculation of three yeasts in Catuaí Vermelho coffee at 600 m using SIAF method. The authors showed that the inoculation of yeasts separately or together in SIAF fermentation process impacted the quality. The best result was obtained by fermentation released with co-inoculation of *C. parapsilosis* and *T. delbrueckii*, whose coffee achieved 85 points in the sensory analysis and cocoa/chocolate, caramelized, molasses, honey, and spice flavors. Oliveira et al. (2021) evaluated cherry coffees harvested in eight points of altitudes between 826 and 1078.08 m. They demonstrated that *S. cerevisiae* inoculation did not guarantee the increase of final score when compared to

the spontaneous fermentation process. Only samples of 969 m showed a significant difference in the sensory score between spontaneous fermentation and inoculated.

The growth and permanence of the inoculum are one of the principles for using this technology. During the fermentation process, the inoculated yeast population is normally larger than those found in the spontaneous process, however, the growth of each strain is variable along the time, and can change depending on the fermentation process applied (Bressani et al., 2020; da Mota et al., 2020; Ribeiro, Ribeiro, et al., 2017). The yeasts inoculums were not able to inhibit bacteria development (Evangelista, Silva, et al., 2014).

Nonetheless, studies have been demonstrating that *S. cerevisiae* is capable of modifying the fungi community regardless of the fermentation time and reducing the richness of the fungi and bacteria community, probably because of the ethanol accumulation (M. C. S. da Silva et al., 2022). The variation in the population of each strain used as inoculum is complex and depends on several factors, as already discussed previously (Bressani et al., 2020; da Mota et al., 2020; Evangelista, Miguel, et al., 2014; Ribeiro, Miguel, et al., 2017). Some studies did not verify the inoculum population along the fermentation, however they accompanied the activity of the microorganism through the difference of metabolites produced (L. L. Pereira et al., 2020).

#### **4.4.Coffee fruit treatment in fermentative process**

In coffee processing, the most common ways of using fruits, called in this review coffee fruit treatments, are the natural and the pulped forms. Studies have been demonstrating that this variable has strong influence on coffee quality, modifying coffee flavor and performance on the SCA sensory evaluation.

The influence of this variable has come to be observed in fermentation studies using inoculants. The same inoculum subjected to the same processing conditions can develop different sensory characteristics and final scores to the beverage depending on the variety and treatment of the coffee. Bressani et al. (2020) evaluated the Bourbon Amarelo (BA) and Canário Amarelo (CA) coffees, pulped and natural, with four yeasts as single inoculation in an open environment fermentation. Each coffee inoculated with *T. delbrueckii* (CCMA 0684) resulted in different sensorial classification: citric fruits and caramelized sugar when inoculated in the BA natural, milk notes when inoculated in the BA pulped, caramelized sugar when inoculated in the CA natural, and red fruits when inoculated in the CA pulped. For Bressani, Martinez, Sarmiento, et al. (2021) fermentation resulted in different characteristics depending on the treatment of the coffee. The natural fermented coffees were characterized by acidity, medium to high sweetness, dense to medium body, and a predominance of malic acid, while the pulped coffee had a predominance of citric acid. The pulped coffee inoculated with *S. cerevisiae*, *C. parapsilosis*, and *T. delbrueckii* showed the perception of creamy, dense bodies and a higher sweetness when compared to the natural coffee treatments. Da Mota et al. (2020) applied the SIAF method with different coffee varieties and regions. These authors found that *S. cerevisiae* inoculation was most suitable for pulped coffee, while *T. delbrueckii* inoculation showed the best performance in natural coffee.

Natural and pulped coffee have different compositions, which may explain the different sensory characteristics observed in the beverage. During the fermentation process, coffee is the substrate. In biotechnological processes, substrate availability is one of the main variables influencing microbiological development and product formation.

Natural coffee is a whole fruit, and its composition has a higher sugar concentration, due to the presence of mucilage and the pulp attached to the peel (Figure 3). Pulped coffee, on the other hand, has lower initial sugar concentrations because it presents only the mucilage as the main source of fermentable sugars (Cardoso et al., 2021). Natural and pulped coffee can be used in all fermentation conditions shown above. The choice of coffee treatment should be based on the availability of farm resources and the final desired sensory characteristics. The use of natural coffee makes the process more accessible and consumes less water. Pulped coffee, on the other hand, requires greater investments in structure to enable pulping the fruits and has a greater generation of wastewater after harvesting. The influence of treatment coffee is still poorly addressed in coffee fermentation studies. Further studies can be developed to evaluate the solubilization of sugars and metabolites in fermentative processes with added water and the influence of the presence of immature fruits during the fermentative process. In addition, other factors such as the influence of compounds present in the coffee peel can also be studied, since they can be solubilized and transferred to the bean during fermentation, or act as inhibitors.

Figure 3 - Technical representation of the layers of the coffee fruit, natural coffee, and pulped coffee.

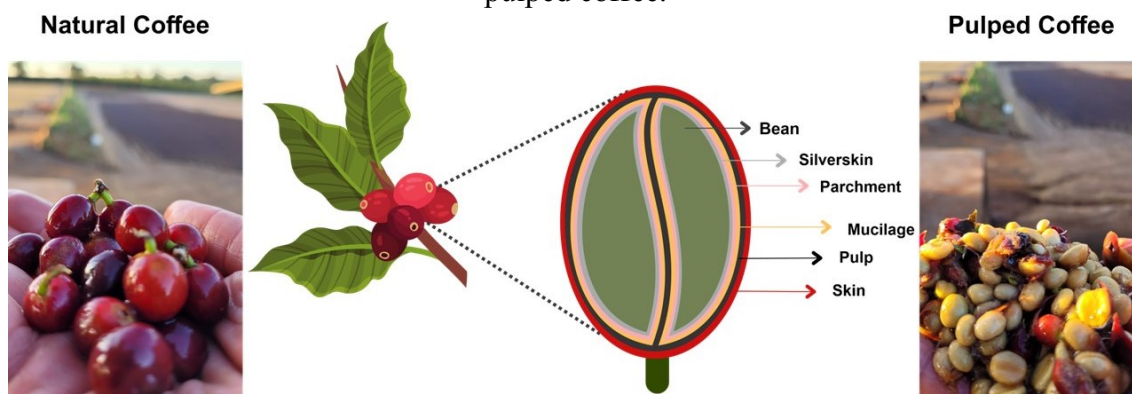


Figure was created by authors.

## 5. Discussion

In recent years, coffee research has been based on mapping microorganisms, compounds produced during the coffee fermentation process and evaluating the effect of fermentation conditions on the physical-chemical composition of coffee and its sensorial characteristics. The researches aim to develop quality coffees that satisfy the global demand for specialty beverages with different sensory profiles. The sensory evaluation system proposed by SCA has been used worldwide to evaluate and classify specialty coffee through grades, determining its market value. Coffee can be classified with the same final grade and still have different sensory profiles, which represents gains for expanding the sales market. In fermentation processes, there is a migration from the use of processes in open environments to closed environments with some anaerobic conditions. However, it is not possible to define a single process capable of increasing coffee quality or developing a specific sensory pattern in any environmental condition. Different fermentation processes are currently used on coffee processing farms. There is

no prevalence of a single process because it depends on the farm infrastructure and objective of each producer.

Coffee fermentation is a multifactorial and rudimentary process, which still does not have process control tools applied effectively, such as temperature control, aeration, and agitation. Each type of processing provides different fermentation conditions, which results in different types of coffee beverages. It is observed that excellent quality coffees can be obtained using spontaneous fermentation, despite the absence of control. However, the reproducibility of the results is difficult since the present microbiota can be altered, responding to climatic differences and cultural practices from one year to another. The application of fermentation as a step before drying can be advantageous due to minimize environmental variations. The type of fermentation used in coffee processing influences the availability of oxygen, the natural microbiota present, the metabolites produced, and consequently, the sensory characteristics obtained in the beverage. In addition to the type of fermentation, the variation in other process conditions, such as the use of natural or pulped fruit, altitude, coffee variety, and starter culture strain influence the result.

The use of starter cultures contributes to the control of the process by promoting the dominance of the microbiota. The inoculum can promote a change in the development of the epiphytic microbiota during fermentation, and it is possible to select those that promote improvement in the final quality of the coffee. Even when it does not cause a final score increase, the use of a starter can develop different sensory profiles, which is interesting to expand the possibilities of serving the consumer market. There are some inoculum strains that are more suitable for certain coffee varieties described under certain fermentation conditions. In addition, the starter culture use can be indicated for fermentative process of coffees from low altitudes, in which spontaneous fermentation were not so efficient in improving quality. Co-inoculation showed promising results in improving coffee quality when compared to simple inoculation. Since there is evidence of differentiation in the growth of each microbiological group (lactic acid bacteria, mesophiles, and yeasts) during spontaneous fermentation, there is the possibility of using a mixed starter, capable of providing a dominant microorganism at each stage of fermentation, allowing greater control of the fermentation process.

Induced fermentation is not mandatory but could be used as a tool by the producer to achieve greater sensory differentiation and quality control of coffee. The application of fermentation protocols with well-defined processing parameters, as we suggest in this review, could be used to increase food safety, predictability, and reproducibility in coffee post-harvest processing. Most current studies relate the compounds and sensory results using different inoculums in not controlled environment conditions. There are few studies that relate the formation of compounds to processing variables. Likewise, studies should be conducted with the intention of mapping the environmental conditions responsible for producing undesirable compounds.

The open environment fermentation is simpler than the SIAF or Anerobic fermentation, and literature results showed that it is possible to obtain high quality coffees, including some qualified as specialty coffees. However, there are some issues that deserve attention. The higher availability of oxygen in this fermentation allows the development of mycotoxigenic fungus, as *Aspergillus*, *Fusarium*, and *Penicillium*. The use of starter cultures may suppress mycotoxin formation, as observed in some studies. Also, the higher availability of oxygen favors aerobic metabolism. On other hand, in the SIAF and Anerobic fermentation methods, the anaerobic metabolism of microorganisms is favored, in which production of flavor precursors can be favored.

Considering production costs, anaerobic fermentation has a disadvantage that is the need of CO<sub>2</sub> acquisition. SIAF and anaerobic fermentation need the acquisition of

bioreactors, that can be of low cost, as the polypropylene drums, or the expensive and sophisticated stainless-steel bioreactors. In submerged fermentations, costs of water and wastewater treatment also have to be taken into account. Starter culture utilization also increases the budget. Regarding the sensorial descriptions and quantitative results of quality, it is difficult to compare between methods, as final quality depends on several factors, as previously discussed. But in the studies included in this review, the highest scores, considering the SCA evaluation method, were 87.09 for solid-state, open environment fermentation of pulped coffee (variety not specified) (L. L. Pereira et al., 2020); 85.15 for solid-state anaerobic fermentation of natural Catuaí vermelho (Brioschi Junior et al., 2021); 87.25 obtained using solid-state SIAF method with natural Bourbon Amarelo and *Torulaspora delbrueckii* (CCMA 0684) as inoculum (da Mota et al., 2020); 91.5 for submerged fermentation using a combination of aerobic and anaerobic conditions, natural Catuaí and *Lactobacillus plantarum* (LPBR01) as inoculum (Carvalho Neto et al., 2018). Few studies observed mycotoxin formation, but in all of them it was observed that starter culture utilization may suppress fungus development. More studies and regulations should be implemented in order to identify contaminations and guarantee that the final product is safe. Currently the quality control tools are mainly performed at the end of the process, making it impossible to make preventive decisions for the quality of the coffee grain. For this reason, more compounds desirable or undesirable should be identified to be used as markers/indicators of quality, as well as analytical techniques should be developed for the on farm quantification of these compounds at different stages of coffee processing.

Investment in control technologies during the fermentation process could help to guarantee the reproducibility of the results obtained over the years. A great improvement for coffee fermentation process was the development of more sophisticated bioreactors, in stainless steel, with mechanical agitation. This allowed a more hygienic operation and homogenization of coffee mass. A temperature controlling system is still not available, and it would be desirable as these variable influences microorganisms' development. The fermentation protocol application using starter culture under controlled conditions of temperature and oxygen availability, would allow for greater precision, reproducibility, and definition of the coffee quality standard verified through sensory analysis of the beverage. From this, it is suggested that more studies in controlled conditions of temperature, agitation, and oxygen concentration is necessary, to determine how the action of starter culture could be modified during the coffee fermentation.

## 6. Conclusion

Important technological advances are being tested in coffee fermentation processes, but due to the complexity of coffee variety and the relationship with soil and climate characteristics, microbiota, and fermentation process conditions based on coffee treatment, oxygen availability, water addition, and starter cultures utilization, much remains to be explored. This review has contributed to the understanding of the several possibilities now available for coffee fermentation, not only the traditional processes related to the dry, semi-dry, and wet methods, as well as the sensory results obtained with different process conditions and different locations.

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## **CAPÍTULO 2 - Impact of immature coffee fruits and water addition during the spontaneous fermentation process of *Arabica coffee* var. Arara: Chemical composition and sensory profile**

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### **Competing interests**

The authors have no competing interests as defined by Springer, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

### **Authors' contributions**

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### **Declarations**

*Ethical Approval*

Not applicable.

## ABSTRACT

The coffee fermentation process influences the final coffee composition and the sensory aspects which define the quality of the beverage. In this study, coffee fruits were submitted to spontaneous self-induced anaerobic fermentation using 0.3 and 11% of immature fruits in submerged and solid-state processing. The effects on physicochemical composition and on the sensorial quality of coffee were evaluated. The percentage of immature fruits significantly altered the initial content of sugars (sucrose, glucose, and fructose), ash, and titratable acidity. The water addition during the fermentative process did not significantly influence final moisture, proteins, citric, and propionic acids concentrations. The coffee fermented with 0.3% of immature fruits showed major production of lactic acid in submerged fermentation (67.44 mg/g), and in solid-state fermentation, major concentrations of ethanol (42.84 mg/g) and glycerol (1.68 mg/g). Compared with solid-state, the submerged process gave rise to coffees with lower concentrations of ethanol, glycerol, ash, lipids, succinic, and acetic acids. All coffees produced were classified as specialty coffees with a score above 84 points. However, the submerged fermented coffee with 11% immature fruit stood out with notes of caramel, brown sugar, honey, orange, lemon, floral, nut, yellow and red fruits.

**Keywords:** Coffee ripeness; unripe coffee; coffee flavor; postharvest; coffee fermentation; non-volatile precursors.

## 1. Introduction

Arabica coffee is the main specie related to the production of superior quality coffees in the world. Many factors are capable to influence the coffee quality, among which there are the genetic characteristics of the plant, cultivation methods, and post-harvest processing [1]. The coffee fruit is composed of skin, pulp, mucilage, parchment, silverskin, and seed [2]. According to Elías [3] and Schwan and Wheals [4], 5% of the fruit corresponds to mucilage on a dry basis. The mucilage is the soft layer found between the peel and the parchment, formed by water, sugars, complex polysaccharides, pectic substances (mainly pectin), organic acids, holocellulose, lipids, proteins, and minerals. Due to its rich composition, it is directly related to the fermentation process [5]. The coffee bean has in its composition up to 53% of insoluble polysaccharides, among them mainly cellulose, up to 18% of lipids, and 12% of proteins [6]. It is important to know the initial composition of coffee fruits because the changes that occur during roasting depend on volatile and non-volatile precursors that can be altered during the post-harvest processing steps [7, 8].

The maturation stage cause impact on the chemical compounds and physical properties of coffee [9, 10]. During the development and ripening of fruits occurs alterations in malic acid, citric acid, oxalic acid, and quinic acid. These acids are found in different concentrations in coffee fruit. Citric acid was at its highest amount in seeds, whereas malic acid was detected highest in pericarps [11]. The mucilage thickness varies according to the maturation of the grain, from 0.5 to 2.0 mm [12]. Harvesting coffee is a complex operation, because in a single coffee crop, several blooms can occur, and this fact result in a harvest with heterogeneous maturation [13]. For this reason, the ripe coffee

fruits are processed with a variable percentage of unripe fruits. The sensory characteristic of green coffee beans is known and considered detrimental to the final quality of the coffee beverage [14]. The immature coffee fruits produce roast coffees with lower scores than those produced from mature cherries according to sensory analyses [15]. However, to the best of our knowledge, the influence of the percentage of immature coffee fruits during a fermentation process is unknown.

Fermentation has been used to promote improvements in coffee quality and sensory differentiation. During a spontaneous fermentation the epiphytic microorganisms from coffee fruit, soil and environment, are responsible to mucilage consumption and metabolites production [16–18]. Different biochemical processes occur by which enzymes produced by yeasts and bacteria degrade sugars, lipids, proteins, and acids present in the mucilage, transforming them into alcohols, acids, esters, and ketones [19]. The postharvest process and the way of conducting the fermentation process influences coffee final composition and sensory characteristics [20–23]. During a Self-induced Anaerobic Fermentation (SIAF), the process can be carried out by conditioning the whole coffee fruits in an appropriate container, with or without the addition of water [24–27]. However, the interference of water during the coffee fermentation process, including its solubilizing role in substrates and products, and its influence on the result of the beverage, is not yet well studied.

There are still no studies that compare the effect of the presence of water and immature coffee fruits together with mature coffee during processing. The sensory aspects that define the quality of a coffee are associated with the chemical composition of the grain. Thus, the present study seeks to carry out the physicochemical characterization and sensory evaluation of Arara coffee fruits from the Cerrado Mineiro, submitted to SIAF spontaneous fermentation using two different percentages immature fruits and in submerged and solid-state process.

## **2. Material e methods**

### **2.1. Coffee fruits**

Coffee cherries of the *Coffea arabica* variety Arara were harvested mechanically at in Patos de Minas, Cerrado Mineiro region, Brazil (latitude 18° 35' 15, altitude 1040 m, and longitude 46° 25'35). Two treatments with two percentages of immature coffee fruits were used (0.3 and 11%). The fruits with 11% of immature fruits were obtained by mechanically separated in a horizontal washing machine (Ecoflex, Pinhalense, São Paulo, Brazil), which promoted the exclusion of over ripe fruits, sticks, stones, and leaves. The ripe coffee fruits (0.3%) were obtained after mechanical separation followed by manual selection to remove the immature fruits.

### **2.2. Coffee fermentation process**

Each fermentation was processed using whole coffee fruits. Fermentations treatments were performed without inoculation, in solid-state (without moisture control) or submerged process, and with different percentages of immature fruits, as described in Table 1. In submerged fermentations, water was added until covering the fruits. Fermentations were carried out using Self-Induced Anaerobic Fermentation (SIAF) process, in a 20L closed polyethylene bioreactors equipped with an airlock valve, for 96

h, in triplicate. The temperature and pH were measured at different coffee mass points at intervals 0, 24, 40, 48, 64, 72, 88, and 96 h. Temperature was measured using a digital rod thermometer (-50 to 300 °C) and pH using portable pH meter Akso. After fermentation, the cherries were transferred to suspended terraces until obtaining 11.0–12.0% moisture. Samples of cherries (100 g) were collected before and after fermentation and frozen until chemical analyses.

**Table 1** Detailed information of the treatments used in the experiment

**Description Table 1 ID = identification of treatments**

| ID    | Process     | Percentage of immature fruits |
|-------|-------------|-------------------------------|
| SB11  | Submerged   | 11 %                          |
| SB0.3 | Submerged   | 0.3%                          |
| SS11  | Solid-state | 11 %                          |
| SS0.3 | Solid-state | 0.3%                          |

### 2.3. Chemical proximate composition analysis

The whole frozen fruit samples were lyophilized and ground in a knife mill (SOLAB) in a fine granulometry of 20 mesh. The physical chemical analyzes were performed with the powder sample according to Association of Official Analytical Chemists, [28]. The following determinations were performed: moisture (method 925.09B), protein content (method 920.87, using 6.25 as correction factor), lipid content (method 920.97), ash content (method 923.03), and titratable acidity (method 942.15).

### 2.4. High Performance Liquid Chromatography (HPLC) Analysis

Organic acids (citric, malic, lactic, succinic, acetic, propionic, and butyric), sugars (sucrose, glucose, and fructose), glycerol and ethanol were analyzed. Samples were extracted using MilliQ water according to Elhalis et al. [29] with modifications: 10 g of fruits were mixed with 100 mL deionized water and blended in a domestic blender (Oster 1400W) for 2 min. The slurry was filtered through two layers of organza polypropylene and then centrifuged in an Heal Force (Neofuge 18R) at 10.000 rpm for 15 min at 17 °C, and the supernatant was retained. The supernatants were filtrated through a 0.45-micron nylon filter before injection into Shimadzu liquid chromatography system (Shimadzu Corp., Japan). A Supelcogel C-610H (7.8 mm × 30 cm) column was used with a 0.1% solution of phosphoric acid, with a flow rate of 0.5 mL per min as the mobile phase. The oven temperature was kept at 30 °C for the analysis of acids, detected with a 210-nm UV detector (SPD), and at 30 °C for the analysis of sugars, detected with a refractive index detector (RID). The quantification of compounds was performed using calibration curves constructed with different concentrations of standard compounds and analyzed using the same conditions as for the samples.

## 2.5. Sensory analyses

The sensory analyses were performed following the recommendations of the Specialty Coffee Association with modifications [30]. A panel of five expert coffee tasters, with a Q-Grader Coffee Certificate, performed the sensory analysis using five cups for each sample (a predetermined ratio of  $8.25 \pm 0.25$  g per 150 mL of water). The attributes evaluated were fragrance/aroma, flavor, aftertaste, acidity, body, balance, sweetness, clean cup, uniformity, overall, and final score, besides the sensory descriptors. Each attribute was scored from 0 to 10, and the final score consisted of the sum of the scores.

## 2.6. Image processing

The number of immature fruits was determined by using image processing techniques using Python. For each pixel in the image, a spectral analysis was performed aiming to identify the predominance of green colors, and then segmentation by pixel. Then, digital filters were applied to identify coffee fruits, and those with predominantly green colors were identified as immature fruits. Finally, the percentage of immature fruits is calculated taking into account the total number of fruits present in the image. Pictures were taken of the total mass of coffee in different positions and the final percentage was averaged over the five image treatments.

## 2.7. Statistical analyses

The data of carbohydrates, organic acids, ethanol, glycerol concentrations, and proximate composition (moisture, ash, protein, lipid, and titratable acidity) were evaluated by analysis of variance (ANOVA) and the Duncan test was used for comparison between means, considering each compound in isolation, considering the initial sample and the final sample after the spontaneous fermentation ( $p < 0.05$ ).

The spontaneous fermentation experiments were carried out in randomized design. A  $2^2$  factorial experimental design was used to analyze the compounds identified at 96h: two types of process (solid-state [SS] and submerged [SB]) and two percentage of immature fruits in sample (0.3% [S0.3] and 11% [S11]). Data were analyzed under the following model:

$$X = \text{mean} + \text{Proci} + \text{Immj} + \text{Pro} \times \text{Immij} + e_{ij}$$

where mean=global mean; Proci =process effect ( $i$  =SS, SB); Immj = immature fruits effect ( $j$  = S0.3 and S11); Proc  $\times$  Immij =effect of interaction between process and immature fruits;  $e_{ij}$  = experimental error. Significance was defined at  $p < 0.05$  level.

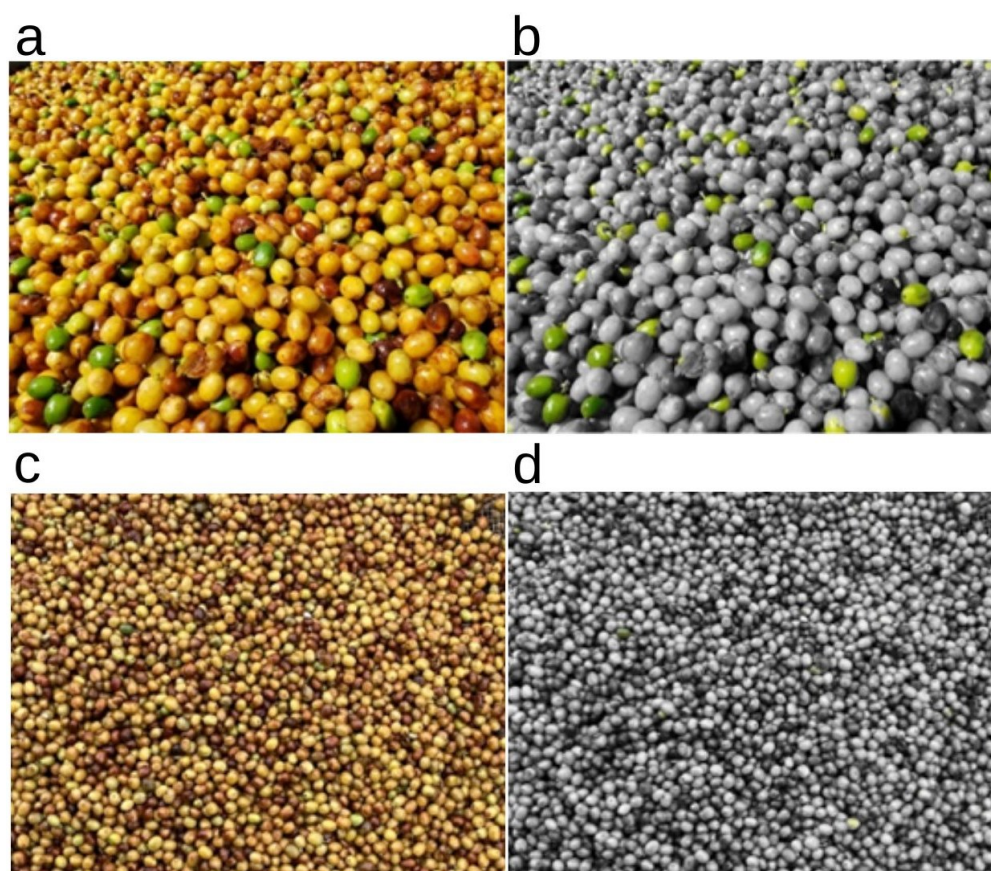
## 3. Results and Discussion

### 3.1. Influence of immature fruits in the initial sample composition

According to the image analysis, after mechanical separation in a horizontal machine, the coffee sample showed 11% immature fruits, and after manual selection to

remove the remaining immature fruits, the coffee showed 0.33% immature fruits (Fig. 1). The maturation stages of the coffee fruit are marked by the color transition from intense green to cane green and finally red or yellow, depending on the coffee variety [11, 31]. Fruit coloring is the simplest way to identify the coffee maturation stage, and this identification is important since a lower incidence of immature fruits and raisins is desirable, as they can play a negative influence in the production yield and the quality of the coffee beverage [32]. Image processing has become a valuable tool for the coffee industry, mainly to characterize coffee fruit ripeness [33]. The present study showed that it is possible to effectively apply image processing tools for determining the percentage of green fruits present in the coffee sample after harvest.

**Fig. 1** Image analysis of Arara coffee fruits treatments, mechanically and manually selected



a: Arara coffee mechanically selected by the washing machine (S11) with an average of 11% immature fruits, photo taken in the sun; b: S11 after image treatment; c: Arara coffee manually selected after the washer (S0.33) with an average of 0.33% of immature fruits, photo taken in the shade; d: S0.33 after image treatment

Propionic acid, butyric acid, lactic acid, ethanol, and glycerol were not found in natural coffee fruits (0 h). It was not identified significant difference in moisture, lipids, protein, succinic acid, and acetic acid between the coffees with different percentages of immature fruits (Table 2). Despite it is not considered a specific acid from coffee, in this study acetic acid was identified in natural coffee (1.12 and 1.26 mg/g, in S11 and S0.3,

respectively). Ribeiro et al. [34] also found acetic acid in initial samples of pulped coffee: 1,6 and 2,3 mg/g, in Ouro Amarelo and Mundo Novo varieties, respectively. There are epiphytic microorganisms that can start fermentation in the fruit still on the tree, as demonstrated by Vilela et al. [12] and Evangelista et al. [35], that found a great variety of bacterial and yeasts species in freshly harvested coffee fruit. Probably the acid detected in the initial sample comes from the metabolism of these microorganisms.

**Table 2** Effects of water adding and immature fruits percentage on carbohydrates, organic acids, ethanol, glycerol, and proximate composition of coffee fruits submitted to SIAF processing

| Compounds and Processing     | Treatments          |          |          | P value / Effect* |                 |        |      |
|------------------------------|---------------------|----------|----------|-------------------|-----------------|--------|------|
|                              | Natural Coffee (0h) | SB (96h) | SS (96h) | Processing (P)    | Immature % (Im) | P x Im | ER** |
| <b>Sucrose (mg/g)</b>        |                     |          |          | <0.01             | <0.01           | <0.01  | 0.08 |
| S0.3                         | 33.15 d             | 0.97 a   | 1.93 ab  | 0.61*             | 1.15*           | 1.57*  |      |
| S11                          | 18.19 c             | 3.69 b   | 1.51 a   |                   |                 |        |      |
| <b>Glucose (mg/g)</b>        |                     |          |          | <0.01             | <0.01           | 0.03   | 0.22 |
| S0.3                         | 49.24 e             | 8.29 a   | 11.29 ab | -4.12*            | 6.14*           | -1.12* |      |
| S11                          | 32.33 d             | 13.31 b  | 18.56 c  |                   |                 |        |      |
| <b>Fructose (mg/g)</b>       |                     |          |          | <0.01             | <0.01           | <0.01  | 0.58 |
| S0.3                         | 76.26 d             | 31.32 a  | 32.48 a  | -6.71*            | 6.09*           | -5.55* |      |
| S11                          | 54.30 c             | 31.86 a  | 44.12 b  |                   |                 |        |      |
| <b>Citric Acid (mg/g)</b>    |                     |          |          | 0.73              | 0.81            | 0.15   | 0.03 |
| S0.3                         | 0.65 a              | 0.64 a   | 0.52 a   | 0.02*             | -0.01*          | -0.09* |      |
| S11                          | 1.06 b              | 0.53 a   | 0.60 a   |                   |                 |        |      |
| <b>Malic Acid (mg/g)</b>     |                     |          |          | <0.01             | <0.01           | 0.062  | 0.04 |
| S0.3                         | 10.11 d             | 0.98 a   | 1.65 ab  | -0.85*            | 0.58*           | -0.18* |      |
| S11                          | 8.80 c              | 1.38 a   | 2.42 b   |                   |                 |        |      |
| <b>Lactic Acid (mg/g)</b>    |                     |          |          | <0.01             | <0.01           | 0.67   | 0.69 |
| S0.3                         | 1.31 a              | 67.44 d  | 51.27 b  | 15.57*            | -11.72*         | -0.60* |      |
| S11                          | 0.00 a              | 55.12 b  | 40.15 c  |                   |                 |        |      |
| <b>Succinic Acid (mg/g)</b>  |                     |          |          | <0.01             | 0.35            | 0.09   | 0.06 |
| S0.3                         | 1.84 c              | 0.41 ab  | 0.78 ab  | -0.61*            | 0.12*           | -0.23* |      |
| S11                          | 1.87 c              | 0.30 a   | 1.14 bc  |                   |                 |        |      |
| <b>Acetic Acid (mg/g)</b>    |                     |          |          | <0.01             | 0.12            | 0.39   | 0.16 |
| S0.3                         | 1.26 a              | 4.15 b   | 5.63 c   | -1.78*            | -0.58*          | -0.30* |      |
| S11                          | 1.12 a              | 3.27 b   | 5.35 c   |                   |                 |        |      |
| <b>Propionic Acid (mg/g)</b> |                     |          |          | 0.26              | 0.51            | 0.39   | 0.01 |
| S0.3                         | 0.00 b              | 0.22 a   | 0.20 a   | 0.03*             | -0.02*          | 0.02*  |      |

|                                            |        |    |        |    |        |    |         |         |        |      |
|--------------------------------------------|--------|----|--------|----|--------|----|---------|---------|--------|------|
| S11                                        | 0.00   | b  | 0.22   | a  | 0.20   | a  |         |         |        |      |
| <b>Butiric Acid<br/>(mg/g)</b>             |        |    |        |    |        |    | -       | -       | -      | -    |
| S0.3                                       | ND     |    | ND     |    | ND     |    | -       | -       | -      |      |
| S11                                        | ND     |    | ND     |    | ND     |    |         |         |        |      |
| <b>Ethanol<br/>(mg/g)</b>                  |        |    |        |    |        |    | <0.01   | <0.01   | 0.65   | 0.75 |
| S0.3                                       | 0.00   | a  | 21.80  | b  | 42.84  | d  | -20.34* | -17.34* | 0.69*  |      |
| S11                                        | 0.00   | a  | 5.16   | c  | 24.80  | b  |         |         |        |      |
| <b>Glycerol<br/>(mg/g)</b>                 |        |    |        |    |        |    | <0.01   | <0.01   | <0.01  | 0.02 |
| S0.3                                       | 0.00   | a  | 0.36   | b  | 1.68   | d  | -1.06*  | -0.60*  | 0.27*  |      |
| S11                                        | 0.04   | a  | 0.02   | a  | 0.81   | c  |         |         |        |      |
| <b>Moisture<br/>(g/100g)</b>               |        |    |        |    |        |    | 0.09    | 0.69    | 0.43   | 0.95 |
| S0.3                                       | 66.59  | b  | 72.20  | a  | 69.59  | ab | 1.82*   | -0.43*  | -0.79* |      |
| S11                                        | 66.30  | b  | 70.99  | a  | 69.95  | a  |         |         |        |      |
| <b>Ash (g/100g)</b>                        |        |    |        |    |        |    | <0.01   | 0.35    | 0.32   | 0.12 |
| S0.3                                       | 6.99   | c  | 5.19   | b  | 6.04   | a  | -0.99*  | -0.12*  | -0.13* |      |
| S11                                        | 5.95   | a  | 4.93   | b  | 6.05   | a  |         |         |        |      |
| <b>Lipid (g/100g)</b>                      |        |    |        |    |        |    | <0.01   | <0.01   | 0.22   | 0.09 |
| S0.3                                       | 4.22   | a  | 3.39   | bc | 4.21   | a  | -0.69*  | -0.41*  | 0.13*  |      |
| S11                                        | 4.27   | a  | 3.11   | b  | 3.67   | c  |         |         |        |      |
| <b>Protein<br/>(g/100g)</b>                |        |    |        |    |        |    | 0.60    | 0.06    | 0.07   | 0.26 |
| S0.3                                       | 9.96   | ab | 9.96   | ab | 10.65  | b  | -0.14*  | -0.58*  | 0.55*  |      |
| S11                                        | 9.89   | a  | 9.93   | a  | 9.53   | a  |         |         |        |      |
| <b>Acidity (mL<br/>NaOH<br/>0,1N/100g)</b> |        |    |        |    |        |    | <0.01   | <0.01   | 0.83   | 2.06 |
| S0.3                                       | 179.47 | b  | 286.98 | e  | 270.78 | a  | 15.73*  | -9.98*  | -0.47* |      |
| S11                                        | 190.06 | c  | 276.53 | a  | 261.27 | d  |         |         |        |      |

Data are presented as mean. For each compound, mean values with different lowercase letters are significant at  $p < 0.05$  by the Duncan test. \*Effects values. \*\*Standard error of the means. SB, submerged fermentation process; SS, solid-state fermentation process; S0.3, coffee sample with 0.3% of immature fruits; S11, coffee sample with 11% of immature fruits. ND: not detected

In this study, the initial percentage of immature fruits significantly affected the initial concentration of sugars (sucrose, glucose, and fructose), ash, titratable acidity, and citric acid ( $p < 0.05$ ). Initial sucrose, glucose, and fructose concentrations were significantly major in S0.3 than in S11 samples ( $p < 0.05$ ) (Table 2). This is expected because in ripe fruits sucrose is found in pulp and mesocarp, and its concentration increases during ripening when hexoses are converted to sucrose [36].

The ash content is related to minerals present in different compositions and concentrations in the different parts of coffee fruit [3, 6]. The major initial ash content  $6.99 \pm 0.30\%$  was observed in the sample with a major percentage of ripe fruits (Table 2). It may have occurred because there are minerals found in the pulp, and the immature

fruits have less mucilage completely formed [37]. Possibly, immature fruits have lower ash content because they did not yet absorb the maximum minerals of the soil.

Regarding the acidity, the highest value was identified for the fruit samples with the highest percentage of immature fruits ( $190.0 \pm 3.11$  mL of NaOH 0,1N/100g (Table 2). This result agrees with Torres et al. [38] that found higher values of total titratable acidity in immature coffee beans when compared to coffee beans originated from the harvest of ripe fruits. The acidity is naturally present in coffee fruits, and it comes from various organic acids mainly citric acid, malic acid, and phosphoric acid [39]. Citric and malic acids are the acids most prominent in green coffee [40]. The initial citric acid concentration was significative higher in S11 treatment (1,06 mg/g), while malic acid was higher in S0.3 treatment (10.11 mg/g). There is concern about the difference in composition of coffee fruits harvested at different degrees of ripeness, and some studies investigate chlorogenic acids and volatile compounds [41, 42]. However, studies about organic acids variation during the ripeness were not found.

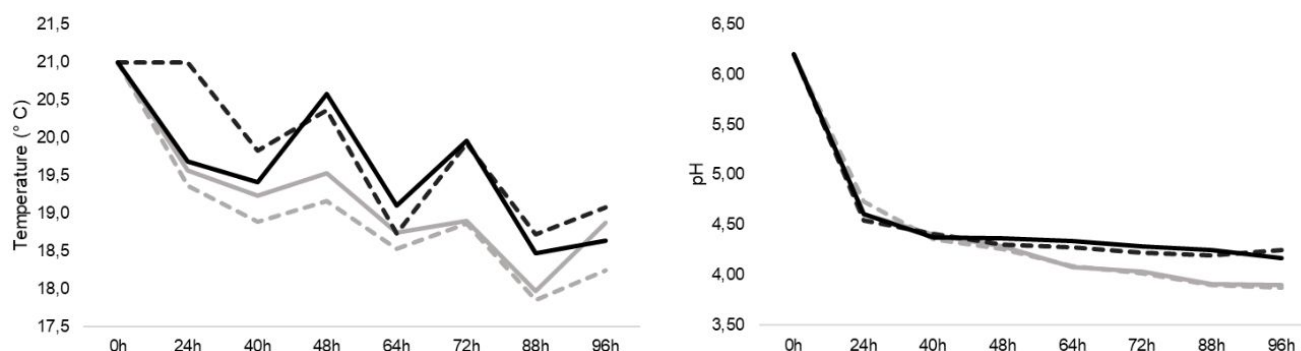
## **3.2. Immature coffee fruits and water influence in coffee fermentation**

### **3.2.1. Physicochemical characterization**

Temperature monitoring during SIAF fermentation is widely applied and can indicate microbiological activity [24, 26]. Fermentation is an exothermic reaction, capable of releasing energy during biochemical reactions to form alcohols and acids [43]. During fermentation, the mass temperature ranged from 17.8 to 21 °C, and the environmental temperature ranged from 16 to 29 °C (night/day). The temperature in the solid-state process remained higher than in the submerged state for most of the fermentation time. Despite the heat loss during the night, it was possible to observe microbiological activity capable of increasing the temperature during the day (Fig. 2). The temperature variation during the fermentative process can be exploited to modulate the concentration of compounds for flavor improvement [1].

The pH of aqueous coffee extract is used to specify its perceived acidity and to indicate the progress of acids production during the fermentation [32]. The end of the fermentation can be evaluated by the reduction in pH levels below 4.5 [1]. There was a reduction in pH during the fermentation time for all treatments. After 64 h, lower pH values were observed for the submerged processes. The final values measured were 3.9 and 4.2 in submerged and solid-state process, respectively. This behavior may be indicative of high acid dissolution in the aqueous phase during the process (Fig. 2).

**Fig. 2** Temperature and pH variation during the fermentative process



Black lines refers to the solid-state process and grey lines refers to the submerged process. Whole lines show the S0.3, and dotted lines show the S11

### 3.2.2. Sugars

In the present study, reductions in sugar concentrations were detected in all types of processing, however no sugar was completely consumed by the end of the fermentations (Table 2). The reduction in sugars is often presented as a result of the fermentative process, because of the microbiological action in the consumption of the mucilage of the grains [18, 29]. The lower final sucrose concentration found in this study indicates the action of the enzyme  $\beta$ -fructofuranosidase, which acts in the generation of monosaccharides, including glucose and fructose [44].

Both factors analyzed had significant effect on the final glucose, and fructose concentration. On average when the process changes from solid-state to submerged, the final concentration of glucose and fructose decrease, and sucrose increase. While increasing the percentage of immature fruit in the initial sample (11%) favored higher residual values in all sugars. After submerged fermentation, the sucrose and glucose residual were significantly lower in S.03 (0.97 and 8.29 mg/g, respectively), and the highest fructose concentration (44.12 mg/g) was identified in solid-state process and S11 (Table 2). Knopp, Bytof and Selmar (2006) found that, after submerged fermentation, only low amounts of fructose and glucose were present, while concentrations of those sugars were significantly higher after solid-state process.

Although the initial fructose concentration was about 1.7 higher than glucose's, the residual fructose was about 3-fold higher than residual glucose (Table 2). That's because glucose is the preferential molecule used in fermentative process. The metabolism of the fruits in post-harvest changes from aerobic to anaerobic pathways, when the lactic and alcoholic fermentation require glucose as the main substrate [29]. The residual sucrose found in this study is probably from the endosperm and it is an interesting result because the sucrose is an import precursor of flavor compounds during roasting and can contribute to sweetness in coffee beverage [29, 45, 46]

### 3.2.3. Organic acids

Organic acids are frequently determined after coffee fermentation process. Citric, malic, and succinic acids are normally naturally found in coffee fruits. On other

hand, acetic, lactic, butyric, and propionic can be produced during the fermentative process [18, 27, 43, 47, 48].

The butyric acid was not identified in any treatment. The final concentrations of citric and propionic acids were not influenced by analyzed factors and did not show a significant difference in any of the four treatments ( $p > 0.05$ ). After the fermentation process, the citric acid concentration ranged from 0.52 to 0.64 mg/g, however, compared to the initial sample, a significant decrease in citric acid was observed only for S11 (Table 2). After coffee fermentation Bressani et al. [49] found that citric acid content decreased after 27 hours of fermentation using natural coffee with the final concentration varying from 0.46 to 1.26 mg/g. Similarly, Bressani et al. [47] after 40h of fermentation of Bourbon amarelo, observed a decrease in citric acid concentrations, which varied from 0.53 to 1.66 mg/g at the end of the fermentation. The reduction can be attributed to the fact that citric acid can serve as a precursor to other acid decomposition products, such as glutaric, fumaric, and maleic acids [40].

Propionic acid, which was not detected in the natural fruit, achieved a concentration of 0.2 mg/g after fermentation in all treatments (Table 2). Propionibacteria and several other genera of anaerobic bacteria such as *Veillonella*, *Selenomonas*, and *Clostridium*, especially *Clostridium propionicum*, produce propionic acid as a main fermentation product. The propionic acid can be produced from lactic acid [50, 51]. Since high concentrations of lactic acid were identified at the end of all treatments the presence of this acid is justified. It is known that butyric acid and propionic acid develop in the later stages of coffee fermentation, however, concentrations below 2.09 mg/g are not considered prejudicial to coffee quality [19, 46]. This may indicate that 96 hours was a satisfactory time to avoid overfermentation in this environment conditions.

The final concentrations of the succinic and acetic acids were only significantly affected by water adding during fermentative process. Succinic acid is a dicarboxylic acid naturally present in coffee fruit and it is also produced by several microorganisms [52]. Succinic acid is an intermediate of the tricarboxylic acid (TCA) cycle and one of the end products of anaerobic metabolism, and it is synthesized in almost all microbe, plants, and animal cells [53]. After fermentation succinic acid decreased significantly in all treatments except in SS11 varying from 0.30 to 1.14 mg/g. On average the final concentration of succinic acid was major in solid-state process. Ribeiro et al. [18] found in initial coffee succinic acid concentrations of 2.61 to 4.42 mg/g and after fermentation, concentrations of 0.74 to 3.85 mg/g. Bressani et al. [47] observed after spontaneous fermentations succinic acid contents varying from 0.18 to 1.01 mg/g.

In this study, there was an increase in the acetic acid content in all types of fermentations. The initial immature fruits percentage did not interfere significantly in the acetic acid formation. On average when the process changes of solid-state to submerged, acetic acid decrease, being the highest concentrations found in solid-state processes. After fermentation the acetic acid increased significantly until 4.15 and 5.63 mg/g (SB0.3 and SS0.3 respectively), and 3.27 and 5.35 mg/g (SB11 and SS11 respectively). Similar findings on the acetic acid increase in SIAF spontaneous coffee fermentation have been reported elsewhere. Bressani et al. [27] found that the highest acetic acid concentration was found in the SIAF spontaneous fermentation (7.59 mg/g). Ribeiro et al. [18] observed an increase of acetic acid in Mundo novo coffee after fermentation, whose concentration changed from 2.23 to 7.0 mg/g. Acetic acid is identified as a beneficial product in some fermented foods, as well as coffee [50]. The presence of acetic acid bacteria has been commonly identified during the spontaneous coffee fermentation [16, 29]. During the spontaneous SIAF process the bacterial sequences most detected in the samples were assigned to genera *Acetobacter* [16]. *Acetobacter* can produce acetic acid by oxidizing

ethanol under aerobic conditions, however, in the SIAF method the presence of oxygen is low and only available for the first few hours [50]. Therefore, the production of acetic acid can be attributed to heterolactic pathway performed mainly by lactic acid bacteria and *Enterobacteriaceae* [19].

Lactic and malic acids had the final concentration affected by the two factors analyzed. Malic acid content decreased significantly in all treatments performed and it was less in submerged process (0.98 and 1.38 mg/g to S0.3 and S11, respectively). On average when the process changes of solid-state to submerged, malic acid concentration decrease. On other hand, when the percentage of immature fruits change of 0.3% to 11%, malic acid concentration increase. Malic acid is naturally present in coffee fruit and is possibly produced by endogenous microorganisms present in the soil [54]. The reduction of malic acid after the application of the coffee fermentation process is reported by several authors [26, 27, 46]. Cassimiro et al. [26] found a reduction of malic acid from 3.48 to 0.39 (mg/g) after 72h of SIAF fermentation without inoculation. Malolactic fermentation is responsible for the conversion of malic acid into lactic acid and CO<sub>2</sub>. This fermentation can occur during or after alcoholic fermentation and is carried out by lactic acid bacteria, which are able to grow at high ethanol levels and low pH values [55].

The lactic acid is not naturally found in coffee fruits [40, 56]. The lactic acid is produced mainly by lactic acid bacteria present in coffee fruits from the harvest, and it significantly influences the final coffee quality [16, 18, 35, 57]. Lactic acid content increased significantly in all treatments performed and it was higher in submerged fermentation with less immature fruits, 67.44 mg/g. On average when the process changes of solid-state to submerged, lactic acid concentration increase. On other hand, when the percentage of immature fruits change of 0.3% to 11%, lactic acid concentration decrease. Just like in this study the lactic acid is a common acid found at the end of the fermentative processes of submerged coffee and SIAF. Pereira et al. [56] found lactic acid was a major end-metabolite of carbohydrate metabolism during submerged fermentation for 24 hours. In the study of Ribeiro et al. [58], lactic acid concentration ranged from 0.71 to 8.14 mg/g after wet fermentation for 48 hours. Bressani et al. [27] in a solid-state SIAF process found 1.27 mg/g at the end of spontaneous fermentation. The lactic acid production was favored by higher sugars concentrations in both processes. The homofermentative LAB usually uses hexose and pentose sugars via the Embden-Meyerhof pathway, (by using glycolysis and pentose phosphate pathways). Homofermentative LAB produces two lactic acid molecules as a major end-product per mole of consumed glucose [59]. In addition to sugars, LAB species have the ability of metabolizing citrate present in high concentration in the coffee pulp [60].

### 3.2.4. Ethanol and glycerol

Although it was not identified in the initial sample, ethanol was identified in all treatments after fermentation. Ethanol accumulation after fermentation has been reported previously by Elhalis et al. [61] and confirmed in our study. The highest final concentration (42.84 mg/g) was found in coffee processed by solid-state fermentation and minor immature fruits (Table 2). On average when the process changes of solid-state to submerged, ethanol decrease. Likewise, when the percentage of immature fruits change of 0.3% to 11% there was a decrease ethanol concentration. The ethanol can be produced by yeasts during the alcoholic fermentation and by LAB in the heterofermentative process [60, 62]. Ethanol is one of the most abundant alcohols found after coffee fermentation, it

has an essential role in the beverage viscosity, and it serves as a solvent for volatiles compounds [61]. Ethanol in coffee beans shows an alcoholic sensory description [17].

Glycerol concentration increased in all treatments after fermentation, except for SB11, in which concentration remained constant (Table 2). The final concentration was 0.36 and 1.68 mg/g (SB0.3 and SS0.3 respectively), and 0.02 and 0.81 mg/g (SB11 and SS11 respectively). The glycerol concentration decreases significantly, when the process changes of solid-state to submerged, and when the percentage of immature fruits change of 0.3% to 11%. According to Elhalis et al. [61] the endosperm did not contain glycerol initially, but it was detected as the fermentation progressed, reaching a maximum concentration of 0.08%. Yeasts are the microbial group responsible to produce glycerol during coffee bean fermentation [61]. High sugar concentration favors glycerol production, because this compound protects yeast from high osmotic stress [62]. The glycerol has an exceptionally role in Maillard reactions and it has a potential to act as an active flavor precursor. In addition to its role of solvent, glycerol actively contributes to the formation of proline-specific compounds in Maillard model systems [63]. Glycerol has a sweet taste and smooth mouthfeel [62]. The highest value of glycerol found in this study occurred in the same process where the highest value of ethanol was found, in SS0.3 (1.68 and 42.84 mg/g, respectively). This may suggest some relationship between the production of these compounds. The water in fermentative process may be responsible for solubilize some compounds.

### 3.2.5. Proximate composition

The type of process and the percentage of immature fruits had no significant effect on the final coffee protein and moisture ( $p>0,05$ ) (Table 2). The moisture increased in all treatments, except in SS0.3. Since the final moisture was statistically equal in all treatments, the application of submerged fermentation may not extend the drying time, when compared to solid-state fermentation.

The final ash content was not affected by the immature fruits during the fermentation. The highest ash concentration was found after solid-state fermentation. After fermentation the final ash concentration was 5.19 and 6.04 mg/g (SB0.3 and SS0.3 respectively), and 4.93 and 6.05 mg/g (SB11 and SS11 respectively). On average when the process changes of solid-state to submerged, ash decrease 0.98 mg/g. It may be because of the solubilization of the minerals of the coffee in the water.

Lipid content decreased in all processes except in SS0.3 (Table 2). After fermentation the final lipid concentration were 3.39 and 4.21 mg/g (SB0.3 and SS0.3 respectively), and 3.11 and 3.67 mg/g (SB11 and SS11 respectively). The lipid composition decreased when the process changes of solid-state to submerged, and when the percentage of immature fruits change of 0.3% to 11%. Lipid consumption by microorganisms was higher in coffee that had fewer sugars as a substrate.

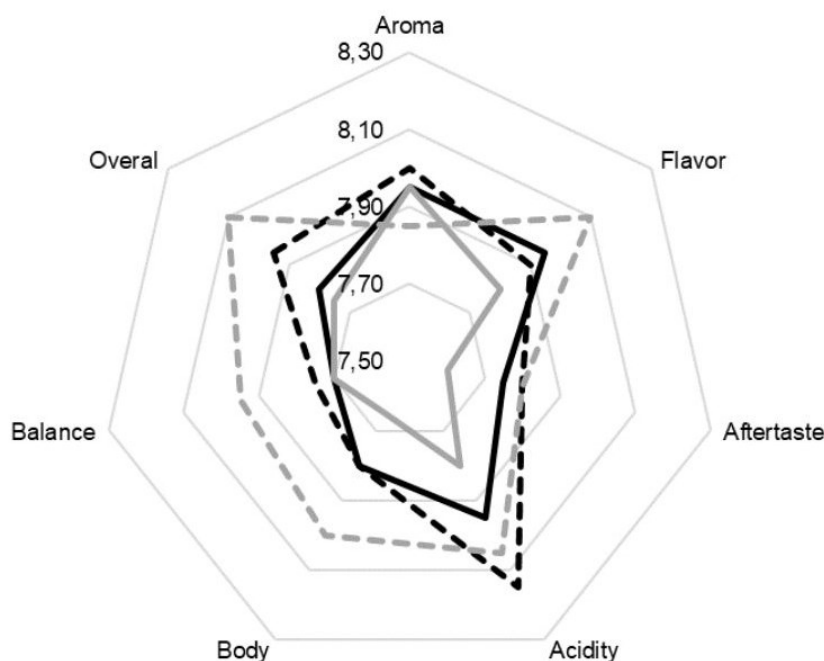
The titratable acidity increased significantly in all samples after fermentation ( $p<0,05$ ). After fermentation the titratable acidity was 286.98 and 270.78 mL NaOH 0,1N/100g (SB0.3 and SS0.3 respectively), and 276.53 and 261.27 mg/g (SB11 and SS11 respectively). Similarly, Elhalis et al. [29] show that the coffee spontaneous fermentation is responsible for increase the titratable acidity. On average the titratable acidity increased when the process changes of solid-state to submerged. On other hand, titratable acidity decreased the percentage of immature fruits change of 0.3% to 11%. The titratable acidity was higher in submerged fermentation and in S0.3 samples. These results are

correspondents with the lactic acid, the main acid quantified in this study, showing the relationship between organic acid production and titratable acidity.

### 3.2.6. Sensory Analyses

Controlled fermentation in coffee processing was able to modify aroma and flavor of coffee beverage [16, 18, 64]. The SIAF spontaneous fermentations were able to modify the sensory descriptions and the final score of the coffees as shown in Fig. 3 and Table 3. According to the SCA protocol, all coffees produced in this study can be classified as specialty coffees, and showed final score above 84 points. The coffee treatment fermented with 11% of immature fruits in the submerged process (SB11) had the best sensory result: aroma (8.0), flavor (7.9), after taste (7.8), acidity (8.15), body (7.8), balance (7.75), and overall (7.95) (Fig. 3). The spontaneous fermentation using SIAF method in solid-state process has been related to specialty coffee production [27, 65]. Different of Cassimiro et al. [26] our results showed specialty coffee production using natural coffee and submerged process in spontaneous fermentation using SIAF method.

**Fig. 3** Average score of attributes evaluated by SCA protocol cupping



All treatments scored 10 for uniformity, sweetness, and clean cup. Treatments: Black lines refers to the solid-state process and grey lines refers to the submerged process. Whole lines show the S0.3, and dotted lines show the S11.

The sensorial analysis results were consistent with the chemical analysis and confirmed that immature fruits and water addition can modify the sensory perceptions on coffee beverage. The changes in the chemical composition of the coffee caused by each process variable analyzed resulted in beverages with a specific sensory profile for each fermentation, such as lemon in SB11, grape, pineapple, apple, and spices in SB0.3, bitter in SS11, and dry fruits, strawberry, and passion fruit in SS0.3. The fruit sensory perception and orange was found in all treatments and probably is related to Arara coffee

variety. The chocolate sensory perception was found only in S0.3 treatments. The concentration of ethanol interferes with the tasters' sensorial perceptions. Only the solid-state treatments presented alcoholic descriptions, as well as the major concentrations of ethanol at final of fermentation (Table 3).

**Table 3** Coffee beverage sensory descriptions and final score by SCA protocol

| Treatment | Sensory Descriptions                                                                 | Final Score |
|-----------|--------------------------------------------------------------------------------------|-------------|
| SB11      | Caramel, brown sugar, honey, orange, lemon, floral, nut, yellow fruits, red fruits   | 86,50       |
| SB0.3     | Chocolate, floral, brown sugar, honey, nuts, orange, grape, pineapple, apple, spices | 84,25       |
| SS11      | Caramel, molasses, wine, red fruits, orange, bitter                                  | 85,35       |
| SS0.3     | Chocolate, nuts, wine, alcoholic, dry fruits, orange, strawberry, passion fruit      | 85,75       |

The acidity in sensory analyses can be related to total titratable acidity [32]. The best score for sensory acidity were found in SS11 and the worst were found in SB0.3 (Fig. 3). The sample with the lowest value of titratable acidity, correspondent to SS11 was more valued during the sensory analysis (261.27 mL NaOH 0,1N/100g). On other hand, the highest value of titratable acidity, correspondent to SB0.3 was penalized during the sensory analysis (286.98 mL NaOH 0,1N/100g). Although the initial titratable acidity was higher in S11 fruits, organic acids produced during fermentation seemed to have a greater influence in the perception of acidity in the sensorial tests.

It is known that the incomplete maturation of coffee beans can be related with a metallic and astringent flavor caused by the high composition of chlorogenic acids and phenolics compounds [31, 66]. However, according to the results found in this study the fraction of 11% of immature fruits during processing may not have a negative influence on the final score of the drink evaluated by the SCA protocol. There is no information in the literature about immature fruits influence during coffee fermentation. This work is thus the first to show that the immature fruits can modifying the results of fermentation process. Therefore, the immature fruits percentage and the fermentation method (solid-state or submerged) influenced the sensory results.

#### 4. Conclusion

The percentage of immature fruits directly influences the initial concentration of glucose, sucrose, fructose, ash, citric acid, and titratable acidity. During the fermentative process, this variable was able to significantly modify the lactic acid, ethanol, and glycerol production. It also altered the final concentration of sugars, lipids, and titratable acidity. The lower percentage of unripe fruits (S0.3) was related to lower consumption/degradation of lipids during fermentation. This shows that the presence of immature fruit during fermentation significantly alters the availability of substrates for fermentation, mainly sugars, and consequently the capacity for metabolite production.

The presence of water during the fermentation process did not significantly influence the final concentration of citric acid, propionic acid, moisture, and protein. The submerged process favored the production of lactic acid and gave rise to coffees with lower concentrations of ethanol, glycerol, malic acid, acetic acid, and succinic acid. These results suggest that water may have a solubilizing action during the coffee fermentation process.

The fraction of 11% of immature fruits during processing may not have a negative influence on the final score of the drink evaluated by the SCA protocol. All coffees produced were classified as specialty coffees with a score above 84 points. Despite of that, there was a great variation in the chemical compounds analyzed. This indicate that the percentage of immature fruits during a fermentative process is an important parameter to control, because they are capable to modify the chemical compounds profile and consequently the sensory description of coffee beverage.

## 5. Declaration of Conflict of Interest

The authors declare that they have no conflict of interest.

## 6. Acknowledgements

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### **CAPÍTULO 3 - Coinoculation of *Saccharomyces cerevisiae* and *Bacillus amyloliquefaciens* in in solid-state and submerged coffee fermentation: Influences in chemical and sensory compositions**

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#### **ABSTRACT**

This study aimed to evaluate the influence of yeast and bacillus as starter cultures in SIAF method applied in solid-state and submerged process. *Saccharomyces cerevisiae* and *Bacillus amyloliquefaciens* were separately and coinoculated into natural coffee in buckets with or without water adding. The fermentations were performed for 96h, and the effects on physicochemical composition and on the sensorial quality of coffee were evaluated. All coffees produced were classified in specialty coffee with final score above 84. The coinoculated coffees obtained the highest sensory scores in submerged process (> 86). Due to the results found, the type of process, submerged and solid-state, showed more influence in chemical and sensory analyses than type of inoculation. Submerged process showed major concentration of lactic acid, while solid-state process promotes major concentrations of ethanol, glycerol and acetic acid. Different sensory descriptions were observed in each treatment. Coffees from the submerged process showed nutty, cocoa, and sweet sensory descriptions, while solid-state process produced coffees with sour and acidic attributes.

**Keywords:** SIAF method; Natural coffee; Induced fermentation; Sensory descriptors; Starter culture; Submerged fermentation; Solid-state fermentation.

#### **1. Introduction**

The quality of the coffee beverage is related to the post-harvest processing of the fruits. After harvesting, the coffee fruits are transformed into green coffee beans, which

have various aromatic and non-aromatic precursors. These precursors are transformed during roasting to determine the final coffee aroma and flavor (Lee et al., 2015; Montavon et al., 2003; G. V. de M. Pereira et al., 2019). The evaluation method developed by the Specialty Coffee Association (SCA) is the most widely used by coffee producers and traders to determine the physical and sensorial quality of coffee. The growing market for specialty coffees has motivated the advancement of post-harvest processing techniques in order to achieve greater commercial value by developing coffees with different sensory descriptions. In recent years, studies have shown that the development of a fermentation step in a closed bioreactor can improve coffee quality and modulate flavor and aroma. Additionally, it is economically viable and can be used as a standardization tool for the process (Elhalis et al., 2023; Magalhães Júnior et al., 2021; Martinez et al., 2021).

In this way, the Self-Induced Anaerobic Fermentation (SIAF) is an emerging technology based on the conditioning of coffee fruits in closed containers, with or without the addition of water. The anaerobiosis is caused by the consumption of O<sub>2</sub> and the production of CO<sub>2</sub> by the metabolism of the fermenting microorganisms. The SIAF method has been capable to improve coffee quality, resulting in higher scores according to the SCA method. This is the anaerobic method more accessible to producers and it is related to changes in microbial behavior, higher lactic acid concentration, modifications in the concentration of volatile compounds, and differentiation in sensory profiles resulting in coffees with more intense fruity attributes (Bressani, Martinez, et al., 2021; Cassimiro et al., 2022; da Mota et al., 2020, 2022; Martinez et al., 2021; T. S. Pereira et al., 2022).

Selected yeasts and bacteria can be used as inoculum in the fermentative process to promote major differentiation, uniformity, and reproducibility of the results, allowing major process control. The mesophilic bacteria are identified on the beginning of coffee fermentation in high diversity of species. The highest bacterial population was normally detected in the beginning or in the middle of the fermentation process. *Bacillus amyloliquefaciens* are widely used to produce fermented foods (Dahake & Dhoble, 2022), as in the development of novel soybean products, to increase peptide yield (Cho, 2019; Wang et al., 2021; Zou et al., 2022), to improve of bioactivity of product (Chi & Cho, 2016), and to flavor development (Lu et al., 2021). *Bacillus* is one of the genera of mesophilic bacteria found during spontaneous coffee fermentation (Elhalis, Cox, & Zhao, 2020; Evangelista et al., 2015; P. M. M. Martins et al., 2020; T. S. Pereira et al., 2022; Ribeiro et al., 2018). Some *Bacillus* species produce cellulases and pectinases that degrade the pulp and mucilage (Vilela et al., 2010).

*Saccharomyces cerevisiae* has been widely used in recent years as an inoculant in various fermentative process conditions. The performance of yeasts during the fermentation process is related to higher final concentrations of isoamyl alcohol, ethanol, acetaldehyde, and ethyl acetate (Elhalis, Cox, Frank, et al., 2020). The addition of yeasts as starter cultures helps to control the fermentation process. *S. cerevisiae* is capable to modify the filamentous fungi community regardless of the fermentation time and to reduce the richness of the fungi and bacteria community, probably because of the ethanol accumulation (da Silva et al., 2022). *S. cerevisiae* is a resistant yeast that has been applied to several types of fermentative processes and was able to exert population dominance. Moreover, several studies show significant difference in the sensory analysis after use of that yeast (Bressani et al., 2020; da Mota et al., 2020; Evangelista et al., 2014; Ribeiro et al., 2017).

As the natural microbiota of coffee is diverse, composed of filamentous fungi, yeasts, lactic bacteria, and mesophilic bacteria, the use of a consortium of microorganisms can be beneficial. That said, an inoculant can be composed of multiple strains and is called a coinoculation process. Bressani, Martinez, et al., (2021) showed that the fermentation process using *Candida parapsilosis* CCMA 0544 and *Torulaspora delbrueckii* CCMA 0684 improved the quality of low-altitude coffees. Cassimiro et al., (2022) found that using lactic acid bacteria (LAB) and yeast as starter cultures in the SIAF process improved coffee quality and produced a sweetness coffee. The microorganisms most used as coinoculants are yeast and LAB, studies using *Bacillus* sp. are still rare (B et al., 2019; Bressani, Martinez, et al., 2021; Cassimiro et al., 2022; Vale et al., 2019).

Over the years many studies have demonstrated that post-harvest processing directly influences the sensorial profile of coffee. It is known that coffees processed by dry, semi-dry, and wet methods show difference on physicochemical and sensory characteristics (Cortés-Macías et al., 2022; da Silva et al., 2022). There are studies that evaluate solid-state fermentation, and others that evaluate submerged fermentation, but there are no studies that conduct the two studies simultaneously in order to compare the effect of the addition of water in a fermentation process conducted with the same oxygen condition. Therefore, the objective of this study was to evaluate the influence of two fermentation process, solid-state and submerged, using natural coffee fruits and coinoculation of yeast and bacteria.

## **2. Material e methods**

### **2.1.Coffee fruits**

*Coffea arabica* L. of the Arara variety from the Chuá farm in Patos de Minas, Minas Gerais, Brazil (latitude 18° 35' 15, altitude 1040 m, and longitude 46° 25'35), was fermented by solid-state and submerged process. The coffee fruits were mechanically harvested, washed, and selected. Only ripe coffee cherries were used.

### **2.2.Starter cultures**

Two starter cultures isolated from coffee were used in this study. The *Saccharomyces cerevisiae* CCMA0543, selected by Nucoffee artisans project and produced by Syngenta, was acquired in liquid form, in a concentration of  $10^6$  cells/mL. This suspension was used as inoculum. The *Bacillus amyloliquefaciens* was obtained from the Culture Collection of Agricultural Microbiology of Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil. Inoculum was prepared as follow: the bacteria cells were reactivated and grown in GYP medium (1% glucose, 0.8% yeast extract, 0.2% soy peptone) at 37 °C with a shaking of 160 rpm according to Chi & Cho (2016) and Wang et al. (2021), until reaching a final concentration of  $10^8$  cells/mL.

### 2.3. Coffee fermentation process

Whole coffee cherries were inoculated with yeast and/or bacteria suspensions resulting in eight treatments. Each treatment was inoculated separately with yeast and bacteria in a single or coinoculation using two processes: i) solid-state (SS) and ii) submerged (SM), as described in Table 1. Fermentations were carried out using Self-Induced Anaerobic Fermentation (SIAF) process, in a closed polyethylene containers of 20 L, equipped with an airlock valve, for 96 h. In the submerged fermentations, water was added until cover the fruits. The room temperature was measured using a glass thermometer and ranged from 16.0 to 29.0 °C (night/day) during the assays. After fermentation, the cherries were washed and transferred to suspended terraces until obtaining 11.0–12.0% moisture. Cherries (100 g) samples were collected before and during fermentation (0, 24, 48, 72, and 96 h) and frozen until chemical analyses. All experiments were development in triplicate.

Table 1 - Treatment experimental design.

| ID    | Process     | Starter Cultures                                                    |
|-------|-------------|---------------------------------------------------------------------|
| SM-C  | Submerged   | None (Spontaneous fermentation)                                     |
| SM-S  | Submerged   | <i>Saccharomyces cerevisiae</i>                                     |
| SM-B  | Submerged   | <i>Bacillus amyloliquefaciens</i>                                   |
| SM-SB | Submerged   | <i>Saccharomyces cerevisiae</i> + <i>Bacillus amyloliquefaciens</i> |
| SS-C  | Solid-State | None (Spontaneous fermentation)                                     |
| SS-S  | Solid-State | <i>Saccharomyces cerevisiae</i>                                     |
| SS-B  | Solid-State | <i>Bacillus amyloliquefaciens</i>                                   |
| SS-SB | Solid-State | <i>Saccharomyces cerevisiae</i> + <i>Bacillus amyloliquefaciens</i> |

### 2.4. Chemical compounds

#### 2.4.1. Chemical proximate composition analysis

The whole frozen fruit samples were lyophilized and ground in in a knife mill (SOLAB) in a fine granulometry of 20 mesh. The physical chemical analyzes were performed with the powder sample according to (Association of Official Analytical Chemists, 1990). The protein content determination was performed according to method 920.87, using 6.25 as correction factor. The lipid content, ash content, and titratable acidity were determined according to method 920.97, method 923.03, and method 942.15, respectively.

#### 2.4.2. High Performance Liquid Chromatography (HPLC) Analysis

Organic acids (citric, malic, lactic, succinic, acetic, propionic, and butyric), sugars (sucrose, glucose, and fructose), glycerol and ethanol were analyzed. Samples were extracted using MilliQ water according to Elhalis, Cox, & Zhao (2020) with modifications: 10 g of fruits were mixed with 100 mL deionized water and blended in a domestic blender (Oster 1400W) for 2 min. The slurry was filtered through two layers of

organza polypropylene and then centrifuged in an Heal Force (Neofuge 18R) at 10.000 rpm for 15 min at 17 °C, and the supernatant was retained. The supernatants were filtrated through a 0.45-micron nylon filter before injection into Shimadzu liquid chromatography system (Shimadzu Corp., Japan). A Supelcogel C-610H (7.8 mm × 30 cm) column was used with a 0.1% solution of phosphoric acid, with a flow rate of 0.5 mL per min as the mobile phase. The oven temperature was kept at 30 °C for the analysis of acids, detected with a 210-nm UV detector (SPD), and at 30 °C for the analysis of sugars, detected with a refractive index detector (RID). The quantification of compounds was performed using calibration curves constructed with different concentrations of standard compounds and analyzed using the same conditions as for the samples.

## **2.5.Sensory analyses**

The sensory analyses were performed following the recommendations of the Specialty Coffee Association (SCA, 2018). A panel of five expert coffee tasters, with a Q-Grader Coffee Certificate, performed the sensory analysis using five cups for each sample (a predetermined ratio of  $8.25 \pm 0.25$  g per 150 mL of water). The attributes evaluated were fragrance/aroma, flavor, aftertaste, acidity, body, balance, sweetness, clean cup, uniformity, overall, and final score, besides the sensory descriptors. Each attribute was scored from 0 to 10, and the final score consisted of the sum of the scores.

## **2.6.Statistical analyses**

The data of carbohydrates, organic acids, ethanol, glycerol concentrations, and proximate composition (ash, protein, lipid, and titratable acidity) from initial and final samples (unfermented and fermented fruits, respectively) were evaluated by analysis of variance (ANOVA) and the Tukey test was used for comparison between means, considering each compound in isolation ( $p < 0.05$ ).

The Matlab software (version 2.3) was used for the Spearman correlation coefficient to calculate the correlations between the aroma, flavor, aftertaste, acidity, body, balance, score, and final score. The Matlab software was also used to evaluate the decision trees (root and branch) based on the treatment experimental design and create algorithm for scores. The accuracy and the “full-training-cross-validation” (FTCV) values were used as performance scores.

## **3. Results**

### **3.1. Proximate composition**

The fermentative process promoted the acidity increase in solid-state and submerged fermentation. The initial value of 179.47 (mL de NaOH 0,1N/100g) increased varying from 254.01 to 293.83 (mL de NaOH 0,1N/100g) in submerged processes, and varying from 253.84 to 276.76 (mL de NaOH 0,1N/100g) in solid-state processes. The initial ash contends of 6.99 (g/100g) decreased significantly after submerged process, ranging from 4.92 to 5.19 (g/100g) (Table 2).

Table 2 - Proximate Composition of coffee fruits after fermentation process at different treatments.

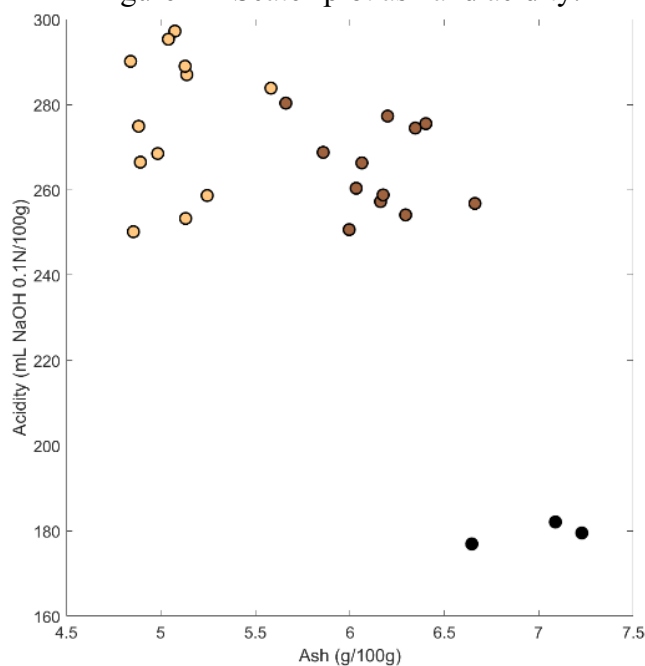
| Processing | Proximate Composition |                |                  |                             |
|------------|-----------------------|----------------|------------------|-----------------------------|
|            | Ash (g/100g)          | Lipid (g/100g) | Protein (g/100g) | Acidity (mL NaOH 0,1N/100g) |
| AS*        | 6,99 ± 0,30 a         | 4,22 ± 0,12 a  | 9,96 ± 0,14 ab   | 179,47 ± 2,58 a             |
| SM-C       | 5,19 ± 0,37 b         | 3,39 ± 0,03 b  | 9,96 ± 0,34 ab   | 286,98 ± 3,15 bc            |
| SM-S       | 5,08 ± 0,04 b         | 3,39 ± 0,09 b  | 8,86 ± 0,75 a    | 293,83 ± 4,30 c             |
| SM-B       | 5,08 ± 0,20 b         | 3,55 ± 0,14 b  | 9,61 ± 0,36 ab   | 254,01 ± 4,30 d             |
| SM-SB      | 4,92 ± 0,06 b         | 3,29 ± 0,01 b  | 10,46 ± 0,60 ab  | 269,95 ± 4,40 e             |
| SS-C       | 6,04 ± 0,17 c         | 4,21 ± 0,33 a  | 10,65 ± 0,64 ac  | 270,78 ± 5,76 e             |
| SS-S       | 6,32 ± 0,33 ac        | 2,81 ± 0,04 c  | 9,81 ± 0,33 ab   | 253,84 ± 3,08 d             |
| SS-B       | 6,12 ± 0,08 c         | 4,10 ± 0,05 a  | 12,36 ± 1,46 c   | 258,78 ± 1,56 d             |
| SS-SB      | 6,14 ± 0,41 c         | 2,79 ± 0,18 c  | 10,84 ± 0,24 bc  | 276,76 ± 3,12 be            |

The assays were performed in triplicate. Lower-case letters show significant differences between different fermentation treatments and was determined by Tukey's test ( $p < 0.05$ ).

\*Coffee fruit at 0 h, before fermentation process.

Regarding the composition of coffee fruits after the different fermentation processes, only the ash and acidity responses were well-defined for the submerged and solid-state conditions. Figure 1 shows the change in ash content and acidity in the fruit after fermentation. It is possible to verify that the ash content in the submerged fermentations decreased more intensely than in the solid-state fermentations, suggesting the influence of water on this reduction. As for the acidity, the increase was similar in some submerged and solid-state processes, suggesting that the water did not influence significantly.

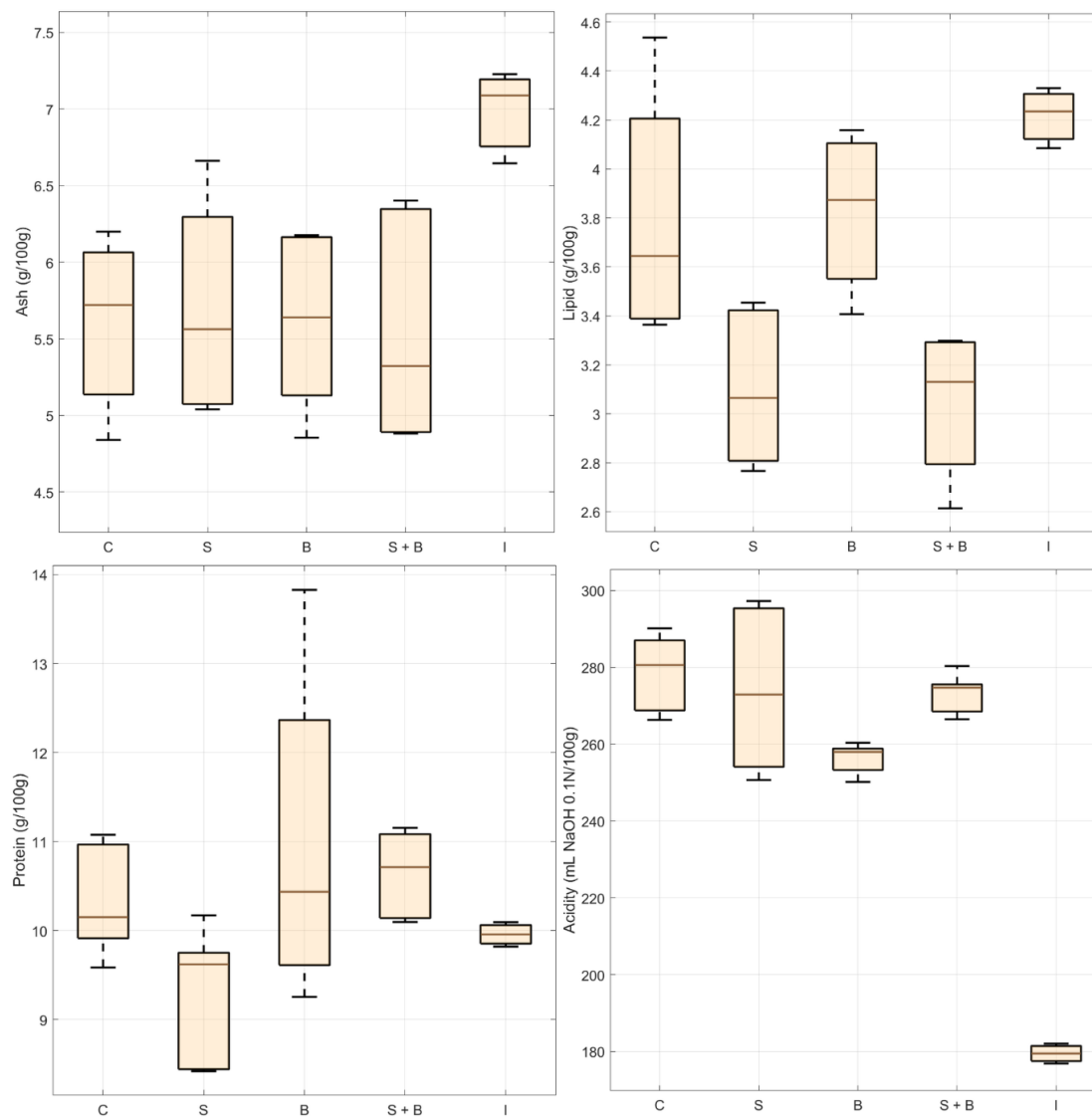
Figure 1 – Scater-plot ash and acidity.



Yellow: submerged process, Brown: solid-state process, and black: initial composition.

The initial lipid concentration decreased significantly after the fermentation process in all treatments except SS-C and SS-B. The type of inoculum did not significantly affect the final lipid concentration in the submerged process. The lowest final concentrations were identified in solid-state processes using yeast as inoculum, SS-S e SS-SB (2.81 and 2.79 g/100g, respectively) (Table 2). Regardless of the type of process, the yeast inoculation decreased the lipid concentration relative to the initial fruit concentration, more accentuated than in the processes using the natural microbiota or the bacteria inoculation (Figure 2b). The protein concentration increased after the fermentation process and it was significantly higher in solid-state processing using bacteria as inoculum, SS-B (12.36 g/100g) (Table 2). In Figure 2c the results of the box plot show that with the use of *Bacillus* the protein content tends to increase relative to coffee fruit and processes using other microorganisms. The acidity of the fruit post fermentation increases independent of the type of inoculum used (Figure 2d).

Figure 2 - Box-plot of composition of coffee fruits after fermentation process.

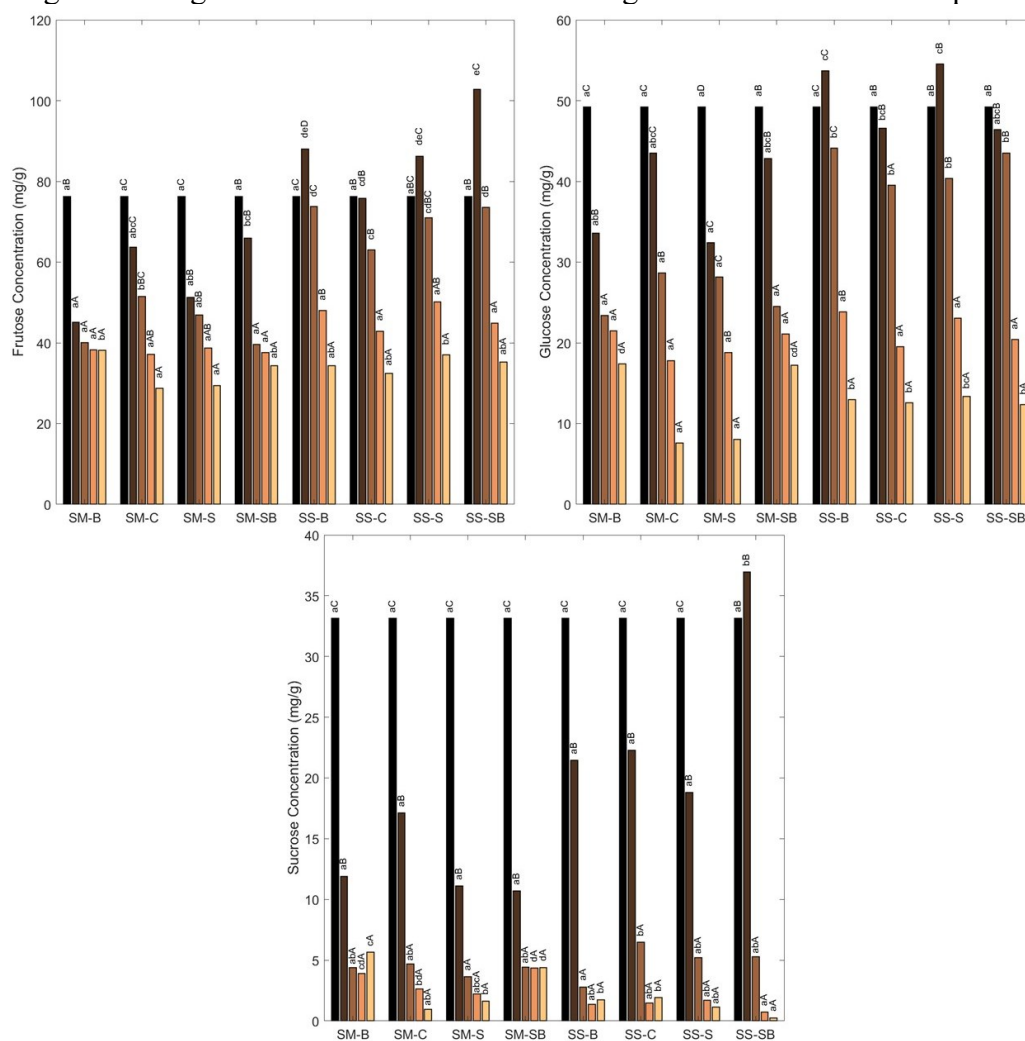


C- Control (Spontaneous fermentation); S: *S. cerevisiae* inoculation; B: *Bacillus* inoculation; I: Initial composition (coffee fruit 0 h).

### 3.2. Concentrations of sugars, organic acids, and ethanol with fermentation time

Fructose and glucose were the most abundant carbohydrates in the whole coffee fruit at the beginning of fermentation (76.26 and 49.24 mg/g, respectively). Sucrose content decreased significantly from the beginning of fermentation until 48h in all treatments, except in SS-SB. After that, there were no significant reductions in concentration. The final sucrose concentration was smaller than the other sugars. The glucose content decreased significantly at 24h in SM-S and SM-B. While fructose increased at 24h in all inoculated solid-state fermentations, suggesting the modulation of microbiological activity by the staters. In 24h of fermentation, fructose decreased only in submerged processes. The smallest concentrations of glucose and fructose at the end of fermentation were found in SM-C and SM-S, 7.59 and 8.06 mg/g for glucose and 28.75 and 29.46 mg/g for fructose. For glucose, the final concentration was the same for all treatments in solid-state. In submerged fermentations with *Bacillus* the highest final glucose concentrations were obtained (17.38 and 17.20, for SM-B and SM-SB respectively). The addition of water during the fermentation process seems to favor faster consumption of carbohydrates. But in 96h of fermentation, both the submerged and solid-state processes reached similar concentrations (Figure 3).

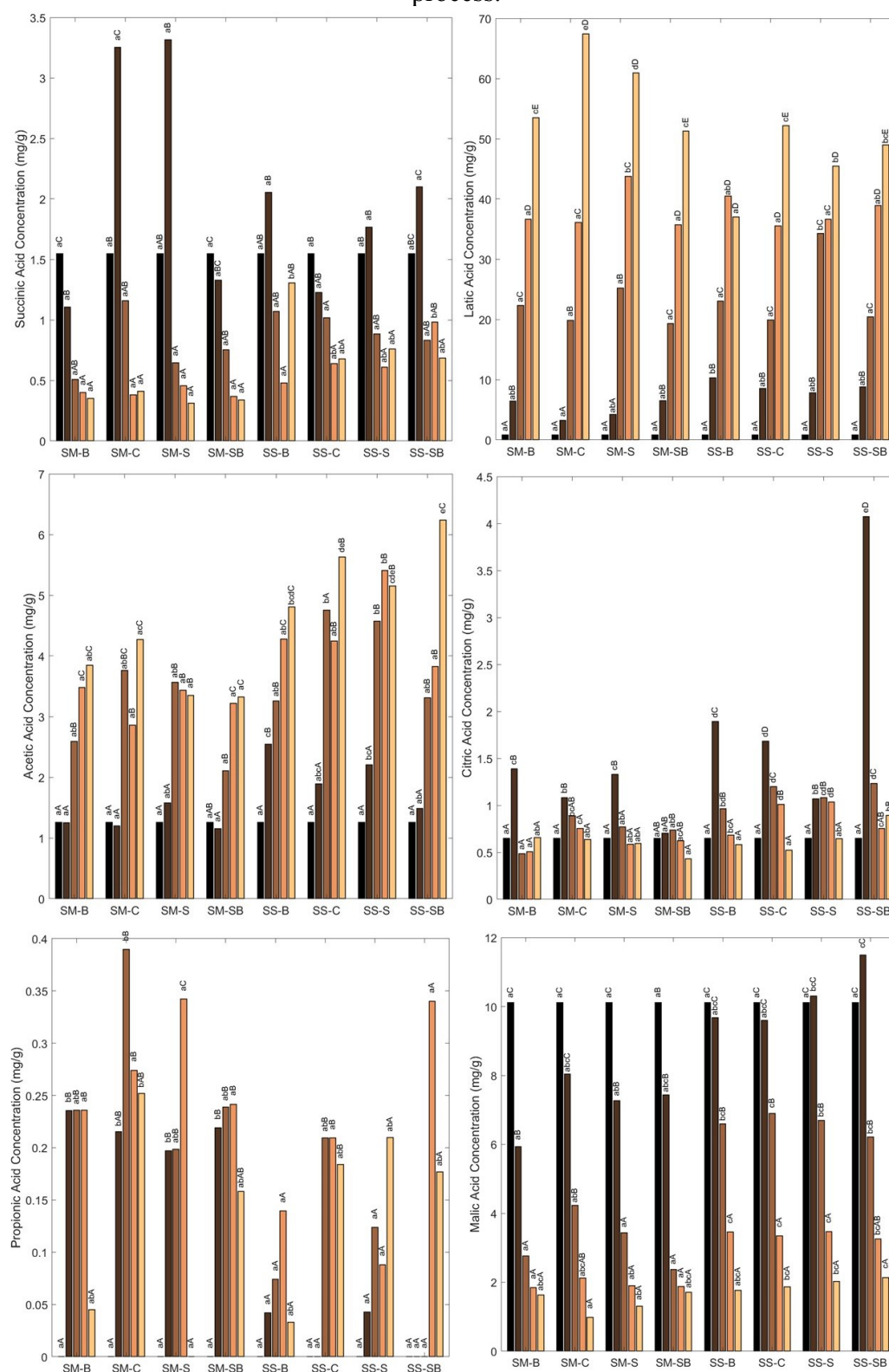
Figure 3 – Sugars concentration variation during the coffee fermentation process.



Compounds concentration along the time for each treatment: (Black) 0h, (Dark brown) 24h, (Brown) 48h, (Orange) 72h, and (Yellow) 96h.

Lactic acid was the main acid produced during the fermentative process for all treatments. An increase in the concentration of this acid was observed after 24h of fermentation, except for the SM-C and SM-S treatments. The production increased up to 96 h of fermentation in all trials except for the SS-B treatment. The highest final concentration was 67.44 mg/g in SM-C and the lowest 37.00 mg/g in SS-B. The spontaneous fermentation showed the highest concentration of lactic acid was obtained in 96 h for both the solid-state and submerged processes compared to the use of inoculum (52.19 and 67.44 mg/g to SS-C and SM-C, respectively). Citric acid concentration increased significantly in 24 h for all treatments except in SM-SB. The highest value was found in SS-SB (4.07 mg/g) in 24h. After 24h the citric acid concentration decreased with fermentation time in all processes. Only the final concentration (96 h) of the SS-SB and SM-C processes was higher than that of the coffee fruit at 0h. Acetic acid concentration increased with fermentation time for all treatments. Malic acid decreased significantly in the first 24 h only for SM-S and SM-B. The lowest final concentration at 96 h was identified for SM-C (0.98 mg/g). The acetic acid increased significantly in 24 h only for SS-B. The highest final concentration was 6.23 mg/g in SS-SB. The highest concentrations of acetic acid were found in the solid-state process. The results of the processes using the natural microbiota were the ones that presented one of the highest concentrations of acetic acid for both the submerged and solid-state. The highest concentration of propionic acid was identified in SM-C at 48 h (0.39 mg/g). Solid-state fermentation favored the production of this acid. The succinic acid variation was not influenced by type of treatment. It was observed a decrease in concentration in all treatments, reaching statistically equal final values, except for SS-B fermentation. Butyric acid was not identified in any treatment (Figure 4 –Variation of organic acids concentration during the coffee fermentation process. Figure 4).

Figure 4 –Variation of organic acids concentration during the coffee fermentation process.

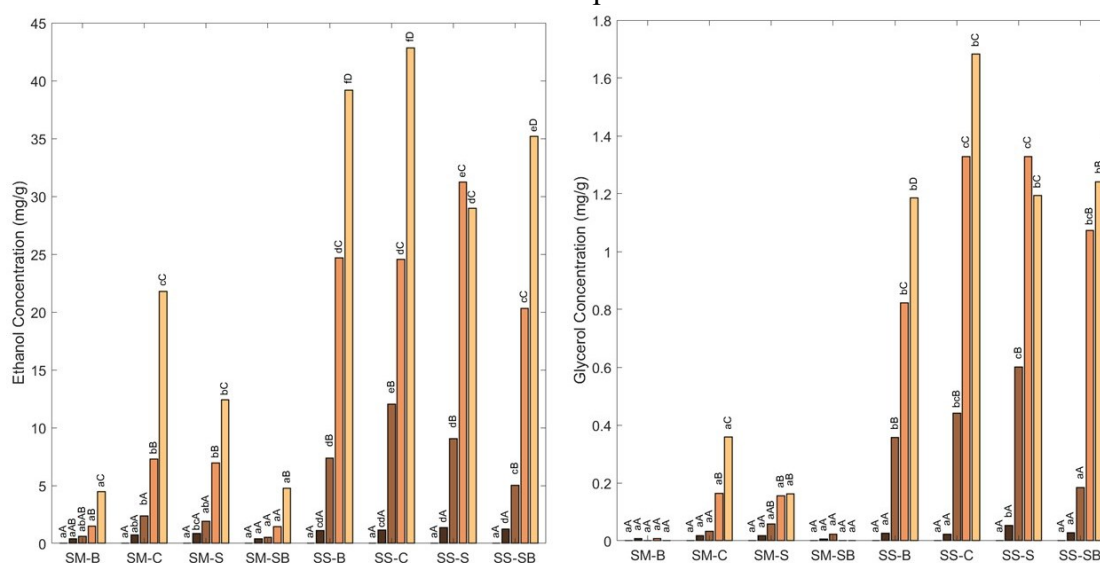


Compounds concentration along the time for each treatment: (Black) 0h, (Dark brown) 24h, (Brown) 48h, (Orange) 72h, and (Yellow) 96h.

From the beginning of fermentation until 24h of fermentation, ethanol and glycerol remained constants in all treatments. After 48h of fermentation, the highest

ethanol concentrations were identified for the treatments performed in solid-state processing. Solid state fermentations presented higher ethanol concentrations than the submerged processes. The highest final concentrations were found for SS-C and SS-B (42.83 and 39.19 mg/g). In the submerged processes, significant increases in ethanol concentrations happened only after 72h of fermentation. The highest final concentration was found for SM-C 21.80 mg/g, while the lowest were found for treatments inoculated with bacteria (4.46 mg/g for SM-B and 4.74 mg/g for SM-SB). In both processes, the final ethanol concentrations were higher for the fermentations without inoculation (SS-C e SM-C). From the beginning to the end of fermentation, there were no significant increases in glycerol concentrations in submerged fermentations inoculated with bacteria (SM-B and SM-SB treatments). After 72h, the highest glycerol concentrations were identified for the treatments performed in solid-state processing (Figure 5).

Figure 5 - Variation of ethanol and glycerol concentrations during the coffee fermentation process.

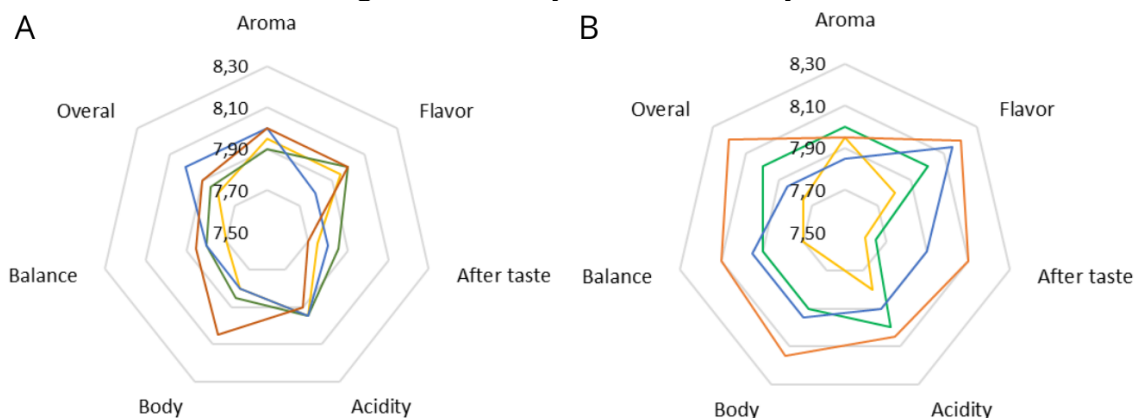


Compounds concentration along the time for each treatment: (Black) 0h, (Dark brown) 24h, (Brown) 48h, (Orange) 72h, and (Yellow) 96h.

### 3.2. Sensory analysis of coffee

Coffees produced by all treatments in this study were classified as special according to SCA protocol (>84 points). The scores variation by attributes is represented in Figure 6. The SM-B and SM-SB treatments had the highest final scores of 86.55 and 86.75, respectively.

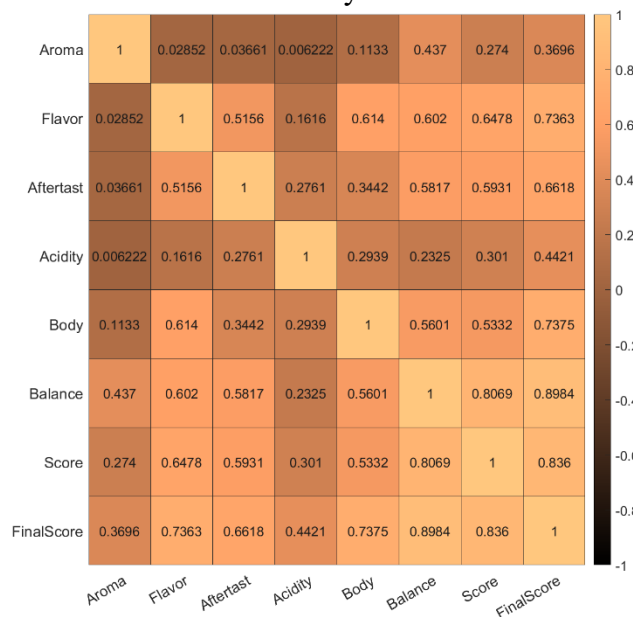
Figure 6 - Sensory attribute intensity.



All treatments scored 10 for uniformity, sweetness, and clean cup. Treatments: (A) Solid-state process; (B) Submerged process. (Yellow) Control; (Green) *S. cerevisiae* inoculation; (Blue) *Bacillus* inoculation; (Orange) *S. cerevisiae* + *Bacillus* coinoculation.

Figure 7 represents the correlation between sensory attributes. A positive strong correlation was obtained to score/balance; final score/flavor; final score/body; final score/balance, and final score/score. The score is the overall grade that the evaluator gives to the coffee, while the final score is calculated from the sum of all 10 attributes evaluated.

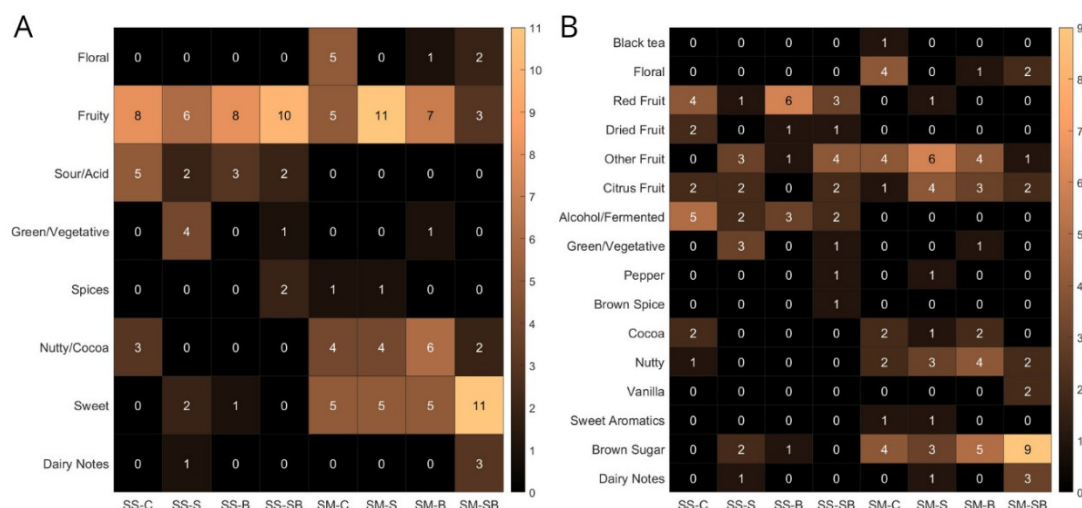
Figure 7 - Correlation between sensory attribute and coffee beverage score



Specific characteristics were identified for each process applied (Figure 8). According to the SCA defined flavor wheel, regarding primary sensory characteristics, floral perceptions were identified only in coffees submitted to submerged processes, while sour and acidic notes were attributed only in coffees obtained with the solid-state fermentation. Also, nutty, cocoa and sweet were more evident in the submerged process. Regarding secondary sensory characteristics, red and dried fruits were perceived in coffees from solid-state fermentations, while citric fruit was more evident in coffees from submerged processes. Nutty was perceived mainly in submerged process. Dairy notes were identified in single inoculation of *S. cerevisiae* coffees and gained evidence in the

submerged process when co-inoculated with the bacteria. In coffees submitted to solid-state process, only SS-S and SS-B showed caramel descriptions corresponding to sweetness. On the other hand, all submerged process showed sweetness attributes like honey and caramelized sugars (Figure 8).

Figure 8 – Sensory descriptions for each treatment according SCA protocol.

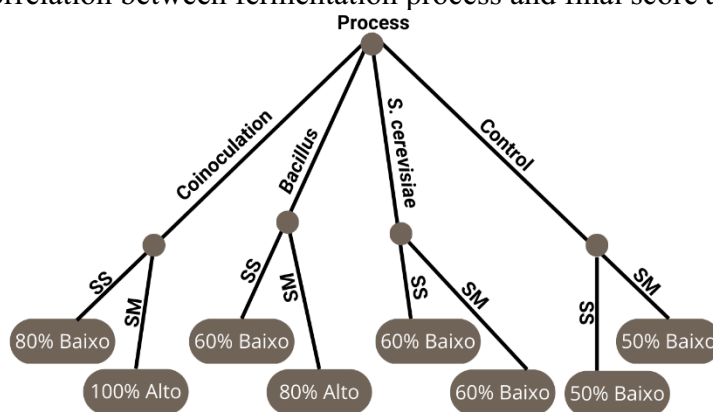


Attribute occurrence during sensory analyses. (A) Primary and (B) Secondary sensory descriptions.

### 3.3. Correlation between fermentation process and final score SCA

Figure 9 shows the possibility of achieving a final score greater than 86 points for all treatments. For the environmental conditions evaluated, the application of *S. cerevisiae* and *Bacillus* coinoculation provided more consistent results with 100% in the submerged process and 80% in the solid-state process.

Figure 9 – Correlation between fermentation process and final score above 86 points.



SS: solid-state process and SM: submerged process.

#### 4. Discussion

This study reports for the first time the use of a *B. amyloliquefaciens* as starter culture to conduct SIAF coffee fermentation during the solid-state and submerged process. *S. cerevisiae* have been used as inoculum during SIAF coffee fermentation to improve sensory quality (Bressani, Batista, et al., 2021; Bressani, Martinez, et al., 2021; Cassimiro et al., 2022; da Mota et al., 2020; Martinez et al., 2021). Mesophilic bacteria have already been used as coffee inoculants. (B et al., 2019) used *Saccharomyces cerevisiae*, *Lactobacillus plantarum*, and *Bacillus sphaericus* during coffee fermentation and found significant difference in demucilization of coffee beans with noticeable improvement in alcohol and pectinase production. During a fermentative process *Bacillus* are able to use the proteins, carbohydrate, and lipid to convert in amino acids, organic acids, nucleosides, alcohols, and other organic classes (Dahake & Dhoble, 2022).

The presence of yeast reduced the lipid content, indicating greater lipid assimilation when compared to the control, and bacteria alone. It was observed that the highest protein concentrations were achieved in the fermentative processes using *Bacillus* as inoculum. *B. amyloliquefaciens* was reported as being able to produce thermostable protease, amylase and lipase (Harwood and Cranenburgh, 2008), and lipopeptides (Alvarez et al., 2012; Ongena and Jacques, 2008; Zhu et al., 2012). The variation in protein content during the fermentation process can alter sensory characteristics of the coffee beverage. Green coffee beans have potential nitrogenous flavor precursors and are represented by free amino acids, peptides, and proteins. These compounds are important aroma precursors for coffee flavor formation during roasting. Proteins and sucrose during roasting are key flavor precursors that participate in Maillard reactions (Lee et al., 2015; Montavon et al., 2003). The melanoidins formation in Maillard reactions influences highly coffee beverage flavor (Gigl et al., 2022). Coffee lipids contribute to the texture and feel of the beverage in the mouth (Cardoso et al., 2021).

The sucrose, glucose, and fructose contents decreased from start to end of the fermentation in all treatments analyzed. For the specialty coffees production, the consumption of sugars during the fermentation stage is important because it prevents the growth of undesirable microorganisms during the drying stage, mainly filamentous fungi (L. L. Pereira et al., 2021). The lower residual value of sucrose can be related to the strong action of  $\beta$ -fructofuranosidase enzymes, which are able to perform the hydrolysis of sucrose into glucose and fructose (Ni et al., 2022). Whole coffee fruits have higher initial sugar values because, besides the mucilage attached to the kernel, they still have the pulp that is also rich in carbohydrates (Santos et al., 2021). The extended fermentation time was not sufficient to reduce the fructose present in the coffee fruits by much more than half. High residual fructose values are often found after coffee fermentation (Cassimiro et al., 2022; da Silva Vale et al., 2023). Probably the endogenous microbiota and starter culture used do not possess fructophilic phenotype (Junqueira et al., 2022).

Due to the characteristic of the SIAF process, during the first hours of the fermentation process, there is a certain amount of oxygen available. This can be related to the increase in citric acid in the first 24h of processing. The synthesis of citric acid results from the first stage of the Krebs cycle, in which the pyruvate resulting from glucose degradation will undergo oxidative decarboxylation and carboxylation, forming Acetyl-CoA oxaloacetic acid. These intermediate products suffer the action of the enzyme citrate synthase and some cofactors that trigger the reaction, causing the accumulation of

citric acid and initiating the tricarboxylic cycle (Angumeenal & Venkappayya, 2013; Vandenberghe et al., 2018).

Lactic acid is produced mainly by lactic acid bacteria. However, it can be produced by *Bacillus* and yeasts capable of producing lactate dehydrogenases. Pleissner et al. (2016) showed lactic acid production from coffee pulp hydrolysate using *Bacillus coagulans*. Lactic acid has been reported to be one of the main organic acids produced in submerged coffee fermentation process (Carvalho Neto et al., 2017; Elhalis, Cox, Frank, et al., 2020). This was also observed in our study. The highest final lactic acid value was identified in the treatment without inoculant, probably due to the strong presence of endogenous lactic acid bacteria. Unlike the results from da Silva Vale et al., 2023 which identified a low prevalence of lactic acid bacteria and yeast, in the SIAF method conducted in the Paraná region. The higher final concentrations of lactic acid in the submerged process may be related to the higher sensory perception of nutty, cocoa, and sweet in the coffee beverage.

Acetic acid was the second most prevalent organic acid identified and the highest values were observed in solid-state processes. The bacteria *Enterobacter* sp. and *Lactobacillus* sp. are dominant in SIAF method and can be responsible for production of acetic acid by heterolactic pathway (Valéria Ulhano Braga et al., 2023). Low acetic acid levels are desirable in coffee beverages and can be related to sweet taste. However, the undesirable vinegary taste can be found in beverages from over fermentation of coffee (Lopez et al., 1990). In our study higher concentrations of acetic acid were found in solid-state process and can be related to sensory perception of sour and acid flavor.

*S. cerevisiae* is widely used in fermentation processes for ethanol production and in food for wine, beer, and bread. It is now also being widely investigated for use in coffee fermentation (Bressani, Martinez, et al., 2021; da Mota et al., 2020; Martinez et al., 2021; L. L. Pereira et al., 2020). Nevertheless, in our study the *S. cerevisiae* inoculum did not play a significant role in increasing ethanol production. Carvalho Neto et al. (2017) found *Saccharomyces* sp. as important yeast found during the spontaneous fermentative process in Cerrado Mineiro region.

The use of bioreactors during the fermentation process allows to conduct the process in two ways: submerged fermentation, which uses substrates solubilized or in suspension in a liquid phase, and solid-state fermentation, which uses solid substrates (Prado Barragán et al., 2016). The fermentation of coffee is a particular case, because as in other food products there is no product recovery, the metabolites become part of the sensorial matrix of the food. However, for coffee, during submerged fermentation we must consider that the water used will be discarded after the process, and with it, due to polarity iterations, several organic compounds produced may not remain in the fruits/coffee beans (C. R. Martins et al., 2013). In our study, the presence of water mainly altered the amounts of ethanol and glycerol present in the fruit after fermentation. Ethanol, which is an infinitely water-soluble molecule, was identified in the fruits that underwent fermentation without water in final values up to 8 times higher than when processed with water. Similarly, glycerol which is a polyol has high water solubility and showed up to 7 times higher values in the solid-state process. In this study, we found that higher concentrations of ethanol may be related to alcoholic perceptions in the coffee beverage.

According to the results obtained the type of process, solid-state or submerged, can influence the composition of the fruit at the end of the fermentation performed in self-induced anaerobiosis. For this reason, the addition of water should be an important

evaluation parameter to be considered for coffee fermentation. Also, using water during fermentation can facilitate the homogenization of the coffee. However, the solid-state fermentation can reduce water consumption by 89% in the coffee post-harvest phase (L. L. Pereira et al., 2021).

## 5. Conclusion

The inoculation of yeast and bacillus separately or together in the fermentation process impacts the quality of coffees. *Bacillus amyloliquefaciens* showed promising result improving beverage quality and process control. The coinoculated coffees obtained the highest sensory scores in submerged process (score > 86). Due to the results found, the type of process, submerged and solid-state, showed more influence in chemical and sensory analyses than type of inoculation. Submerged process showed major concentration of lactic acid, while solid-state process promotes major concentrations of ethanol, glycerol, and acetic acid. Coffees from the submerged process showed Nutty/Cocoa and Sweet sensory descriptions, while solid-state process produced coffees with Sour/Acidic attributes. Besides, further studies should be carried out on coffees of different varieties and altitudes.

## 6. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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