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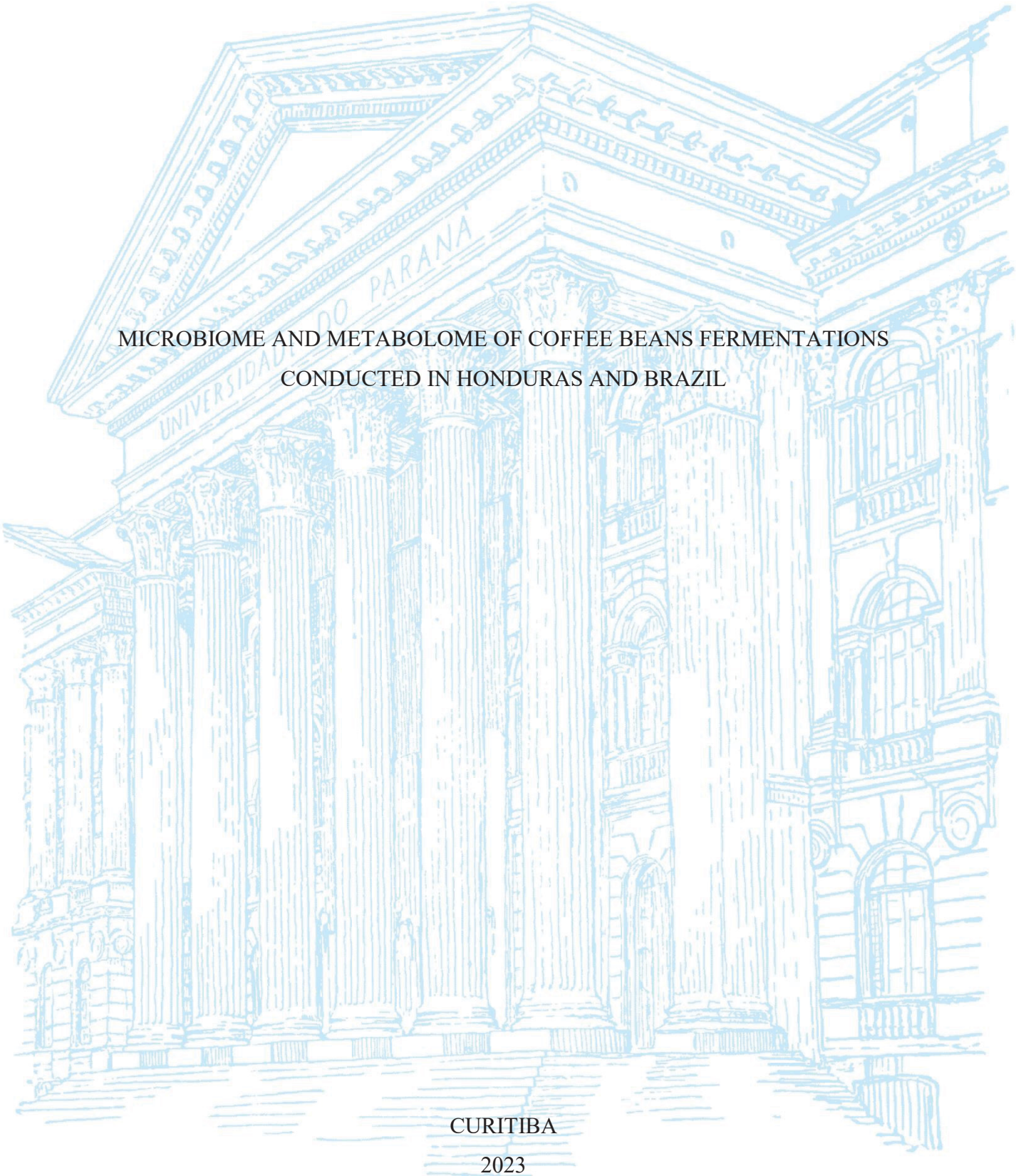
ALEXANDER DA SILVA VALE



MICROBIOME AND METABOLOME OF COFFEE BEANS FERMENTATIONS CONDUCTED IN HONDURAS AND BRAZIL

CURITIBA

2023

ALEXANDER DA SILVA VALE

MICROBIOME AND METABOLOME OF COFFEE BEANS FERMENTATIONS CONDUCTED IN HONDURAS AND BRAZIL

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

Orientador: Prof. Dr. Gilberto Vinícius de Melo Pereira

Coorientador: Prof. Dr. Carlos Ricardo Soccol

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ALEXANDER DA SILVA VALE** intitulada: **Microbiome and metabolome of coffee beans fermentations conducted in Honduras and Brazil**, sob orientação do Prof. Dr. GILBERTO VINICIUS DE MELO PEREIRA, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 27 de Janeiro de 2023.

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GILBERTO VINICIUS DE MELO PEREIRA
Presidente da Banca Examinadora

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ADRIANE BIANCHI PEDRONI MEDEIROS
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

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JULIO CESAR DE CARVALHO
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

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MARIA GIOVANA BINDER PAGNONCELLI
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“É impossível para um homem aprender aquilo que ele acha que já sabe”
Epictetus

RESUMO

Vários estudos têm reportado que o processamento pós-colheita, em particular a via úmida, apresenta um grande impacto sobre a qualidade da bebida final. No processamento por via úmida, os frutos de café são despolpados e transferidos para tanques abertos contendo grandes volumes de água. Este ambiente microaerófilo associado com os açucares e aminoácidos provenientes da polpa de café favorecem o crescimento de microrganismos com metabolismo fermentativo. No entanto, este processo ainda é realizado de maneira rudimentar, uma vez que as fermentações são realizadas de forma espontânea e sem o controle de parâmetros fermentativos, como agitação e temperatura. Portanto, no primeiro capítulo deste trabalho foi construída uma revisão sobre a perspectivas do uso de biologia sintética para o desenvolvimento de novas culturas iniciadoras. O uso de algumas ferramentas (construção de promotores sintéticos e CRISPR-cas) são propostas como possíveis estratégias para a criação de cepas com os fenótipos desejados. Embora estas abordagens apresentem uma série de vantagem em relação aos métodos tradicionais de seleção microbiana, nenhum estudo focado em café foi reportado até o momento, mostrando que ainda há muitos desafios e um novo campo para exploração industrial. O segundo capítulo avaliou a hipótese não confirmada que há uma conexão entre o microbioma da fazenda e os processos fermentativos tradicionais. Foram identificados mais de 300 grupos microbianos a partir do solo, folhas, frutos, grãos despolpados, água utilizada para o despolpamento e fermentação. Em geral, os principais gêneros identificados na fermentação (*Leuconostoc, Gluconobacter, Pichia, Hanseniaspora* e *Candida*) são oriundos dos frutos despolpados e foram responsáveis pela produção de ácidos orgânicos e compostos voláteis. Além disso, este foi o primeiro trabalho que reportou *Gluconobacter* como grupo dominante durante a fermentação dos grãos de café. Por outro lado, deve-se evitar que os frutos "passa", folhas e solo tenham contato com tanque de fermentação, pois microrganismos não desejados (Enterobactérias, *Colletotrichum)* foram identificados com alta frequência. O terceiro capítulo avaliou a estrutura da comunidade microbiana e as alterações químicas em diferentes camadas de um tanque de fermentação estático de grãos de café (denominada fermentação anaeróbica autoinduzida) em diferentes momentos (24, 48, e 72h). A composição taxonômica microbiana compreendia uma alta prevalência de Enterobacteriaceae e Nectriaceae e baixa de bactérias lácticas e leveduras, o que difere muito do processo tradicional realizado em tanques abertos. Não foi observada uma grande variação na diversidade bacteriana e fúngica entre as camadas inferior, média e superior do tanque de fermentação. No entanto, o metabolismo destes microrganismos variou significativamente, mostrando um maior consumo de açúcar da polpa e produção de metabolitos nas camadas inferior e média em comparação com a parte superior do tanque de fermentação. Processos estendidos (48 e 72h) permitiram uma maior produção de metabolitos-chave durante a fermentação (por exemplo, 3-octanol, acetato de etilo, e acetato de amila), acumulação em grãos de café torrados (ácido acético, pirazina, metilo, 2-propanona, 1-hidroxi), e diversos perfis sensoriais da bebida de café em comparação com 24 h de processo de fermentação. Este estudo mostrou a importância da agitação do tanque de fermentação e a diversificação do tempo de fermentação para a entrega de uma bebida de café de alta qualidade.

Palavras-chave: fermentação de café, biologia sintética, metagenética, análise sensorial.

ABSTRACT

Several studies have reported that post-harvest processing, particularly wet processing, has a major impact on the quality of the final beverage. In wet processing, coffee fruits are pulped and transferred to open tanks containing large volumes of water. This micro-aerophilic environment associated with the sugars and amino acids coming from the coffee pulp favors the growth of microorganisms with fermentative metabolism. However, this process is still performed in a rudimentary way, since fermentations are carried out spontaneously and without control of fermentative parameters, such as agitation and temperature. Therefore, the first chapter of this work built a review of the perspectives on the use of synthetic biology for the development of new starter cultures. The use of some tools (construction of synthetic promoters and CRISPR-cas) is proposed as a possible strategy to create strains with the desired phenotypes. Although these approaches present several advantages over traditional microbial selection methods, no study focused on coffee has been reported so far, showing that there are still many challenges and a new field for industrial exploration. The second chapter of this work assessed the unconfirmed hypothesis that there is a connection between the farm microbiome and traditional fermentative processes. More than 300 microbial groups were identified from soil, leaves, fruits, pulped beans, and water used for pulping and fermentation. In general, the main genera identified in the fermentation (Leuconostoc, Gluconobacter, Pichia, Hanseniaspora, and Candida) originated from the pulped fruits and were responsible for the production of organic acids and volatile compounds. Moreover, this was the first work that reported Gluconobacter as a dominant group during the fermentation of coffee beans. On the other hand, one should avoid over-ripe fruits, leaves, and soil having contact with the fermentation tank, as unwanted microorganisms (Enterobacteriaceae, Colletotrichum) were identified with high frequency. The third chapter evaluated the microbial community structure and chemical changes in different layers of a static coffee beans fermentation tank (named self-induced anaerobic fermentation - SIAF) was conducted at different times (24, 48, and 72h). The microbial taxonomic composition comprised a high prevalence of Enterobacteriaceae and Nectriaceae and low of lactic acid bacteria and yeast, which greatly differs from traditional process performed in open tanks. No major variation in bacterial and fungal diversity was observed between the bottom, middle, and top layers of the fermentation tank. On the other hand, the metabolism of these microorganisms varied significantly, showing a higher consumption of pulp sugar and production of metabolites in the bottom and middle layers compared to the top part of the fermentation tank. Extended processes (48 and 72h) allowed a higher production of key-metabolites during fermentation (e.g., 3-octanol, ethyl acetate, and amyl acetate), accumulation in roasted coffee beans (acetic acid, pyrazine, methyl, 2-propanone, 1-hydroxy), and diverse sensory profiles of coffee beverage compared to 24 h of fermentation process. This study highlighted the importance of the revolving of coffee fermentation tank and the diversification of fermentation time for delivering high-quality coffee beverage.

Keywords: coffee fermentation, synthetic biology, metagenetic, sensory analysis.

SUMÁRIO

Introduction

Due to its pleasant taste and unique sensory attributes, coffee has been ranked as one of the most consumed beverages on the planet, as well as being the second most traded commodity in the world in financial terms (HAMEED *et al.*, 2018; LEE *et al.*, 2015b). However, it is known that the prices of this beverage are significantly correlated with the chemical composition of the coffee beans, as these compounds modulate the aroma of the coffee, a fundamental characteristic that defines the quality of the final product (OTERO *et al.*, 2018). In general, these characteristics are highly influenced by the growing environment (geographical face, altitude of the site, arrangement of plants in the area), genetics of the coffee tree, harvesting method, and post-harvest processing used, in addition to the roasting profile and extraction of the beverage(FERREIRA *et al.*, 2022; FILETE *et al.*, 2022; PEREIRA *et al.*, 2019), demonstrating the complexity in producing cups of coffee with high-quality standards.

Among the variables highlighted, post-harvest processing has been associated as one of the main factors linked to beverage quality (MAGALHÃES JÚNIOR *et al.*, 2021), and although there are peculiarities in the processing method of each producing country, they can be classified into three distinct methods, called dry, semi-dry and wet processing (HAMEED *et al.*, 2018). In dry processing, coffee fruits are harvested and sun-dried for approximately 30 days until they reach 12% humidity. On the other hand, wet processing is relatively more complex, as the fruits are mechanically pulped and placed in open tanks containing water for natural, spontaneous fermentation to occur for about 24 hours. This process was developed to remove the layer of mucilage adhered to the coffee beans, which facilitates and reduces drying time. Finally, semi-dry processing features steps of the dry and wet methods, where coffee fruits are mechanically pulped and then subjected to sun drying (HAMEED *et al.*, 2018; PEREIRA *et al.*, 2019).

Recent studies using high-throughput sequencing (HTS) have shown that more than 100 prokaryotic and eukaryotic genera are involved in the fermentation of coffee beans produced in South and Central America. However, these fermentations are generally dominated by lactic acid bacteria (e.g., *Leuconostoc, Lactobacillus, and Pediococcus*), acetic acid bacteria (e.g., *Gluconobacter* and *Acetobacter*), yeasts (e.g., *Pichia, Candida,* and *Saccharomyces*) and filamentous fungi (e.g., *Fusarium*) (CRUZ-O'BYRNE; PIRANEQUE-GAMBASICA; AGUIRRE-FORERO, 2021; JUNQUEIRA *et al.*, 2019; PREGOLINI *et al.*, 2021; VALE *et al.*, 2021a; ZHANG *et al.*, 2022). In addition, it is known that these microorganisms can contribute to the sensory

characteristics of the beverage due to metabolites produced during fermentation (PEREIRA *et al.*, 2019). Therefore, understanding the microbial diversity and dynamics involved in coffee fermentation is essential to propose new processing methods that have consistency and reproducibility.

1. Chapter 1: Perspective on the use of synthetic biology in rudimentary food fermentations

This chapter was published as a scientific article in 2022 in the journal Systems Microbiology and Biomanufacturing: https://doi.org/10.1007/s43393-022-00131-6.

1.1 Abstract

Rudimentary food fermentation can be defined as a spontaneous process of conversion of food components through enzymatic action. A great variety of fermented foods are produced using spontaneous approaches; however, cocoa and coffee represent the most important agricultural commodities on international markets. As a manner to increase the efficiency of these processes, starter cultures have been developed and applied under field conditions. The selection process begins with the recovery of microbial strains from spontaneous fermentation through phenotypic and metabolic traits. Next, mutation-based breeding is used to develop and improve well-adapted starter cultures. With advances in synthetic biology, especially in the last decade, the development of robust cellular fabrications with high fermentative capacity has become easier—largely due to the development of genomic approaches, such as next-generation sequencing techniques, CRISPR-Cas system and bioinformatics tools. This review brings prospects on the use of synthetic biology to design new robust strains for use in cocoa and coffee fermentations, but which can be extended to other rudimentary foods. In addition, metabolic traits and target genes (e.g., *UvrA*, *RecA*, *GPD1*, and *GPP2*) are proposed as a starting point for the improvement of cocoa and coffee starters. Finally, the regulatory and safety requirements for these food crops are addressed. This review aims to stimulate research on the process of fermentation and the associated synthetic biology tools to produce fermented food efficiently and sustainably.

1.2 Introduction

The production of fermented foods and beverages dates back as far as 10,000 B.C. in the Neolithic period (LIU *et al.*, 2019). This process certainly began as a favorable outcome of recurrent microbial contamination of plant and animal materials, which resulted in last-longing fermented products. The replication of environmental conditions of these accidental events using pottery or handcrafted vessels allowed humans to ensure a minimum safety standard of the fermentation process, which relied on either *i)* the spontaneous growth of desirable microbiota present on the raw material; or *ii)* the addition

of portions from previous, well-succeeded fermented products to a fresh substrate, known as backslopping (TAMANG *et al.*, 2020).

The adoption of fermentation by hunter-gatherer societies had the main objective of nutritional preservation (CRAIG, 2021; SHODA, 2021). However, other characteristics, such as increased flavor, texture, and biological functionalities, stimulated the use of fermentation in several societies. Remarkably, the usage of the rudimentary fermentative process occurred independently, but simultaneously, on every continent based on cultural aspects and accessibility to raw substrates (TAMANG *et al.*, 2020). For instance, in the Middle East and Europe, natural dairy products (e.g., kefir, *koumiss*, and *airag*) are often consumed; while in East Asia and Oceania—regions with eating habits based on cereals, vegetables, and fish—fermented products, such as *kimchi*, *natto*, and *sikhae*, continue to be part of the daily diet (ANAL, 2019; PEREIRA *et al.*, 2022a; SONI; DEY, 2014). Although rudimentary fermented products play a pivotal role in regional nutrition and economy, chocolate and coffee beverage are among the few commodities that achieved worldwide acceptance and economic relevance. Cocoa and coffee production is mostly performed in developing countries (*e.g.,* Brazil, Vietnam, Colombia, Indonesia, and Ethiopia) and natural fermentations are used to achieve the final commercialization of the products. Other spontaneously produced fermented foods include sauerkraut, kimchi, and pickles, but are usually produced in small-scale settings and developing countries.

The microorganisms present in cocoa and coffee fermentations are introduced by processing water, fruit husks, leaves, soil, and human contact (PACHECO-MONTEALEGRE et al., 2020; PREGOLINI et al., 2021; VALE et al., 2021). However, selected starter cultures are now being selected and applied to control these processes. The use of selected microorganisms in the food industry is quite common, especially in the production of wine, beer, cheese, bread, and yogurt. In practice, the main objective of using starter cultures is to enable the standardization of the fermentation process, increase safety parameters, and optimize processing time (PEREIRA et al., 2020; TAMANG, Jyoti P.; WATANABE; HOLZAPFEL, 2016). As the market for fermented food products continuously requires the implementation and diversification of available products, most industrial fermentations require process maximization and hardly all desirable characteristics will be found in a single wild strain.

Therefore, synthetic biology has emerged as an alternative for developing strains with desirable phenotypes and new functions. One of the major challenges in designing

these new microbial phenotypes is to understand the stress factors faced during the fermentation process (CARDINALE; ARKIN, 2012). In cocoa and coffee fermentations, for instance, several physicochemical changes occur, such as osmolarity, temperature, pH, and changes in microbial population density and diversity. These fluctuations affect the physiology of microorganisms in complex ways and their functions often undergo unpredictable interactions. The development of next-generation sequencing platforms has been an important ally of synthetic biology, as the large amount of DNA sequence generated enables the identification of biological parts (bioparts), which include promoters, genes, terminators, and ribosomal binding sites (CHEN *et al.*, 2018; KUNJAPUR; PFINGSTAG; THOMPSON, 2018).

In recent years, synthetic biology has been used to engineer microorganisms for applications in several areas, including vaccines, diagnostics, immunotherapy (TAN *et al.*, 2021), chemicals and biofuels (VAVITSAS *et al.*, 2021), and the food industry (LV *et al.*, 2021). However, only classical breeding methods have been used for the development of new starters for cocoa and coffee. Thus, in this review, we discuss how synthetic biology can be used to design new robust strains for use in cocoa and coffee fermentation.

1.3 Cocoa fermentation

Cocoa (*Theobroma cacao* L.) is a perennial tree cultivated in tropical and subtropical regions (between latitudes 20ºN and 20ºS) at elevations of up to 600 m, with rainfalls well-distributed throughout the year, and temperatures ranging from 24 to 28 ºC (PREDAN; LAZĂR; LUNGU, 2019). Cotê D'Ivoire is the major cocoa producer, responsible for 2,105 mi tons of the 2019/20 crop, followed by Ghana, Ecuador, and Cameroon (INTERNATIONAL COCOA ORGANIZATION (ICCO), 2021). On-farm cocoa processing begins with fruit opening process (manually using club, knife, or machete) (VÁSQUEZ *et al.*, 2019). Cocoa beans are then distributed in wooden boxes, baskets, or banana leaves (heap) for further fermentation (VIESSER *et al.*, 2021). The most traditional methodology used in West Africa is the allocation of cocoa beans into heaps coverage with banana leaves, while countries of South America traditionally use wooden boxes(AFOAKWA, 2010).

The microorganisms responsible for the cocoa beans fermentation are originated from the fruit itself, the surface of tools, human contact, or impurities from previous fermentations (FIGUEROA-HERNÁNDEZ *et al.*, 2019; PAPALEXANDRATOU *et al.*,

2019). Cocoa fermentation is performed during three distinct phases characterized by a temporal succession between yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) (DE VUYST; LEROY, 2020; DE VUYST; WECKX, 2016).

During the first phase (initial 24 h), pulp acidity (pH 3.0 ~ 4.0), allied with high concentrations of fermentable sugar and reduction of oxygen mass transfer, creates a prone environment for the development of yeasts (SCHWAN; WHEALS, 2004). Several species belonging to the genus *Candida* (*C. aaseri, C. butyri, C. diversa, C. ethanolica, C. friedrichii, C. glabrata, C. humilis, C. inconspícua, C. jaroonii, C. orthopsilosis, C. pseudotropicalis, C. stellimalicola, C. temnochilae, C. tropicalis* e *C. zemplinina*), *Hanseniaspora* (*H. guilliermondii, H. opuntiae, H. uvarum* e *H. vineae*), *Kluyveromyces* (*K. marxianus* e *K. lactis*), *Pichia* (*P. barkeri, P. fermentans, P. kluyveri, P. kudriavzevii, P. manshurica, P. membranifaciens, P. rarassimilans* e *P. sporocuriosa*) and *Saccharomyces* (*S. cerevisiae*) are commonly reported in cocoa fermentation (DANIEL *et al.*, 2009; HAMDOUCHE *et al.*, 2019; ILLEGHEMS *et al.*, 2012; JESPERSEN *et al.*, 2005; MOTA-GUTIERREZ *et al.*, 2018; NIELSEN *et al.*, 2007; PAPALEXANDRATOU *et al.*, 2019; PEREIRA; MAGALHÃES-GUEDES; SCHWAN, 2013; SERRA *et al.*, 2019; VIESSER *et al.*, 2021). The yeasts are mainly responsible for ethanol and CO_2 production, which is partially diffused into the cotyledons and results in a complex cascade reaction causing embryo death (HERNÁNDEZ-HERNÁNDEZ *et al.*, 2016). In addition to alcoholic fermentation, yeasts also play crucial roles in the production of volatile aroma precursors, such as aldehydes, ketones, esters, and higher alcohols, which significantly contribute to chocolate flavor formation (FIGUEROA-HERNÁNDEZ *et al.*, 2019)

The second phase (24 ~ 48 h), characterized by the presence of a microaerophilic environment, high concentrations of ethanol, and an abundance of citric acid and fructose, results in a dominance shift to LAB. Species belonging to the *Leuconostoc* (*Leu. mesenteroides, Leu. gasicomitatum,* and *Leu. pseudomesenteroides*), *Fructobacillus* (*F. pseudoficulneus* and *F. tropeaoli*), *Pediococcus* (*P. acidilactici* and *P. pentosaceus*), and *Lactobacillus* (*L. brevis, Lacticaseibacillus casei* [former *L. casei*], *L. pentosus, Lactiplantibacillus plantarum* [former *L. plantarum*], *Limosilactobacillus fermentum* [former *L. fermentum*], among others) are frequently reported in the literature (HAMDOUCHE *et al.*, 2015; ILLEGHEMS *et al.*, 2012; LEFEBER *et al.*, 2012; MOTA-GUTIERREZ *et al.*, 2018; NIELSEN *et al.*, 2007; SERRA *et al.*, 2019). During this step, LAB are metabolically capable of *i)* producing lactic acid from hexoses; *ii)* converting

citric acid into lactic or acetic acid, and/or flavor precursors, such as diacetyl, acetoin, and 2,3-butanediol; *iii)* and reduce fructose to mannitol (ADLER *et al.*, 2013; CAMU *et al.*, 2007; DE VUYST; WECKX, 2016).

With the depletion of fermentable sugars and accumulation of ethanol, acetic acid bacteria (AAB), such as *Acetobacter ascendens, A. ghanensis*, *A. indonesiensis*, *A. pasteurianus, Gluconobacter frateurii, G. oxydans*, *Komagataeibacter hansenii, K. oboediens*, become prevalent (VIESSER *et al.*, 2021). The conversion of ethanol into acetic acid is an exothermic reaction which increases the temperature of the fermentation mass to approximately 50 °C. This temperature rise is crucial to aid in the death of the embryo and allow microbial metabolites to diffuse into the cocoa seed. In parallel, alkaloids and other astringent compounds are leached out of the cocoa bean, allowing the development of the flavor and color for chocolate production (FIGUEROA-HERNÁNDEZ *et al.*, 2019; HO; ZHAO; FLEET, 2015).

1.4 Coffee bean fermentation

The coffee tree (*Coffea* L.) is a tropical woody shrub native to the Ethiopian highlands, which was then widespread across Central and South America during the 17th century (OESTREICH-JANZEN, 2013). Harvested coffee fruits must undergo several unitary processes to reduce fruit humidity from an initial value of 65% to 10~12% for storage and transportation (PEREIRA *et al.*, 2016) . Usually, the fruits are sun-dried with all their constituent parts (*e.g.,* husks, mucilage, pulp, parchment, silverskin, and beans), known as dry processing. In this process, the fruits are spread out in cement terraces and revolved during to ensure a homogeneous drying (PEREIRA *et al.*, 2019). Although cost-effective, this process is inefficient in regions with high rainfall (e.g., Colombia and Hawaii). Thus, microbiological degradation (coffee beans fermentation process) is an alternative method used to improve the drying process. This method, known as wet processing, consists of the removal of the outer layers of the fruit to expose the mucilaginous layer adhered to the beans. Consecutively, the fruits containing the adherent mucilaginous layer are submerged in cement tanks (approximately 4.5 m^3) and filled with water, in a 1:1 proportion, where they are subjected to a microbial fermentation for 24 - 48 h. Variations of this process have been recently proposed, consisting of changes in coffee:water proportion (ELHALIS; COX; ZHAO, 2020; VALE et al., 2021), creation of an anaerobic environment through the imprisonment of the microbial-derived CO_2 (PEREIRA *et al.*, 2022b) and extended fermentation periods (POTHAKOS *et al.*, 2020b).

After the interruption of the fermentative process, coffee beans are submitted to mechanical or sun-drying. The absence of the outer layers significantly reduces the time required for drying (3 – 5 days).

Unlike cocoa beans, the fermentation process is not strictly necessary to obtain a coffee beverage. However, microbial activity can improve the organoleptic characteristics of coffee, such as acidity, aroma, and flavor (LEE *et al.*, 2015). During the initial 12 h, the high availability of reducing sugars (*e.g.,* glucose and fructose) stimulate the fast development of LAB, which is found in populations above 10^4 CFU/ml (EVANGELISTA *et al.*, 2015). The high production of lactic acid by homo- and heterofermentative LAB results in a pH decrease, preventing the growth of spoilage microorganisms, assisting in the hydrolysis of pectin, and creating a favorable environment for the development of yeasts (AVALLONE *et al.*, 2002; DJOSSOU *et al.*, 2011). In addition, the diffusion of this organic acid promotes changes in the organoleptic perception of important coffee attributes, such as acidity and body, assigning lactic notes to the final beverage (CARVALHO NETO *et al.*, 2018; PEREIRA *et al.*, 2016). These microorganisms also have a versatile secondary metabolism and are associated with the production of a wide range of volatile compounds, including esters, higher alcohols, and ketones, through the catabolism of citric acid and amino acids present in coffee pulp (PEREIRA *et al.*, 2020b).

During the second phase (12 ~24 h), non-*Saccharomyces* species, such as *Pichia* (*P. fermentans, P. kluyverii, P. guilliermondii, P. anomala, P. acaciae, P. ciferii, P. caribbica*), *Kluyveromyces marxianus, Candida* (*C. glabrata, C. glucosophila, C. quercitrusa*), *Torulospora delbrueckii*, *Schizosaccharomyces* sp., *Debaromyces* sp., and *Hanseniaspora* (*H. uvarum, H. opuntiae*, and *H. vineae*) are frequently reported (ELHALIS; COX; ZHAO, 2020; POTHAKOS *et al.*, 2020a; VALE *et al.*, 2021b). Carbohydrate metabolism leads to the formation of ethanol, acetaldehyde, and acetic acid as primary metabolites. Although ethanol and acetic acid are associated with *off-flavors* in the coffee beverage ("fermented" and "burned taste", respectively), non-*Saccharomyces* yeasts found in coffee fermentation bear a low activity of the enzyme alcohol dehydrogenase, resulting in the accumulation of acetaldehyde (PEREIRA *et al.*, 2019). This metabolite, alongside with other higher alcohols synthesized via Ehrlich pathway (HAZELWOOD *et al.*, 2008) and esters produced through the condensation of free fatty acids and alcohols, are responsible for desirable sensory notes on the final beverage.

1.5 Perspective on the use of synthetic biology for genetic improvement of cocoa and coffee starter cultures

The first genetic improvement programs for microbial strains were created in the early 1950s and combined random mutagenesis with targeted selection protocols (BACHMANN *et al.*, 2015). Later, the development of molecular tools (*e.g.,* recombinant DNA technology) enabled researchers to perform point gene edits, which led to a shift in research focus to metabolic engineering (BACHMANN *et al.*, 2015). However, the application of a genetically modified organism (GMO) in the food industry was not well accepted by consumers, which eventually only encouraged the use of "classical" techniques—including evolutionary engineering, induced mutations, cytoduction, sexual hybridization, asexual hybridization, and artificial hybridization — to produce starter cultures with food-grade, desired phenotype and, most importantly, classified as non-GMO (BIZAJ *et al.*, 2012; STEENSELS *et al.*, 2014). For instance, Schwan et al. (SCHWAN; COOPER; WHEALS, 1997) used chemical methods to develop mutant yeasts with high pectinolytic activity and potentially reduce the fermentation time of cocoa beans. Leal et al. (LEAL *et al.*, 2008) used inbreeding between two strains of *Kluyveromyces marxianus* to generate a hybrid strain that also exhibited high pectinolytic activity. On the other hand, Meersman et al. (MEERSMAN *et al.*, 2015) hydrated a native (Y927) and industrial (Y115) *S. cerevisiae* parental to generate a thermotolerant strain with moderate pectinolytic activity. These strains were used in a series of hybridization assays to generate new parents that presented superior phenotypes to the parental strains.

In recent decades, the genomic revolution has enabled the rise and development of multiple areas. Due to the increased demand for microorganisms with novel properties, synthetic biology has become an important biotechnological tool in building strains with novel phenotypes and high fermentative capacity for industrial purposes (CHOI *et al.*, 2019; LIU *et al.*, 2017a; MARKHAM; ALPER, 2018). Surprisingly, synthetic biology and/or genetic engineering approaches have not yet been used to develop starter cultures for cocoa and coffee. Thus, we will report models from other microbial sources as a potential strategy for improving starter cultures for these two important commodities.

In synthetic biology, the more recent approaches involve a number of mechanisms and strategies capable of suitably reprogramming the cellular machinery towards a better outcome for industrial purposes. Synthetic biology workflow is based on the design-build-test-learn cycle (OPGENORTH *et al.*, 2019). It allows the creation or improvement of new biological circuits in living cells. The flux of information generated by

accomplishing the design-build-test-learn cycle can guarantee the success of a given improvement platform. By engineering the already characterized microbial cultures, or even creating new microbial cell factories, synthetic biology tools comprise several techniques and biological parts studied in molecular biology, fundamentals of engineering, and computational approaches. In demanding industrial times, where food supply needs to match the populational growth, synthetic biology is suitably applied to engineer starter cultures for different fermentation processes.

Many strategies based on synthetic biology are now available for cell engineering. However, adopting a plan to attend to industrial demands requires precise and meticulous design, as each process results in different final products. In the face of this, synthetic biology approaches now play a pivotal role in the ascension of methodologies able to generate high-added-value products at an industrial scale, mainly those comprising organoleptic properties of biomolecules. We revise here some of the most promising approaches provided by synthetic biology that can be adapted for cocoa and coffee fermentations.

1.6 Promoter engineering

The expression and overexpression of genes or metabolic pathways are suitable for increasing product availability. In this pursuit, promoter engineering has wide application in the scientific community because of its capabilities of regulating expression either constitutively or inducingly. Due to their importance in synthetic biology, they are one of the most studied biological parts for engineering living cells. Promoters are sequences of DNA located upstream of the coding region of a given gene in which the transcription machinery is anchored by the recognition of transcription factors binding-regulatory elements. As it is already known, the interaction between transcription factors and promoters is a pivotal point to start and drive transcription in cells, as it could result in gene expression activation or repression. Because of this, synthetic biology has focused its efforts on engineering and creating synthetic promoters to modulate transcriptional signals in cells.

The promoter study is suitable for engineering cells because it can lead to the expression or over-expression of relevant biomolecules in living organisms. The expression of important enzymes required for vegetal layers' deconstruction and the subsequent fermentation of coffee and cocoa could be achieved by applying transcriptional regulation exerted by promoters designed by synthetic biology

approaches. Furthermore, in this search, boosting the expression of biomolecules related to the production of fine chemicals can generate high value-added products, applying promoter regulation in a rationally designed biological system.

In this sense, constitutive promoters, previously identified through RNA sequencing analysis, were characterized by KONG et al., (2019) in *Streptococcus thermophilus*, *Lactobacillus casei*, and *Escherichia coli* (KONG *et al.*, 2019). As an assessment test, some of these promoters were used to express superoxide dismutase. The results showed an increase in the enzyme activity for all bacteria tested. In addition, a system of overexpression of the exopolysaccharide biosynthesis proteins, *eps*A, and *eps*E, were constructed based on a dual-expression vector using two strong constitutive promoters (KONG *et al.*, 2019). This is a remarkable strategy to engineer LAB for industrial purposes, especially because the use of constitutive promoters does not rely on the presence of inducer signals capable of modulating transcription activity.

This same strategy can be applied to engineer yeasts and LAB from cocoa and coffee fermentations targeting pectinolytic enzyme activity. It can reduce fermentation time and aid in the formation of metabolites by catalyzing the polysaccharide complexes present in cocoa and coffee pulp. To date, only conventional methods of chemical agent mutation and hybridization have been used to increase the production of pectinolytic enzymes from yeasts isolated from cocoa fermentation (LEAL *et al.*, 2008; MEERSMAN *et al.*, 2015; SCHWAN; COOPER; WHEALS, 1997). Although constitutive promoters are a reliable alternative for protein overexpression, their use could generate protein accumulation inside the cells resulting in toxicity to the starter culture itself (SON; JEONG, 2020). To overcome this bottleneck, a precise expression system design may be necessary to guarantee a low toxicity rate for cocoa and coffee starters.

Promoter architecture is also a fundament that needs to be considered when transcriptional modulation is desired. It means that configuration, disposition, and the number of binding sites for transcription factor interactions in promoter sequences may influence transcriptional responses in each system. In this sense, Monteiro et al. (MONTEIRO; ARRUDA; SILVA-ROCHA, 2018) showed that the position distribution of *cis*-regulatory elements for *E. coli*'s transcription factors CRP and IHF interaction could influence promoter activity and even create emergent properties in these promoters (MONTEIRO; ARRUDA; SILVA-ROCHA, 2018). Similarly, the arrangement and distances of binding sites interaction for the transcriptional factor XYR1 were also

relevant for cellobiohydrolase I (CBHI) promoter strength in the filamentous fungus *Trichoderma reesei* (KIESENHOFER; MACH; MACH-AIGNER, 2018).

Synthetic or modified promoters are a source for molecular engineering and could be used for engineering cocoa and coffee starters. As aforementioned, promoter engineering might rely on the arrangements of *cis*-regulatory elements to boost promoter strength that regulates enzymes which, for example, are primary players in fermentation processes. Since many molecular biology methods could easily generate promoters, the applications of synthetic biology in starter cultures could result in gains for protein titer tuning. In addition, metagenomics seems to be an ally for promoter mining since those sequences might be retrieved from environmental samples and further characterized for use, which could potentially contribute to genetic engineering approaches (WESTMANN *et al.*, 2018).

1.7 De novo design of biosynthetic pathways

Genetic engineering is a relevant ally for generating high-added-value compounds through the modification of micro-organisms. One of the most elegant strategies designed for this purpose was proposed by LEE et al., (2016). The authors' approach resulted in significant production of the raspberry ketone, a valuable flavoring agent classified as phenylpropanoid derived from p-coumaric acid, in an industrial strain of *Saccharomyces cerevisiae*. The de novo biosynthetic route was constituted for phenylpropanoid production through metabolic steps played by the heterologous enzymes benzalacetone synthase, cinnamate-4-hydroxylase, coumarate-CoA ligase, and phenylalanine/tyrosine ammonia-lyase from different microbial species. In addition, in the same study, fusion proteins were also designed and further studied regarding ketone (raspberrylike flavor) yield. Synthetized codon-optimized enzymes were expressed in the yeast and the results showed that the engineered strain was able to significantly produce the phenylpropanoid in Chardonnay grape juice (LEE *et al.*, 2016). This same approach can be applied to cocoa and coffee fermentation which favor-active compounds (*e.g.,* butanal, 2-phenyethanol, isoamyl acetate, acetaldehyde, 1-pentanol, ethyl acetate, and 2-phenylacetaldehyde) are used to select yeasts and bacteria capable of influencing the final product quality (PEREIRA *et al.*, 2019; SALEM *et al.*, 2020).

1.8 Genome editing

Genome editing techniques have been gaining prominence in the scientific community due to the promising strategies that can be achieved with their applicability. CRISPR (Clustered regularly interspaced short palindromic repeats)-Cas (associated proteins) is currently the most studied molecular tool. It is defined as a defense system in bacteria and most archaea against invaders by which invaders' exogenous DNA is integrated into CRISPR loci for further transcription (BARRANGOU; MARRAFFINI, 2014). The subsequent transcription of such loci results in RNAs (some of them known as guide RNA – gRNA) that can be driven by nucleases towards matching to complementary DNA sequences, resulting in cleavage (BARRANGOU; MARRAFFINI, 2014). As this machinery represents an enormous biotechnological potential, it is expected to be used in synthetic biology for distinct purposes since CRISPR-Cas systems can generate mutations, insertions, deletions, and even regulate gene expression (SONG *et al.*, 2020). Despite the tremendous results obtained with CRISPR-Cas, off-target effects can occur in experimental methodologies that use this system (VICENTE *et al.*, 2021; ZHANG *et al.*, 2015).

In this sense, SHEN et al., (2019) used a CRISPR-Cas12 system to engineer *Zymomonas mobilis* for lactate production. The authors constructed an expression system composed of a *Bacillus coagulans'* codon-optimized lactate dehydrogenase (*LdhBc*) under the control of an alcohol dehydrogenase native promoter (P*adhB*). After transformation, the recombinant strain was selected. With this approach, the titers of lactate reached 2.21 g/L (SHEN *et al.*, 2019).

CRISPR-Cas approaches that elicit homology-directed repair are also suitable for engineering living cells. In this sense, Muysson et al. (MUYSSON *et al.*, 2019) designed a CRISPR-Cas9 system capable of generating an Icewine yeast, *Saccharomyces cerevisiae* K1-V1116, that lacks STL1, an H^+/glycerol symporter (MUYSSON *et al.*, 2019). The recombinant strain ΔSTL1 presented high glycerol production in an Icewine fermentation, which could be helpful to the yeast to avoid stress caused by the high amount of sugars in Icewine juice (MUYSSON *et al.*, 2019). Transporters are a suitable target for research on strain improvements because they are related to the internalization of substrates which could be directed to the generation of final products. In this sense, blocking some transporters would helpfully protect cells from warning environments. The opposite scenario might be equally profitable, depending on the nature of the transporter. Starter cultures could be benefited from the use of rational engineering as uptake of

substrates would likely result in product generation. In this sense, the metabolization of substrates that will turn into intermediates through biosynthetic routes could yield better titers of aromatic compounds, for instance.

Although Cas applications tend to be used to cleave or disrupt DNA sequences, genetic engineering approaches have also modified this system to activate or repress transcriptional systems. In these cases, Cas systems can also play the role of transcriptional regulators when transcriptional effectors are fused to a deficient Cas (dCas) driven by guide-RNAs (CHAVEZ *et al.*, 2015; JENSEN *et al.*, 2017). The mechanism by which transcription is modulated is based on the positioning of the Cas in the regions surrounding the transcription start site of the target genes, as activation or repression could occur depending on the effector character (CHAVEZ *et al.*, 2015; JENSEN *et al.*, 2017).

Although many studies have described the utilization of CRISPR-Cas for a series of bioprocesses, coffee and cocoa fermentation remain poorly investigated. This approach can be used for a series of processes regarding fermentation, such as the construction of systems for disruptions of potential inhibitors of pectinases' activity or other enzymes required for fermentation for the transcriptional activation of networks that coordinate fermentation enzyme expression. Lastly, CRISPR-Cas systems could also be used to overexpress enzymes necessary for fermentation through the design of expression systems regulated by synthetic promoters.

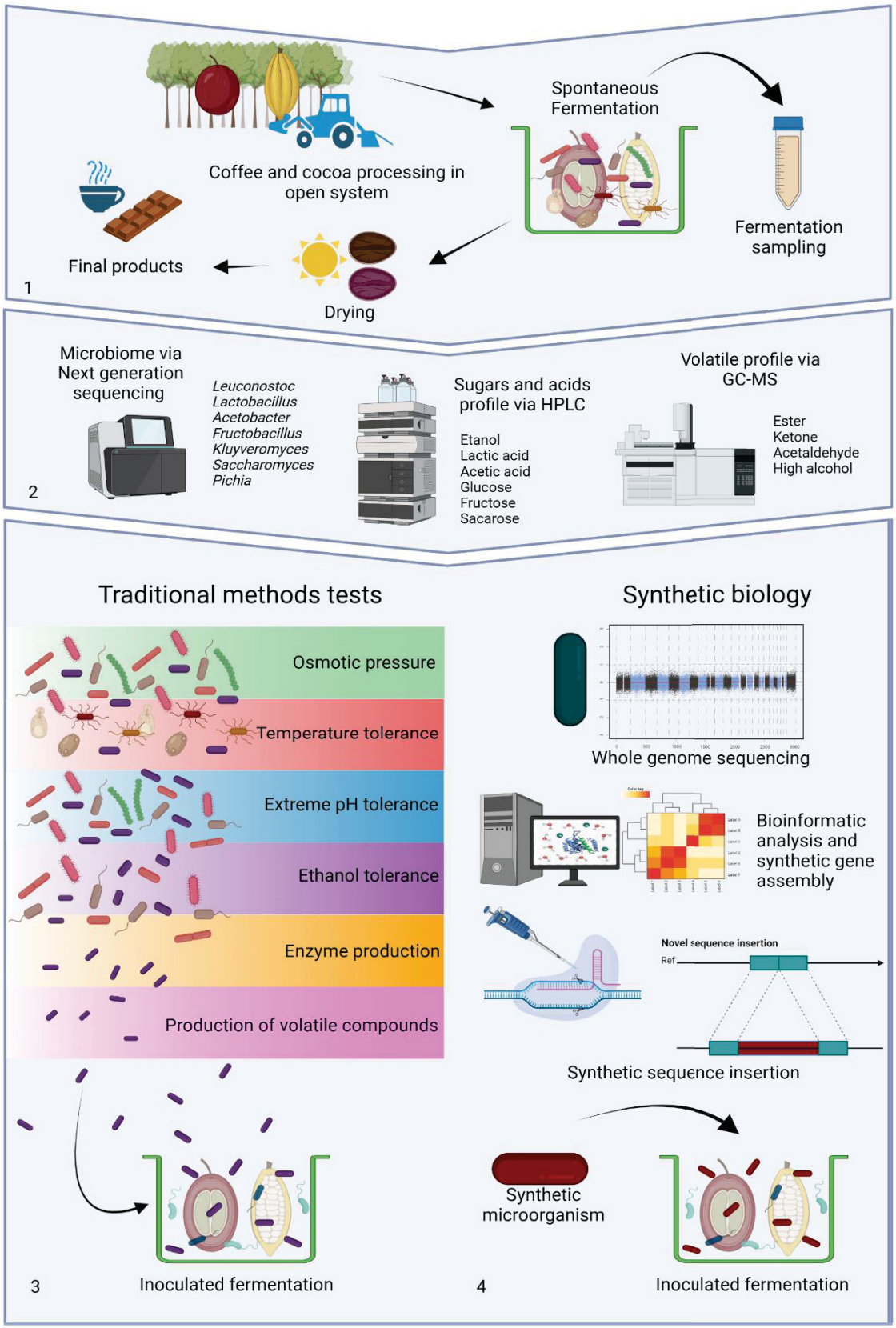
Despite the promising approaches that may be achieved with CRISPR-Cas systems, it is still necessary that research shed light on starter culture engineering. For instance, scientific efforts keep developing strategies capable of interchanging approaches between the useful common industrial microorganisms and starter culture strains. In any case, a complete genomic sequence with few or no gaps is the "gold standard" for a reference genome, but incompletely characterized genomes, are also useful. Large-scale, genome-diversity data on several microorganisms have been generated, including some isolated from cocoa and coffee fermentations (ILLEGHEMS; DE VUYST; WECKX, 2013, 2015; MUYNARSK *et al.*, 2019). This information has provided insights into the genetic and molecular basis of these strains and can serve as a starting point for the creation of breeding programs using synthetic biology focused on starter cultures for cocoa and coffee. In addition, other methods and tools commonly used in synthetic biology to promote the design-build, test-learn cycle for building cell factories for application in the production of fermented foods (e.g., wine, soy sauce, and

pickles) have recently been reviewed by LV et al., (2021) and can be applied to cocoa and coffee.

1.9 Starter cultures selection by traditional methods associated with gene expression and enhancement

Knowledge about the physiological and metabolic parameters of cocoa and coffee starters is the first step in any genetic improvement program. This section reviews the criteria widely used for the selection of cocoa and coffee starters based on traditional procedures. Usually, the selection process starts with many microorganisms recovered from spontaneous fermentation. Thus, a systematic approach is necessary to gradually reduce the number of microbes candidates, as shown in Figure 1. This screening can be divided into four main steps: (1) resistance to fermentation stresses (*e.g.,* osmotic, thermal, and acid/base); (2) enzyme production (3) production of key metabolites (*e.g.,* alcohol, organic acids, and volatile compounds) and (4) kinetic analysis in a simulation medium. Subsequently, characterization of technological parameters can be evaluated, such as large-scale production capacity, resistances to drying, packaging, and storage processes (PEREIRA *et al.*, 2020a). The next topics will address some parameters that should be considered in improvement platforms for cocoa and coffee starters.

Figure 1: Schematization of the improvement of spontaneous cocoa and coffee fermentations through synthetic biology 1— On-farm cocoa and coffee processing; 2—microbiome and strategies for the study of metabolic activity of spontaneous process; 3—desirable metabolic traits for starter culture selection; 4—synthetic biology in the design of new microbial strains.



Source: Author (2022)

1.9.1 Tolerance to stress conditions

The beginning of cocoa and coffee fermentation is constituted by a high concentration of carbohydrates in the pulp. This sugar-rich environment results in high osmotic pressure for starter culture growth. High sugar condition also leads to reduced turgescence pressure and decreased water availability (BAUER; PRETORIUS, 2000). To determine the ability of candidate strains to tolerate these stress conditions, the microorganisms can be grown on a basal medium with different concentrations of glucose and/or fructose (*e.g.,* 5, 15, 30, and 50%). Thus, the isolates showing the strongest growth are selected (ELHALIS *et al.*, 2021; PEREIRA *et al.*, 2012). For example, VISINTIN *et al.*, (2016) showed that some strains belonging to the species *Saccharomyces cerevisiae, Candida ethanolica, Hanseniaspora uvarum, Pichia manshurica, Schizosaccharomyces pombe*, and *Torulaspora delbrueckii* isolated from cocoa fermentation were able to grow in medium containing up to 30% hexoses. The ability of these microorganisms to grow in sugar-rich environments may be associated with a genetic adaptation mechanism that makes the strains resistant to osmotic stress. This mechanism was recently identified in a strain of *S. cerevisiae* isolated from cocoa (DÍAZ-MUÑOZ; DE VUYST, 2021) and, according to the authors, this system is similar to that found in *S. cerevisiae* used in wine fermentation processes (GONÇALVES *et al.*, 2016). Genes involved in long-term adaptation to osmotic pressure in *S. cerevisiae* include GPD1 and GPP2. They are signaled via mitogen-activated protein (MAP) kinase cascade by the High Osmolarity Glycerol (HOG) pathway and results in the production of intracellular glycerol, adapting to hyperosmotic conditions (KOSTOPOULOU *et al.*, 2018).

As the fermentative process develops, the microorganisms produce a wide range of organic acids, ethanol, and other end-metabolites, resulting in a sharp decrease in pH (from 6.0 - 5.5 to 4.5 - 4.0) in coffee fermentations (PEREIRA *et al.*, 2019). On the other hand, the cocoa pulp has a naturally acidic pH (4.0 - 3.0), which tends to increase during the initial stage of fermentation but reduces towards the end of the process due to lactic and acetic acid production (VIESSER *et al.*, 2021). To simulate this hostile environment, the growth of the isolates can be evaluated in synthetic culture media by adjusting the pH in different ranges. These assays should preferably be performed over a wide pH range (2.5 - 7.0). In addition, the tolerance of each strain to organic acids can be evaluated by adding acetic acid or lactic acid to a basal medium at a final concentration of 1.0 to 4.0%. Ethanol tolerance can also be determined by adjusting the final ethanol concentration in

the medium to a range between 2.0 and 15% (ELHALIS *et al.*, 2021; PEREIRA *et al.*, 2015)

Due to the lipophilic properties of some compounds such as organic acids, can lead to a reduction of the intracellular pH and cause the release of purines and pyrimidines generating damage in the genome (VAN DE GUCHTE *et al.*, 2002). Microorganisms have developed some highly complex physiological and genetic mechanisms of protection, and one of these processes is nucleotide excision repair (NER). Several genes, including RecA and UvrA are shown to be important for tolerance to acidic conditions (GRINHOLC *et al.*, 2015). Recently, ZHENG et al., (2018) evaluated the effect of overexpression and deletion of this gene on the fermentative capacity of *Acetobacter pasteurianus* and found that the presence of acetic acid in the medium induced the production of this gene and was able to protect the genome of the strain. Genome sequencing of the *Pediococcus acidilactici* LPBC161 strain isolated from the coffee fermentation process also revealed the presence of more than 70 unique genes encoding enzymes associated with oxidative stress, such as catalase, thiol peroxidase, glutathione reductases, alkaline shock proteins (MUYNARSK *et al.*, 2019). Other examples of stress tolerance genes are found in Table 1 and can be used as a starting point in the application of synthetic biology tools in the development of strains resistant to the fermentative process in cocoa and coffee.

1.9.2 Production of enzymes

The ability of cocoa and coffee starters to produce pectinases is highly desired since this characteristic can accelerate the fermentative process (MEERSMAN *et al.*, 2015; SAMAGACI *et al.*, 2016). Pectinase production can be measured using different methodologies. For example, Silva et al. (SILVA *et al.*, 2013) evaluated the polygalacturonase (PG), pectin lyase (PL), and pectin methylesterase (PME) activity of 127 yeasts and 189 bacteria isolated from coffee fermentation. Briefly, the microorganisms were grown on plates containing mineral medium (SCHWAN; COOPER; WHEALS, 1997) and the specific substrate for each enzyme was analyzed. The production of pectinases by each strain was indicated by the formation of a clear halo around the colonies, indicating that the substrate had been hydrolyzed. Thus, isolates that showed halos greater than 30 mm in diameter were preselected (SILVA *et al.*, 2013). Usually, these semi-quantitative tests are used when there are still a high number of candidates. After this selection, it is recommended that the enzyme activity is determined

quantitatively. For instance, Pereira et al. (PEREIRA *et al.*, 2014) cultivated nine pre-selected yeasts in a liquid medium containing synthetic pectin for 24 h. After this period, the cells were removed, and the supernatant was used to determine pectinase activity by measuring the increase in reducing sugars released from pectin hydrolysis using the 3,5-Dinitrosalicyclic Acid DNS method. The results showed that *Saccharomyces* sp. YC8.10, *Saccharomyces* sp. YC9.15 and *P. fermentans* YC8.8 had high enzyme activity (PEREIRA *et al.*, 2014). Importantly, although many of the studies evaluate only the production of pectinases, similar methods can be used to determine the activity of other classes of hydrolytic enzymes, such as cellulases, laccases, lipases, and xylanases with potential benefits for cocoa and coffee fermentations (DELGADO-OSPINA *et al.*, 2020; ELHALIS *et al.*, 2021).

1.9.3 Production of key metabolites

In cocoa fermentation, high production of ethanol and acetic acid is an important criterion to be considered. However, the high accumulation of these metabolites for coffee fermentation is still unclear and, generally, these parameters are neglected during the selection of coffee starter. On the other hand, lactic acid production is important for both processes (PEREIRA *et al.*, 2020b; VIESSER *et al.*, 2021). The ability of microorganisms to produce these compounds can be assessed by monitoring the pH. However, it is always important to use analytical methods such as high-performance liquid chromatography (HPLC) and gas chromatography (GC) to identify and quantify which acids are being produced. Furthermore, it is known that the production of secondary metabolites (*e.g.,* esters, aldehydes, ketones, and higher alcohols) mainly by yeasts and lactic bacteria can contribute significantly to modulating the sensory profile of cocoa and coffee(FIGUEROA-HERNÁNDEZ *et al.*, 2019; PEREIRA *et al.*, 2020a, 2019). Thus, several studies have reported the selection of new starter cultures based on the production of these volatile molecules (FIGUEROA-HERNÁNDEZ *et al.*, 2019; JUNQUEIRA *et al.*, 2022; PEREIRA *et al.*, 2015). Since on-farm fermentations are performed without temperature control and agitation, it is important to determine the production of volatiles over a wide temperature range (PEREIRA *et al.*, 2014)

Table 1: Use of molecular tools for the genetic improvement of microorganisms

Microorganism	Gene	Genetic tools	Objective	Application	Reference
Saccharomyces cerevisiae BY4741	*COX20* O	Recombinant DNA technology	Developing an *S. cerevisiae* tolerant to acetic acid and oxidative stress	Second-generation bioethanol production	(KUMAR *et al.*, 2015)
Acetobacter pasteurianus AC2005	*uvrA* O - Δ	Recombinant DNA technology	Evaluate the effect of the uvrA gene on the tolerance of the strain to acetic acid during acetic fermentation	Acetic acid production	(ZHENG *et al.*, 2018)
Lactococcus lactis ML23	*Shsp* O	Recombinant DNA technology	Use a food-grade vector to grow a *Lactococcus lactis* strain expressing the gene (shsp) encoding a heat shock protein from *Streptococcus thermophilus* and evaluate growth in the different stress environments	Starter cultures for the dairy industry	(TIAN *et al.*, 2012)
Saccharomyces cerevisiae BY4742	I*AH1* and *TIP1* Δ	CRISPR-Cas	Production of esterases by the yeast results in the degradation of esters formed during fermentation. Thus, deletion of these genes resulted in an increased relative abundance of esters compared to wild strains	Beer production	(DANK; SMID; NOTEBAART, 2018)
Saccharomyces cerevisiae	*Rt PAL, AtC4H, At4CL1, Pc4CL2* and *RpBAS* O	Recombinant DNA technology	Insert a new biosynthetic route to synthesize a raspberry-flavored ketone	Wine production	(LEE *et al.*, 2016)
Saccharomyces cerevisiae AWRI1631	*GDP1* O	Recombinant DNA technology	To develop a strain with the ability to produce low concentrations of ethanol during fermentation, 41 genetic modifications were performed. The best result was obtained after overexpression of the glyceraldehyde-3-phosphate dehydrogenase gene (*GPD1*)	Wine production	(VARELA *et al.*, 2012)
Saccharomyces cerevisiae T73-4	*GES* O	Recombinant DNA technology	Expression of the *Ocimum basilicum* (sweet basil) geraniol synthase (GES) gene in an *S. cerevisiae* was able to substantially modify the terpene profile of wine produced from a non-aromatic grape variety.	Wine production	(PARDO *et al.*, 2015)

Saccharomyces cerevisiae x *Saccharomyces eubayanus hybrid*	*FDC1* ^{IM}	CRISPR-Cas	Developing a CRISPR-based gene editing strategy that allows the systematic and meticulous introduction of a natural mutation in the FDC1 gene resulting in decreased phenolic off-flavor in lager-type beers	Beer production	(MERTENS *et al.*, 2019)
Saccharomyces cerevisiae AWRI1631	*ATF1* and *GPD1* ^{O}	CRISPR-Cas	Overexpression of ATF1 and GDP1 significantly increased the levels of acetate ester and glycerol, respectively. And simultaneous overexpression of these two genes led to a decrease in acetic acid concentration.	Wine production	(VAN WYK *et al.*, 2020)

Δ deletion, *O* overexpression, *IM* induced mutation

1.9.4 Kinetic analysis in a simulation medium

The success of starter cultures is highly influenced by the environment and/or agricultural practices of the country where cocoa and coffee are produced. Therefore, simulating these variations can also aid in the selection of strains well adapted to these processes (FIGUEROA-HERNÁNDEZ *et al.*, 2019). For coffee, one alternative is to prepare an aqueous extract from the hulls, pulp, and/or mucilage of the cherries and use it as a medium to simulate the fermentative process. Due to the addition of this extract, growth factors and inhibitors that are present in natural fermentation can be added to the medium and mimic field conditions. However, to support the growth of microorganisms this medium should be supplemented with glucose, fructose, sucrose, and pectin as carbon sources, while yeast extract and soy peptone can be used as nitrogen sources (ELHALIS *et al.*, 2021; PEREIRA *et al.*, 2014; SILVA *et al.*, 2013). Cocoa pulp simulation medium (PSM) can also be used to simulate the cocoa fermentation process. PSM is composed of fructose, glucose, sucrose, pectin, citric acid, yeast extracts, soy peptone, potassium sulfate, magnesium sulfate, manganese sulfate, and Tween 80 (OUATTARA; ELIAS; DUDLEY, 2020). However, the composition and concentration of the medium components can vary depending on the microbial group being selected (LEE *et al.*, 2019). For example, to evaluate the fermentative profile of AAB, the addition of ethanol to the medium is recommended (LEFEBER *et al.*, 2010). Recently, Lee et al. (LEE *et al.*, 2019) added unfermented cocoa beans to the PSM medium to make the process even similar to on-farm conditions. Therefore, these methodologies can be adapted according to the microorganisms being evaluated.

After formulating the culture media, the strains that were selected based on the previous tests are inoculated and evaluated for their fermentative capacity. During fermentation on a laboratory scale, several factors can be determined, including consumption of carbon sources, microbial growth, enzyme activity, and production of organic acids and volatile compounds. Among all these parameters, the production of aromatic molecules is one of the most important factors, since these compounds can diffuse into the cocoa and coffee beans modulating the sensory profile of the final product. Furthermore, it is possible to perform co-inoculation between the strains that presented the best results and to verify if there is any kind of positive interaction between starter cultures that could be interesting for fermentation. After this screening, the strains that present the greatest number of desired characteristics, without the presence of negative traits, are selected and evaluated in the field.

1.10 Regulatory and safety requirements for food cultures

The development of food cultures (FC) through synthetic biology has drawn attention due to being a dual-use discipline also known as DURC (Dual-use research of concerns). Different countries around the world follow distinct regulations and guidelines for the use and application of synthetic biology. These are based on international conventions, protocols, or agreements that seek to guarantee biosafety and its correct use.

Food cultures need to be safe because they are live microorganisms; it is done through quality management and control with standards, such as HACCP (Hazard Analysis and Critical Control Point) and GMP (Good Manufacturing Practice) (LAULUND *et al.*, 2017). Assessment of food safety should be carried out at the strain level during its production and during the shelf life of the food as a whole. The EU Commission regulates these criteria using the General Food Law in Europe (LARANJO; ELIAS; FRAQUEZA, 2017). Additionally, the EFSA (European Food Safety Authority), the IDF (International Dairy Federation), and the EFFCA (European Food and Feed Cultures Association) use tools that help maintain FC safety such as QPS (Qualified Presumption of Safety). The QPS used by EFSA consists of evaluating panels for microorganisms only and considers aspects such as taxonomy, use, pathogenicity, and virulence, among others (PEREIRA *et al.*, 2020a)

The regulation in the USA is made by the FDA (Food and Drug Administration) through GRAS (Generally Recognized As Safe) status for all ingredients without the existence of a specific regulation. FC need to be characterized to meet food safety requirements as with GRAS strains, which makes them promise in synthetic biology (LIU *et al.*, 2017b). In Asia, FC is considered a food additive without specific regulation. Although fermented foods are widely consumed, they must be as safe for consumers as any regular food. This is because fermentation vary between batches and it is susceptible to pathogenic microbial contamination or the generation of harmful metabolites or toxins (TENG *et al.*, 2021). Thus, the FAO (Food & Agricultural Organization) established four fundamental steps in the analysis of food risk: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment and risk characterization, and (iv) management and risk communication.

Most countries follow the regulation and management for GMOs (Genetically Modified Organisms) when it comes to the biosafety risks of synthetic biology in FC, but this varies depending on the country and has gaps and absence of measures in the international community (LI *et al.*, 2021). One of the tools of synthetic biology, such as

CRISPR, would allow designing genes without introducing DNA from other microorganisms (SAJID; STONE; KAUR, 2021). Countries such as Argentina, Germany, and Sweden already have regulations that differentiate GMOs from DNA edited by CRISPR-Cas9 (JAGTAP *et al.*, 2017).

Despite the possible rejections of current consumers for the use of GMOs, the acceptance of FC in fermented products is a matter of time. Researchers and companies have sought to develop the first synthetic yeast of *S. cerevisiae*. Some genetic modifications such as the production of lactic acid in *S. cerevisiae* by insertion of lactate dehydrogenase gene in the production of sour beer were approved for sale only in the USA by the company Lallemand under the brand Sourvisiae® (ALPERSTEIN *et al.*, 2020; LALLEMAND, 2019).

Some approaches are important to consider. The requirement for natural metabolite compounds (e.g., fragrances, flavors) in fermented foods does not exclude genetic engineering, as there is no modified genetic material in the final product even if it has been produced by bioengineered yeasts; therefore, it does not need to be declared on the label (MORRISSEY *et al.*, 2015). This is similar to the production of ingredients derived from GMOs (e.g., vitamins, enzymes), but they are metabolites that will be separated and purified for use in food, feed, additives, and supplements (SHUKLA, 2019; VAN WYK; KROUKAMP; PRETORIUS, 2018). Therefore, there is still a lack of establishment of specific regulations for the use of FC through synthetic biology. These advances could clarify the proper use of these microorganisms seeking food safety and guaranteeing high-quality products for the whole world.

1.11 Conclusion and Perspectives

The rapid advancement of high-throughput DNA synthesis and synthetic biology techniques have facilitated the creation of microbial strains with robust phenotypes for industrial applications. Therefore, this review shows several characteristics and metabolic traits (*e.g.,* tolerances to different types of stresses, production of enzymes, and key metabolites) that can be exploited by synthetic biology to develop starter cultures for cocoa, coffee and other rudimentary fermentations. Some strategies, such as synthetic promoters, de novo design of biosynthetic pathways, and genome editing are proposed to overexpress enzymes and other key-metabolites involved in cocoa and coffee quality. The use of the CRISPR-Cas tool to activate or repress transcriptional systems is highlighted. However, one of the bottlenecks in synthetic biology for the development of these strains

is understanding how robustness can be achieved and maintained. Depending on the context, a synthetic circuit can behave in diverse ways and, since many of these failures are difficult to predict, a deep understanding of its genetics is required. Furthermore, the more complex these circuits are the more resources (metabolic load) will be exerted on the chassis, potentially leading to a slower adaptation process. Thus, in each case, it is necessary to perform a thorough analysis of the genetic network to be built to determine the potential and need for robustness (DAVENPORT; TICA; ISALAN, 2022; RANDALL et al., 2011).

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2. Chapter 2: Facility-specific “house” microbiome ensures the maintenance of functional microbial communities into coffee beans fermentation: Implications for source tracking

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2.1 Abstract

This work aimed at studying the unconfirmed hypothesis predicting the existence of a connection between coffee farm microbiome and the resulting spontaneous fermentation process. Using Illumina-based amplicon sequencing, 360 prokaryotes and 397 eukaryotes were identified from coffee fruits and leaves, over-ripe fruits, water used for coffee de-pulping, depulped coffee beans, soil, and temporal fermentation samples at an experimental farm in Honduras. Coffee fruits and leaves were mainly associated with high incidence of Enterobacteriaceae, *Pseudomonas*, *Colletotrichum*, and *Cladosporium*. The proportion of Enterobacteriaceae was increased when leaves and fruits were collected on the ground compared to those from the coffee tree. Coffee farm soil showed the richest microbial diversity with marked presence of *Bacillus*. Following the fermentation process, microorganisms present in depulped coffee beans (*Leuconostoc*, *Gluconobater*, *Pichia*, *Hanseniaspora*, and *Candida*) represented more than 90% of the total microbial community, which produced lactic acid, ethanol, and several volatile compounds. The community ecology connections described in this study showed that coffee fruit provides beneficial microorganisms for the fermentation process. Enterobacteria, *Colletotrichum*, and other microbial groups present in leaves, fruit surface, over-ripe fruits, and soil may transfer unwanted aromas to coffee beans, so they should be avoided from having access to the fermentation tank.

2.2 Introduction

Coffee processing is an agricultural practice employing traditional production techniques, dedicated facilities, and specialized equipment. The process begins with the removal of the outer layers of the fruits during on-farm processing by dry or wet method (ELHALIS; COX; ZHAO, 2020). In the dry process, whole fruits are allocated in cement terraces for sun-drying or mechanical dryers, until they reach the desired water content. Wet processing, in contrast, involves a relatively complex series of steps, including mechanical removal of the coffee skin and pulp, microbial degradation (fermentation) of

the mucilage layer and, finally, water removal by sun-drying. The fermentation step is spontaneously performed in tanks containing large volumes of water, where the microaerophilic environment and nutritional selectivity of coffee pulp cause the development of microorganisms with fermentative metabolism, mainly yeast and LAB (PEREIRA *et al.*, 2020). During this process, microbial activity degrades coffee pulp sugars and produces several flavour-active metabolites with a complementary function in the formation of taste and flavour precursors of coffee beverages (LEE *et al.*, 2015; PEREIRA *et al.*, 2020, 2019).

In any fermentation process, the impact of the native microflora is hugely important. What is now known as microbial *terroir*, which posits that 'regional flavors and aromas in fermented products may be in part due to regionally structured microbial diversity' (MIURA *et al.*, 2017; MORRISON-WHITTLE; GODDARD, 2018), has been explored to achieve geographic signatures of many agricultural products. Accumulated evidence on wine, sourdough, and cheese has reflected that the adoption of traditional fermentation practices, such as cleaning procedures, temperature control, oxygenation, water quality, and adjunct ingredients, can modulate the fermentative microflora (KNIGHT *et al.*, 2015; PAXSON, 2010). During the harvest, transport and pulping stages, coffee beans become contaminated with a variety of microorganisms, many of which contribute to the subsequent fermentation. The coffee fruit is the most obvious potential source of microbial diversity. However, nearby managed ecosystems (e.g., soil, water, coffee leaves, outer surface of the fruits, and air) have very high populations of a diversity of yeast, fungal, and bacterial species that can impact on the microbial ecology of the overall fermentation process (JUNQUEIRA *et al.*, 2019). However, the interplay between fermentation microbial communities and coffee processing environments remains poorly understood, and studies on microbial source tracking are limited (BOKULICH *et al.*, 2013; ZHANG; LIU; LIU, 2009). Contaminating organisms can compete with fermentative yeasts and LAB for substrate during fermentation and produce off-flavour compounds compromising coffee quality (PEREIRA *et al.*, 2016).

This work aimed at studying the relationship between microbial communities from coffee farm processing and their resulting spontaneous fermentation to surrounding managed and natural habitats. This was tackled by next-generation DNA sequencing of 16S, 18S, and ITS amplicon libraries produced from coffee fruits and leaves, over-ripe fruits, water used for coffee de-pulping, depulped coffee beans, coffee farm soil, and temporal fermentation samples at an experimental farm in Honduras. In addition, the

metabolites produced by the microorganisms during fermentation process were monitored to determine their influence on the coffee bean metabolite profile over time.

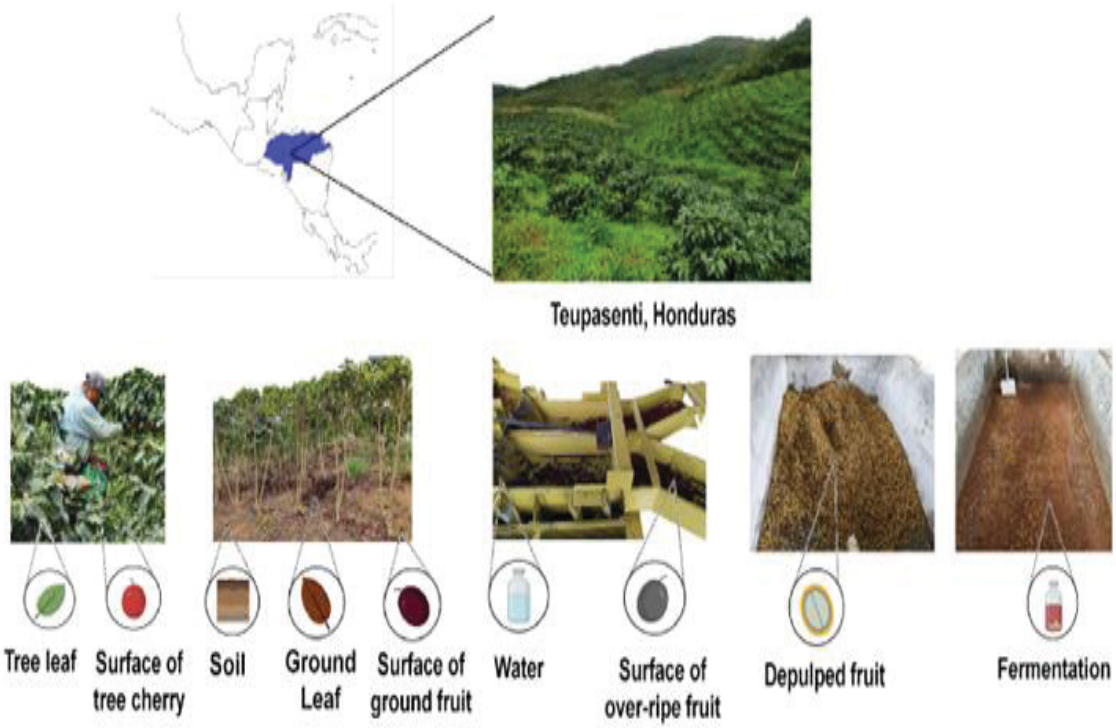
2.3 Materials and methods

2.3.1 Area of study and sampling procedure

The coffee farm (Fortune Mountain Specialty Coffee) sampled in this study is located in Teupasenti, El Paraiso, Honduras (14°11′10.6” N 86°46′21.5” W). This coffee farm is at 1250 m altitude and produces prized, high-quality Arabica coffee beans under strictly controlled conditions. The environmental samples, collected in triplicates, comprised of: (i) 100 g of soil collected inside an aera of 1 m^2 and 10 cm of depth inside the tree canopy; (ii) 100 g of leaves collected from the branches at 30 cm from the apical region; (iii) 100 g of leaves collected from the soil surface inside tree canopy aera (1 m^2); (iv) 20 ripe (cherry) and over-ripe fruits collected from the branches at 30 cm from the apical region; (v) 20 fruits collected from the soil inside tree canopy aera (1 m^2); (vi) 50 ml of water used for de-pulping coffee beans; and (vii) 100 g of mechanically depulped beans. The experimental design illustration and sampling flow are reported in the Figure 1. All samples were stored in sterile Falcon tubes (50 ml) and transported to the Center of Agro-industrial Biotechnology of Paraná (CENBAPAR, Curitiba, Brazil) under refrigeration and kept at −20°C until further analysis.

Subsequently, coffee fruits (*Coffea arabica* var. Catuaí) were manually harvested from the same field and transferred to the fermentation plant. Coffee fruits were mechanically pulped and approximately 100 kg of depulped beans were allocated in cement tanks containing about 50 l of water, where a spontaneous fermentation occurred over 24 h. Aliquots of 50 ml from the liquid fraction of the fermenting coffee pulp-bean mass were collected at 0, 12, 16, 18, 20, and 24 h. All samples were collected in triplicate and frozen at −20°C for appropriate transportation to CENBAPAR, Curitiba, Brazil. The pH of the fermenting mass was measured at each sampling point using a portable pH meter (KASVI, São José dos Pinhais, Brazil).

Figure 1: Flowchart showing the origin of the samples collected at the coffee farm.



Source: Author (2022)

2.3.2 Total DNA extraction and Illumina high-throughput sequencing

Total DNA extraction from environmental samples was performed according to DE BRUYN et al., (2017), where an initial detachment of microbial cells present in the thawed samples was performed by mixing 5 g of each sample with 10 ml of saline solution (9% [w/v]; Merck, Darmstadt, Germany) by vortexing (two treatments for 2 min with a 15-min interval). The suspension was filtered through a 20-µm pore size Polycarbonate (PCTE) membrane filter (Sterlitech, Kent, WA, USA) to eliminate impurities. Water (environmental sample) and fermentation samples were not subjected to the initial cell detachment procedure. Total DNA was extracted using organic (Phenol–Chloroform) method described by CARVALHO NETO et al., (2018) and quantified with a Nanodrop 2000 instrument (Thermo Fisher Scientific, Waltham, MA, USA). Twenty nanograms of DNA, containing complementary adaptors for the Illumina platform (CAPORASO *et al.*, 2012), were used to amplify the V3/V4 region of the 16S rRNA gene using 515F-806R primer pair, and the18S (515F and 806R) and ITS (ITS1 and ITS2) rRNA genes. Bar-coded amplicons were generated by PCR under the following conditions: 95°C for 3 min, and 18 cycles at 95°C for 30 s, 50 for 30 s, and 68°C for 60 s, followed by a final extension at 68°C for 10 min. Samples were sequenced in the MiSeq platform using the 500 V2 kit, following standard Illumina protocols.

2.3.3 Bioinformatic analysis and data availability

Chimeric sequences detection, removal of noises from pre-cluster, and taxonomic attribution were performed using standard parameters of QIIME software package, version 1.9.0. Applying the UCLUST method (EDGAR, 2010), sequences presenting identity above 97% were considered the same operational taxonomic units (OTUs) according to the SILVA and UNITE databases (NILSSON *et al.*, 2019; QUAST *et al.*, 2013).

Illumina reads were deposited on NCBI's GenBank under the BioProject ID PRJNA666881 and the BioSamples IDs SAMN16399020 (coffee beans fermentation), SAMN16399021 (soil), SAMN16399022 (coffee leaves), SAMN16399023 (coffee fruits), and SAMN16399020 (water).

2.3.4 Analysis of fermenting coffee pulp

The concentration of reducing sugars (glucose and fructose) and organic acids (lactic, citric, succinic, acetic, and propionic acids) in the liquid fraction of the fermenting coffee pulp-bean mass was determined by high-performance liquid chromatography (HPLC). Sample preparation and analysis was performed according to CARVALHO NETO et al., (2017), where aliquots of 2 ml were centrifuged (6000*g* for 10 min) and filtered through a 0.22 μm pore size hydrophilic polyethersulfone (PES) membrane (Millipore Corp., Burlington, MA, USA). Filtered samples were injected into the HPLC system equipped with an Aminex HPX 87 H column (300 × 7.8 mm; Bio-Rad, Richmond, CA, USA) and a refractive index (RI) detector (HPG1362A; Hewlett–Packard Company, Palo Alto, CA, USA). The column was eluted in an isocratic mode with a mobile phase of 5 mM H2SO4 at 60°C and a flow rate of 0.6 ml min^{-1}.

A carboxen/poly (dimethylsiloxane) (DVB/CAR/PDMS) type 75 μm SPME fibre (Supelco, Bellefonte, PA, USA) was used to extract volatile constituents from the headspace of the fermenting coffee pulp–bean mass using the method of PEREIRA et al., (2016). The SPME fibre was thermally desorbed into a gas chromatographer coupled to mass spectrophotometry connected to an autosampler (GCMS2010 Plus, TQ8040, AO 5000; Shimadzu, Tokyo, Japan). The injection parameters used were according to the procedures described by (JUNQUEIRA *et al.*, 2019).

2.3.5 Statistical analysis

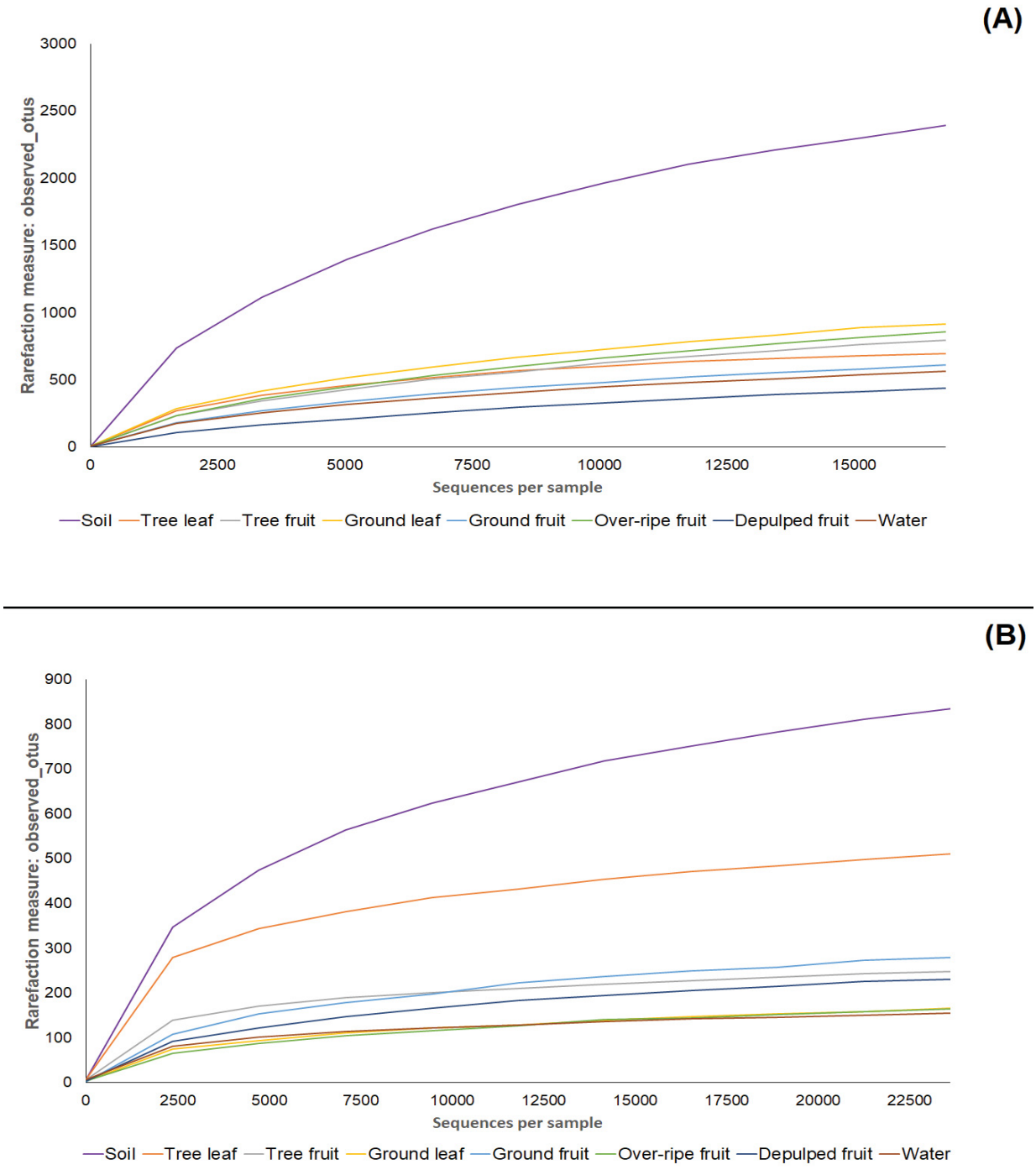
Means and standard deviations of reducing sugars, organic acids, and volatile compounds were analysed by ANOVA test followed by a post hoc comparison of means using Tukey's test. Analyses were performed using the Statistica program, version 10.0 (Statsoft, Tulsa, OK, USA). Level of significance was established using a two-sided *P*-value < 0.05. A principal component analysis (PCoA), based on Weighted UniFrac Distances, was constructed using data on relative microbial prevalence at the family and gender level. Analyses were performed using the Statistica program, version 10.0 (Statsoft).

2.4 Results and discussion

2.4.1 Alpha and beta diversity

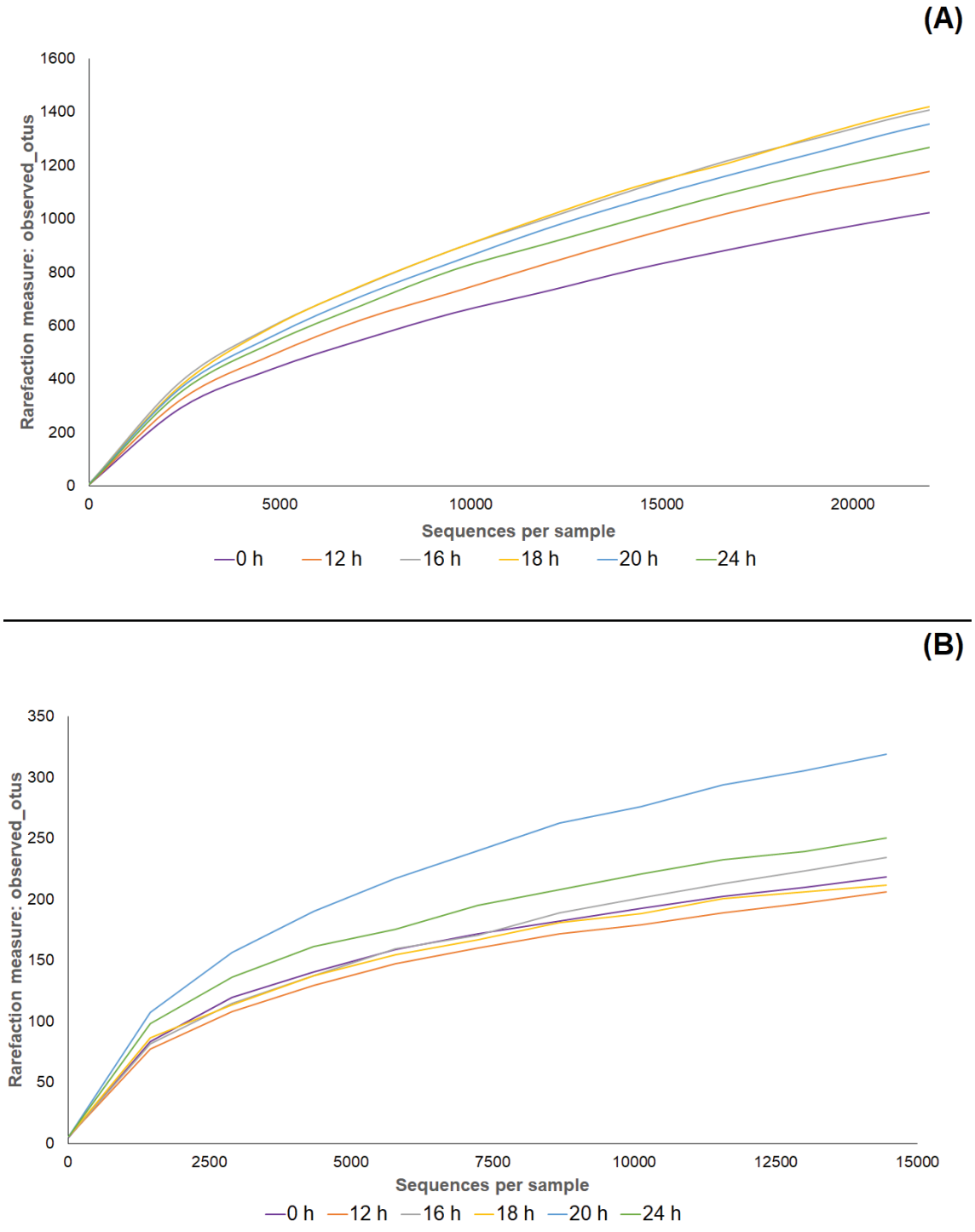
Targeting 16S and 18S small subunit (SSU) rRNA genes, and the fungal Internal Transcribed Spacer (ITS) rDNA sequences, 360 prokaryotes and 397 eukaryotes were identified in the coffee farm microbiome. Alpha diversity allowed studying the richness and evenness of communities from sequencing data between different environments, as shown in the Figures 2 and 3. The trends of rarefaction curves grow rapidly as more common species are detected; however, the curves are not stabilized or reach a plateau. The curve stabilization would show the rarest species found in the samples. Targeted sequencing with ITS rDNA primers revealed additional fungal diversity (397 fungal groups) compared to 18S rRNA sequences (16 fungal groups). It is not an unexpected result, since 18S rRNA primers did not exclusively target microeukaryotes and have fewer hypervariable domains in fungi compared to ITS (HADZIAVDIC *et al.*, 2014; SCHOCH *et al.*, 2012). Thus, the subsequent eukaryotic analysis is based on ITS rDNA sequences, while 18S rRNA gene reads are reported in the Figure 4.

Figure 2: Alpha rarefaction curves of observed OTUs (operational taxonomic units) from the Honduran environmental samples. **A.** Bacterial analysis, **B.** Fungal analysis.



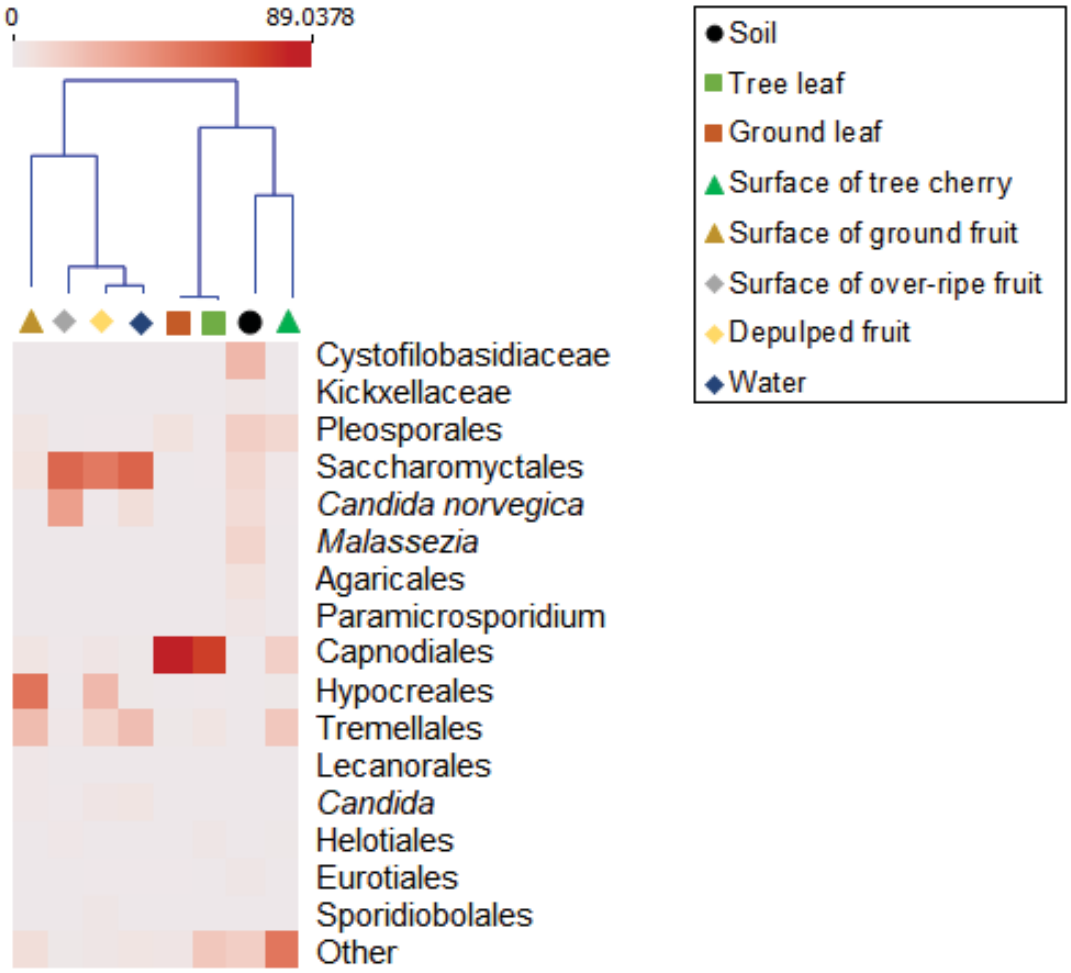
Source: Author (2022)

Figure 3: Alpha rarefaction curves of observed OTUs (operational taxonomic units) from the fermentation temporal samples. **A.** Bacterial analysis, **B.** Fungal analysis.



Source: Author (2022)

Figure 4: 18S rRNA gene high-throughput sequencing of environmental samples collected at an experimental coffee farm in Honduras.



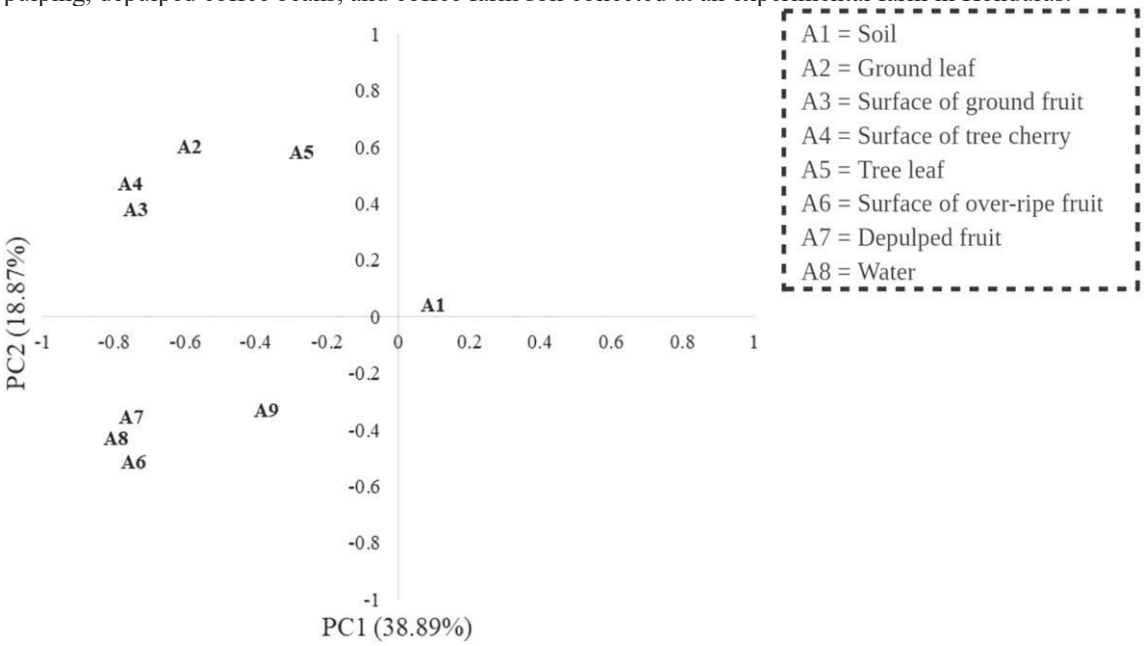
Source: Author (2022)

2.4.2 Farm microbiome

PCA analysis grouped the coffee farm samples in three distinct clusters, according to microbial diversity and abundance, comprising (1) soil; (2) leaves and coffee fruits; and (3) depulped coffee beans, over-ripe coffee fruits, water used for depulping coffee beans, and fermentation sample (Figure 5). The presence and abundance of the major bacteria and fungi, defined as taxa with proportional abundance ≥5%, are reported in Figure 6. Coffee farm soil was grouped in a separate clustering for having the richest microbial diversity, with 299 bacteria and 221 fungi. It was reported the presence of several soil-borne bacteria, including *Corynebacterium, Micrococcus, Candidatus Solibacter, Candidatus,* and *Nitrososphaera* (CHEN *et al.*, 2020; MALIQUE *et al.*,

2019). *Bacillus* (22.52%) markedly dominated soil bacterial composition followed by Gaiellaceae (6.12%) and the uncultured bacterium 0319-6A21 (5.87%). *Bacillus* species are known to dominate soil communities due to their good adaptation to soil pH, moisture, and organic matters promoted by agricultural management practices (MENEGHINE *et al.*, 2017; WEST *et al.*, 1985). Within soil eukaryote community, the Orders Pleosporales (16.18%) and Hypocreales (15.04%) were dominant (Figure 6B). The soil acts as a reservoir of these eukaryotic organisms, which can further migrate into the plant *via* xilema (ZHANG *et al.*, 2012). Fungi members within the Orders Pleosporales and Hypocreales have been reported as endophytes of coffee plant in different producing regions, including Brazil, Colombia, Ethiopia, Hawaii Mexico, and Puerto Rico (DUONG *et al.*, 2020; SETTE *et al.*, 2006; VEGA *et al.*, 2010).

FIGURE 5: Principal component analysis, based on Weighted UniFrac Distances, according to microbial diversity and abundance present in coffee fruits and leaves, over-ripe fruits, water used for coffee de-pulping, depulped coffee beans, and coffee farm soil collected at an experimental farm in Honduras.



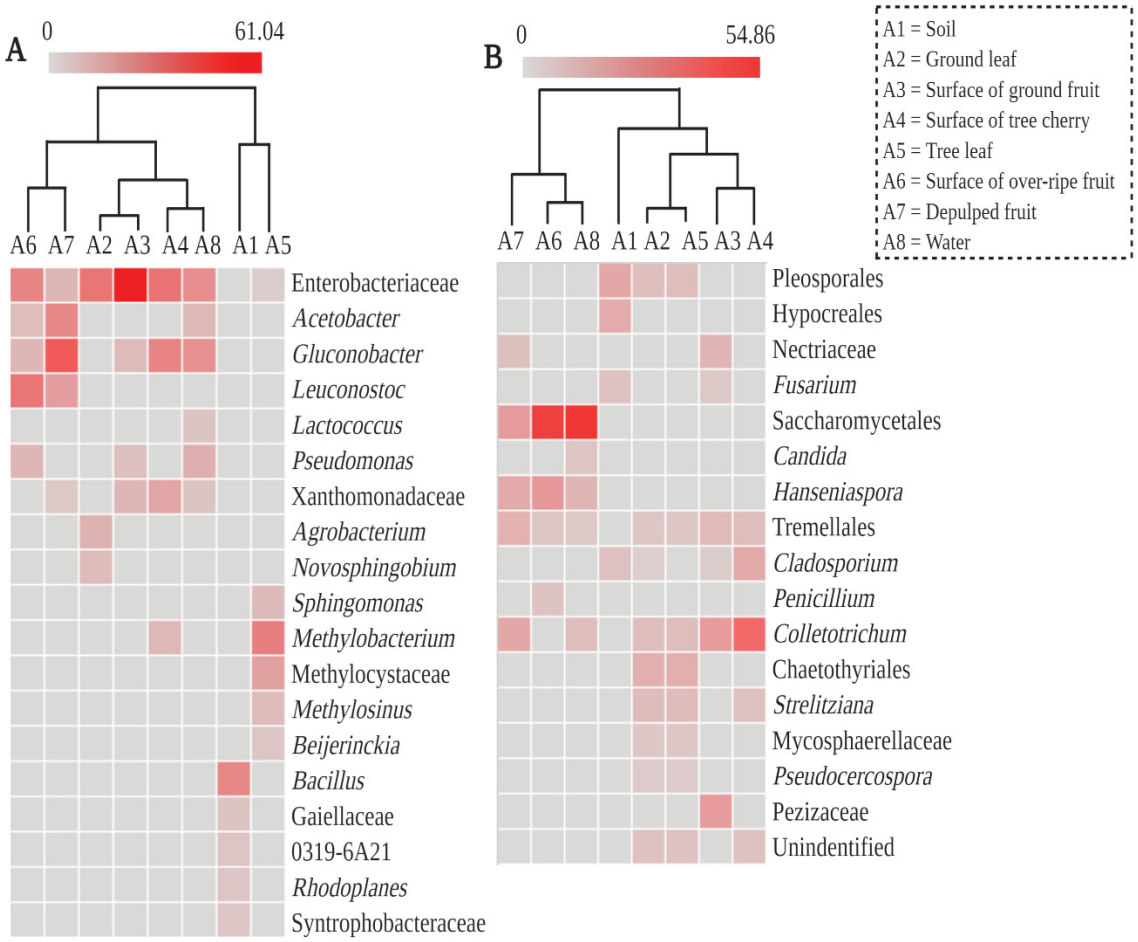
Source: Author (2022)

The second clustering consisted of samples with a high incidence of Enterobacteria, which included fruits and leaves collected from the coffee tree and on the ground (Figure 6). The proportion of Enterobacteriaceae was increased when leaves and fruits were collected from the ground (28.21% and 61.04%, respectively) compared with those from the coffee tree (3.93% and 28.64%, respectively) (Figure 6A). Some Enterobacteriaceae genera, such as *Erwinia* and *Serratia*, produce hydrolytic enzymes enabling their growth in decaying organic matter (MERLINI *et al.*, 2018; SILVA *et al.*,

2000). Interestingly, *Gluconobacter* (23.60%) was the subdominant group of bacteria found in coffee fruits collected from the tree, also reported in freshly harvested coffee cherries in Ecuador (DE BRUYN *et al.*, 2017). The presence of Enterobacteriaceae and *Gluconobacter* on the fruit surface is likely due to horizontal contamination promoted by fruit that commonly infest coffee plantations (ADAIR *et al.*, 2018; DEUTSCHER *et al.*, 2018). The eukaryotic community in this clustering was mainly represented by phytopatogenic and decomposing fungi, such as Pezizaceae, *Colletotrichum*, Nectriaceae, and Tremellales. Leaves collected from the tree also showed high incidence of the bacteria *Methylobacterium* (25.15%) and Methylocystaceae (15.86%), and the fungal Orders Chaetothyriales (13.60%) and Pleosporales (8.69%) (Figure 6). These microbial groups have been reported to co-exist as epiphytes, endophytes, or parasites on plant tree leaves in the tropics (DUONG *et al.*, 2020; ZHANG *et al.*, 2012).

The bacterial structure of over-ripe fruits, depulped coffee beans, water used for de-pulping coffee beans, and fermentation sample changed significantly, allowing the third cluster in the PCA analysis (Figure 5). These samples were mainly characterized by high presence of *Leuconostoc* and *Gluconobacter* within the prokaryote group, and Saccharomycetales (50.86%) and *Hanseniaspora* (20.55%) in eukaryotes. The over-ripe fruits were characterized by a superior dominance of *Leuconostoc* (28.17%), followed by Enterobacteriaceae (23.42%), *Pseudomonas* (10.10%), and *Gluconobacter* (10.01%). *Leuconostoc* inhabits decaying coffee fruits due to its extensive enzymatic machinery (secretion of invertase, proteases, and lipases) and production of bacteriocins against other bacteria (PEREIRA *et al.*, 2020). Water used for de-pulping coffee beans showed a prokaryotic composition similar to over-ripe fruits and depulped coffee beans, suggesting that the major bacteria found in water are derived from the fruit. Serratia was the only bacterium intrinsic in water sample, which is commonly reported from natural water bodies (KÄMPFER; GLAESER, 2016). Regarding eukaryotic group, it was observed a superior prevalence of Saccharomycetales (19.83%) and *Hanseniaspora* (15.13%) in the coffee beans after depulping compared to the surface of the coffee fruits (≤ 1.0%) (Figure 6). The same was observed for *Leuconostoc*, with 17.06% and ≤1.0% incidence in depulped coffee beans and on the coffee fruit surface, respectively. This indicates a probable endophytic nature of LAB and fermentative yeasts in coffee fruits, as already observed in strawberry, soy, sugar beet, pumpkin fruit, and grapes (COMPANT *et al.*, 2008; RODRÍGUEZ *et al.*, 2011).

Figure 6: Composition of bacteria (A) and fungi (B) from coffee farm samples. Only microorganisms with prevalence superior to 5% are showed. The complete list of minor microbial groups is reported in Appendix 1.



Source: Author (2022)

The least-found sequences were commonly associated with microbial groups originating from native soil (*Edaphobacter*, *Niastella*, *Ramlibacter*, and *Adhaeribacter*), tropical freshwater bodies surrounding the farms (*Arthrospira*, *Limnohabitans*, and *Lophiostoma*), alimentary and digestive canal of coffee pests (*Chryseobacterium*, *Acinetobacter*, *Faecalibacterium*, and *Ochrobactrum*), and human contact (*Micrococcus*, *Aspergillus*, *Veillonela*, *Corynebacterium*, and *Malassezia*) (KOCH *et al.*, 2008; WELCH; MACIAS; BEXTINE, 2015), of which 223 have not been previously reported associated with coffee farm (Appendix 1). The presence of these microorganisms differs from those found in coffee plantations from Colombia and Mexico (CABRERA-RODRÍGUEZ *et al.*, 2020; JUNQUEIRA *et al.*, 2019), indicating an intrinsic microbial diversity and activity for each *terroir*. This microbial *terroir* concept has already been observed in wine, where significant

discrepancies on the bacterial and fungal composition of vineyard soil, vine bark, and grape were observed between producing regions within a same country (BOKULICH *et al.*, 2013; MORRISON-WHITTLE; GODDARD, 2018).

2.4.3 Fermentation diversity and dynamics

The core microbiome of coffee fermentation in Honduras, defined as the phylotypes detected in all fermentation samples with at least one proportional abundance equal or superior to 1%, revealed the presence of 11 bacteria and 7 fungi (Figure 7 and 8). *Gluconobacter*, *Leuconostoc*, and *Acetobacter* within the prokaryotic group and *Candida*, *Pichia*, *Fusarium*, and *Hanseniaspora* in the eukaryotes were the most prevalent microorganisms. Although Enterobacteriaceae was the family with most representants within the core microbiome (*Enterobacter, Lelliottia, Kosakonia, Kluyvera,* and *Pantoea*), its maximum proportional abundance only reached 4%. The low frequency of Enterobacteriaceae members was also reported in coffee fermentations conducted in Brazil, Colombia, and Ecuador (CARVALHO NETO *et al.*, 2018; JUNQUEIRA *et al.*, 2019; POTHAKOS *et al.*, 2020). Despite enterobacteria were found in high proportions on the aerial parts of coffee plant (Figure 6), their growth is limited during coffee beans fermentation due to the production of lactic acid and other antimicrobial metabolites produced by lactic acid bacteria (PEREIRA *et al.*, 2016). This relationship of microbial antagonism is desirable to inhibit the production of off-flavour metabolites produced by enterobacteria species (NDAYAMBAJE *et al.*, 2019).

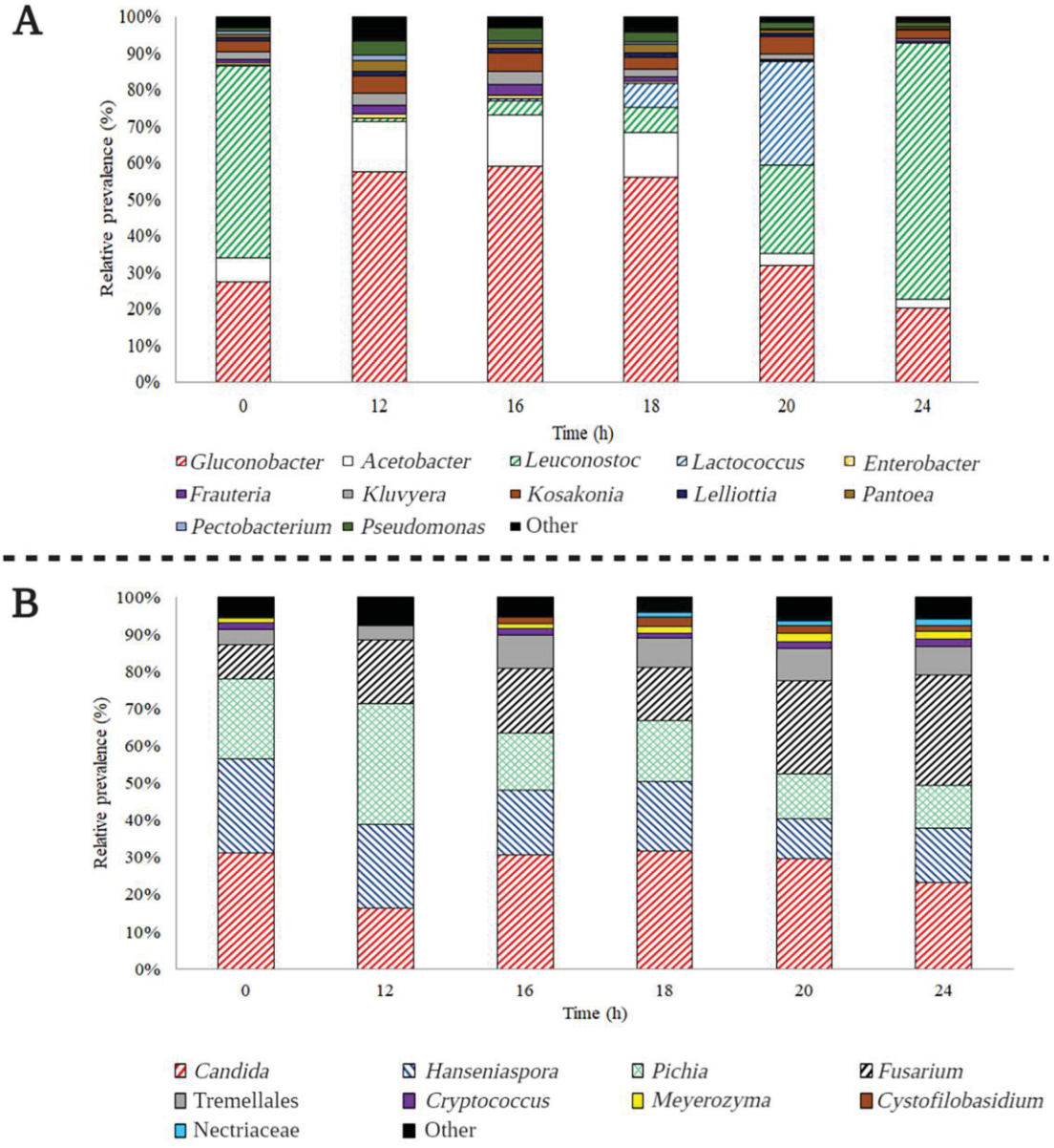
Temporal analysis shown in Figure 8 revealed that, despite the high bacterial diversity found in the coffee farm environment, several microorganisms are suppressed by the growth and dominance of LAB and AAB. *Leuconostoc* (52.47%) was dominant at the beginning of the fermentation process, followed by *Gluconobacter* (27.20%) and *Acetobacter* (6.75). After 12 h, OTU reads assigned to *Gluconobacter* increased significantly to 57.40%, suppressing the growth of *Leuconostoc* and other species. At the end of the process, *Leuconostoc* reads increased to 69.92% sharing dominance with *Gluconobacter* (20.16%). High frequency of *Leuconostoc* and other LAB have been detected in coffee fermentation performed in Brazil, Colombia, Ecuador, and China (CARVALHO NETO *et al.*, 2018; JUNQUEIRA *et al.*, 2019; POTHAKOS *et al.*, 2020; ZHANG *et al.*, 2019), while *Gluconobacter* was for the first time reported as part of the dominant microflora. *Gluconobacter* was recently identified as the main microbial group in the faeces of the civet cat (*Paradoxurus hermaphroditus*) used to produce *kopi*

luwak coffee, which is generated by the cleaning, drying, and roasting the coffee beans that were excreted in faeces by this animal. The authors suggested that the colonization of AAB in the gut of civet cat was related with the diet based on ingestion of coffee cherries containing these microorganisms (WATANABE *et al.*, 2020). *Kopi luwak* coffee is considered one of the most expensive commodities among the coffee matrix, and metabolomic approaches revealed the presence of discriminant markers commonly produced by *Gluconobacter*, such as glycolic and malic acid (JUMHAWAN *et al.*, 2013; PUTRI; FUKUSAKI, 2015; TURKIA *et al.*, 2010; WEI *et al.*, 2009). Further studies should address the relationship between *Gluconobacter* and coffee quality. The farm microbiome suggests that depulped fruit was the major source of this microorganism (Figure 6). These results open new avenues for the implementation of 'Protected Designation of Origin' in coffee beans according to their discriminatory microflora, as already observed in other fermented products(PIRAINO *et al.*, 2005).

The fermentation process was characterized by constant dominance changes between three major species in the fungal core microbiome – i.e., *Candida*, *Pichia*, and *Hanseniaspora* (Figure 7B). These species have been frequently isolated in spontaneous coffee fermentations performed in Australia, Brazil, Colombia, China, Ecuador, and Tanzania (EVANGELISTA *et al.*, 2015; JUNQUEIRA *et al.*, 2019; PEREIRA *et al.*, 2014; POTHAKOS *et al.*, 2020; ZHANG *et al.*, 2019, 2022). *Pichia* and *Hanseniaspora* are associated with flavour-active ester production impacting distinct floral and fruity notes in coffee beverages, while *Candida* species are known to assist in the process of coffee removing mucilage through the production of pectinolytic enzymes (GIORELLO *et al.*, 2019; MASOUD; JESPERSEN, 2006; MASOUD; POLL; JAKOBSEN, 2005; PEREIRA *et al.*, 2014; VALE *et al.*, 2019). In addition to yeasts, filamentous fungi belonging to the Order Tremellales and *Fusarium* were detected within the core microbiome (Figure 7B and 8B). These saprophytic organisms are commonly found in the soil and rhizosphere of important crop commodities, including coffee, soy, sorghum, and corn (LI; HARTMAN, 2003; MUSOLI *et al.*, 2008). The unusual growth of these fungal groups during fermentation can be attributed for microbial interaction with rhizobacteria identified in this work (*Serratia*, *Bacillus*, and *Stenotrophomonas*). A study performed by MINERDI et al., (2008) revealed that these bacteria are linked to the fungal hyphae via fibrillar-like structures, reducing hyphae hydrophobicity, and promoting fungi growth in liquid medium (SIQUEIRA; LIMA, 2012). In addition, the presence of bacterial proteins

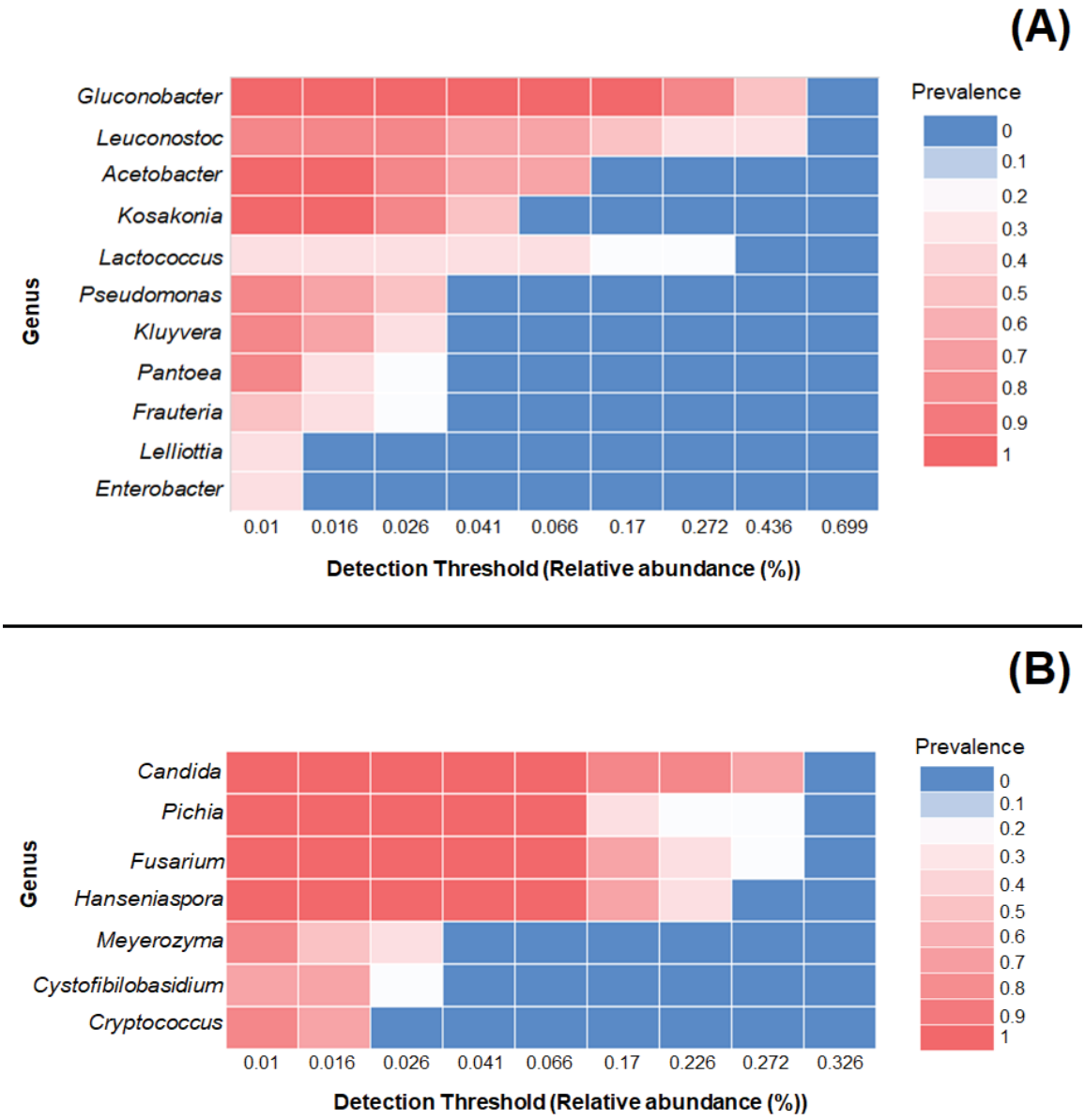
involved in nutrient-uptake in this consortium can favors microbial nutritional exchange (MORETTI *et al.*, 2010). Finally, the metagenetic analysis revealed a great diversity of least prevalent eukaryotes during fermentation process, with over 110 genera of filamentous fungi and yeasts that are commonly isolated from soil and aerial parts of coffee plant. The complete list of genera and families identified in this study can be found in the Appendix 2

Figure 7: Prevalence and detection threshold of the highly persistent bacteria (A) and fungi (B) detected during coffee beans fermentation in Honduras. Only microorganisms with prevalence superior to 1% are showed. The complete list of minor microbial groups is reported in Appendix 2.



Source: Author (2022)

Figure 8: Prevalence and detection threshold of the highly persistent bacteria (A) and fungi (B) in coffee beans fermentation.



Source: Author (2022)

2.4.4 Sugar consumption and target metabolite analysis

The changes in major non-volatiles (sugars, organic acids, and ethanol) and volatiles metabolites were monitored during the fermentation (Table 1). Coffee pulp has 0.42 g l^{-1} of glucose, 1.07 g l^{-1} of fructose and a pH of 5.96. It is possible to observe a significant increase in the content of reducing sugars during the initial 16 h of fermentation. This observation is associated with the action of microbial-derived enzymes that hydrolyze the complex coffee pulp carbohydrates (pectin, cellulose, sucrose) into their monomers (AVALLONE *et al.*, 2001; SRINIVAS MURTHY; M. MADHAVA

NAIDU, 2011). After this initial increase, both glucose and fructose were partially consumed until the end of the process. Lactic acid was the major organic acid produced, achieving a concentration of 0.93 g l^{-1} at 24 h (Table 1). This primary metabolite is mainly produced by the central carbon metabolism of homo-and heterofermentative LAB (PEREIRA, et al., 2020). Lactic acid is well-known for attributing desirable lactic sensorial notes and contributing for the acidity and body of the final beverage (PEREIRA *et al.*, 2016). Ethanol showed a steady increase throughout the fermentation, indicating a high metabolic activity of the non-*Saccharomyces* yeasts(ELHALIS *et al.*, 2020; ELHALIS; COX; ZHAO, 2020). Despite the dominance of *Gluconobacter* during the fermentation, acetic acid production was inferior to 1 g l^{-1} (Table 1), similar to spontaneous coffee fermentation conducted in Brazil, Colombia, and China (FENG *et al.*, 2016; JUNQUEIRA *et al.*, 2019; PEREIRA *et al.*, 2016). The low acetic acid yield can be associated with the central carbon metabolism of *Gluconobacter*, which is able to incorporate only 9% of glucose and fructose into the cytoplasmatic matrix. Due the absence of several genes involved in the Embden–Meyerhof–Parnas (EMP) pathway and the tricarboxylic acid (TCA) cycle, *Gluconobacter* has a unique and complex carbon metabolic system characterized by the incomplete oxidation of a wide range of sugars, alcohols, and polyols (BRINGER; BOTT, 2016). In this central carbon metabolism, most of the glucose and fructose (~ 91%) is oxidized in the periplasm *via* a membrane-bound, pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase, generating a flux of electrons that enters directly in the respiratory chain to produce ATP (RICHHARDT; BRINGER; BOTT, 2013). Although this oxidative pathway results in a low cell yield (0.09 $g_{cdw}/g_{glucose}$) (KRAJEWSKI *et al.*, 2010), the dispensability of sugar phosphorylation reactions and the low nitrogen requirement may have provided the significant energetic and nutrient-competitive advantages for *Gluconobacter* during coffee fermentation (RAUCH *et al.*, 2010). In addition to the high energetic efficiency, the oxidative metabolism of glucose and alcohols produces intermediate products, such as 2-methyl-butanoic acid and gluconic acid, that are precursors of several flavour-active compounds associated with desirable fruity aroma(KELIANG; DONGZHI, 2006). Finally, the detection of citric acid only at 24 h can be resulted of the incomplete tricarboxylic acid (TCA) cycle in *Gluconobacter* due to the lack of succinate hydrogenase, which leads to the accumulation of citrate in the extracellular matrix (WATANABE *et al.*, 2020).

Table 1. Concentration of volatile compounds (area*10^5), organic acids, ethanol, and reducing sugars (g l^{-1}) during spontaneous coffee beans fermentation in Honduras.

Compounds	Fermentation assay (h)					
	0	12	16	18	20	24
GCMS						
Higher alcohols (3)						
1-Butanol, 3-methyl	ND	0.27 ± 0.19^a	0.18 ± 0.05^a	0.28 ± 0.04^a	0.20 ± 0.00^a	0.19 ± 0.00^a
1-Butanol, 2-methyl	ND	0.15 ± 0.00^a	0.12 ± 0.00^a	0.24 ± 0.01^b	0.20 ± 0.00^c	ND
1-Propanol	ND	ND	ND	1.25 ± 0.34	ND	ND
Ester (4)						
Ethyl acetate	3.03 ± 0.59^a	11.77 ± 0.65^b	12.50 ± 0.73^b	21.87 ± 1.07^c	27.93 ± 0.48^d	33.44 ± 3.00^d
Methyl acetate	4.09 ± 0.88^a	2.50 ± 0.85^a	1.85 ± 0.41^a	4.10 ± 0.46^a	4.71 ± 0.67^a	2.58 ± 1.81^a
Propanoic acid, ethyl ester	ND	0.86 ± 0.23^a	1.58 ± 0.19^a	3.45 ± 0.16^b	3.93 ± 0.36^b	3.08 ± 0.00^b
Butanoic acid, ethyl ester	ND	ND	0.11 ± 0.00^a	0.25 ± 0.15^a	0.15 ± 0.00^a	0.42 ± 0.00^a
Organic acid (1)						
Acetic acid	1.71 ± 0.60^a	2.40 ± 0.03^a	4.81 ± 0.40^b	5.01 ± 0.02^b	4.81 ± 1.10^b	5.25 ± 0.00^b
Aldehyde (1)						
Benzaldehyde	ND	ND	ND	ND	ND	0.56 ± 0.00
HPLC						
Glucose	0.42 ± 0.15^a	2.26 ± 0.40^b	2.39 ± 0.06^b	2.15 ± 0.06^{bc}	1.74 ± 0.03^c	0.97 ± 0.11^d
Fructose	1.07 ± 0.03^a	5.07 ± 0.08^b	5.44 ± 0.10^c	5.27 ± 0.04^{bc}	4.59 ± 0.08^d	4.52 ± 0.12^d
Lactic acid	ND	0.27 ± 0.01^a	0.44 ± 0.01^b	0.60 ± 0.01^c	0.67 ± 0.02^d	0.93 ± 0.04^e
Acetic acid	ND	0.18 ± 0.01^a	0.27 ± 0.01^b	0.32 ± 0.01^b	0.38 ± 0.01^c	0.41 ± 0.04^c
Citric acid	ND	ND	ND	ND	ND	0.21 ± 0.00
Ethanol	ND	0.19 ± 0.03^a	0.28 ± 0.02^b	0.42 ± 0.02^b	0.42 ± 0.02^b	0.71 ± 0.06^c

ND = not detected.

Source: Author (2022)

A total of nine flavor-active compounds were detected during the fermentation process, including higher alcohols (three compounds), esters (five compounds), organic acids (one compound), and aldehydes (one compound). Among these, ethyl acetate showed a greater variation, ranging from 3.03 to 33.44. Other ester compounds also increased during the fermentation process. These results are similar to those found in Brazil (PEREIRA *et al.*, 2015, 2016), but it was only in Honduras that ethyl acetate was found in higher proportions then other organic compounds. The high accumulation of this acetate ester may be associated with the presence of different dominant yeasts during the fermentation process (Figure 7). *Candida*, *Pichia*, and *Hanseniaspora* have been reported in literature as responsible for the ethyl acetate production in maize dough, coffee, and wine fermentations, respectively (ANNAN *et al.*, 2003; MOREIRA *et al.*,

2008; PEREIRA *et al.*, 2014). The diffusion and persistence of ethyl acetate into the beans contributes for the development of desirable fruity notes and grape/cherry nuances in the coffee beverage (PEREIRA *et al.*, 2019).

Both diversity and concentration of higher alcohols (e.g., 1-butanol, 3-methyl, 2-methyl-1-butanol, and 1-propanol) were significantly inferior when compared to spontaneous coffee fermentations conducted in Colombia and China (JUNQUEIRA *et al.*, 2019; ZHANG *et al.*, 2019, 2022). This low accumulation can be explained by the superior production of esters, since higher alcohols are important precursors in the esterification reaction conducted by yeasts (PEREIRA *et al.*, 2019). These findings are in agreement to those found in spontaneous wine fermentations, where the strong dominance of *Candida*, *Pichia*, and *Hanseniaspora* during the initial 3–4 days resulted in a superior production of esters and beverages with improved aroma complexity (DAVID *et al.*, 2014; PORTILLO; MAS, 2016).

2.5 Conclusion

Ripe coffee fruits harbour beneficial microorganisms (LAB, AAB, and aroma-producing yeasts) for the fermentation process, and cleaning procedures can prevent the growth of enterobacteria, *Colletotrichum*, *Bacillus*, and other unwanted microbial groups presents in leaves, fruit surface, over-ripe fruits, and coffee farm soil. The Honduran spontaneous coffee beans fermentation process was characterized by the dominance of *Leuconostoc*, *Gluconobacter*, and three different yeasts (*Candida*, *Pichia*, and *Hanseniaspora*). The metabolic activity of these microbial groups resulted mainly in the production of lactic acid, ethanol, ethyl acetate, and methyl acetate. Interestingly, *Gluconobacter* was first reported as dominant in the coffee fermentation process. The farm microbiome revealed that this microbial genus was originated from the surface of coffee beans and may be associated with the production of high-quality coffee through the production of glycolic and malic acids. These data contribute to a better understanding of microbiome composition and open perspectives on their application in the enhancement of coffee fermentation process, such as production of high-quality coffee beans, selection of specific microbial groups for flavour modulation, and potential candidates for biological markers of Honduran coffees. However, the microbial *terroir* from different coffee producing regions should be deeply investigated before their practical application.

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3. Chapter 3: Understanding the effects of self-induced anaerobic fermentation on coffee beans quality: microbiological, metabolic, and sensory studies

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3.1 Abstract: In this study, an investigation of the microbial community structure and chemical changes in different layers of a static coffee beans fermentation tank (named self-induced anaerobic fermentation—SIAF) was conducted at different times (24, 48, and 72 h). The microbial taxonomic composition comprised a high prevalence of Enterobacteriaceae and Nectriaceae and low prevalence of lactic acid bacteria and yeast, which greatly differs from the traditional process performed in open tanks. No major variation in bacterial and fungal diversity was observed between the bottom, middle, and top layers of the fermentation tank. On the other hand, the metabolism of these microorganisms varied significantly, showing a higher consumption of pulp sugar and production of metabolites in the bottom and middle layers compared to the top part of the fermentation tank. Extended processes (48 and 72 h) allowed a higher production of key-metabolites during fermentation (e.g., 3-octanol, ethyl acetate, and amyl acetate), accumulation in roasted coffee beans (acetic acid, pyrazine, methyl, 2-propanone, 1-hydroxy), and diversification of sensory profiles of coffee beverages compared to 24 h of fermentation process. In summary, this study demonstrated that SIAF harbored radically different dominant microbial groups compared to traditional coffee processing, and diversification of fermentation time could be an important tool to provide coffee beverages with novel and desirable flavor profiles.

3.2 Introduction

The sharp rise in the specialty coffee market has been partly driven by the continuous growth of out-of-home consumption in Europe, the United States, Brazil, and Australia (BARBOSA *et al.*, 2020; FERREIRA; FERREIRA, 2018; TELES; BEHRENS, 2019). To reach this market, companies have intensified research to diversify the sensory profile of coffee beverages. The influence of the coffee tree genotype, the climate of the producing region (precipitation, humidity, temperature, and radiation), edaphic factors (acidity/alkalinity and soil fertility), and postharvest processing has been widely studied (GOMES *et al.*, 2022; PEREIRA *et al.*, 2019; SCHOLZ *et al.*, 2018; SIMMER *et al.*, 2022). The combination and interaction of these factors are unique to each region, and

small changes have a major impact on the sensory profile of the final beverage (ALEX *et al.*, 2016; CHENG *et al.*, 2016).

In recent years, new post-harvest processing methods have been developed to modulate the chemical structure of coffee beans. The greatest diversification has occurred in the coffee bean fermentation process, in which the natural sugars in the coffee cherry are converted into alcohol, lactic acid, and a range of other secondary metabolites (PEREIRA *et al.*, 2019; VALE *et al.*, 2021a). Although this process is often associated with coffee bean mucilage removal, several studies have shown that microbial metabolites diffuse into the beans and alter the quality of the final beverage (LEE *et al.*, 2015b; SALEM *et al.*, 2020; ZHANG *et al.*, 2019, 2022). CARVALHO NETO et al., (2018b, 2020) recently proposed the use of a stirred-tank reactor (STR) and starter culture to standardize coffee fermentation, which is usually carried out spontaneously in open tanks. Fermentations conducted in STR were able to improve coffee beverage quality by about 8 points (82.25–91.5) when compared to the traditional process. This increase was associated with temperature and agitation control enabled by the STR method. However, a techno-economic analysis conducted by MAGALHÃES JÚNIOR et al., (2021) showed that, for STR implementation, an investment of about USD 1.4 million is required, which limits the access of this technology to small coffee producers.

Recently, a new low-cost fermentation method (called self-induced anaerobiosis fer-mentation—SIAF) performed in a cylindrical polyethylene tank has been proposed as an alternative for coffee growers (MOTA *et al.*, 2022) Several processing configurations have been evaluated. For example, the so-called “natural” fermentation method, which consists in fermenting the whole fruit (i.e., without pulping) or with pulped fruit in static and closed fermentation tanks, without (MARTINEZ *et al.*, 2022; PEREIRA *et al.*, 2022b) or with (CASSIMIRO *et al.*, 2022) the use of water. Regardless of the configuration used, this new processing method has resulted in a significant improvement in the final beverage when compared to conventional processing. It is speculated that the anaerobic environment can positively modulate the fermentative performance of the indigenous microbiota, as well as increase the production of desired compounds, such as lactic acid, esters, aldehydes, and ketones. However, the microbial succession of fermentations performed by the SIAF method has been little explored, and dedicated studies have characterized bacterial and fungal communities only at the end of the fermentation process (MARTINEZ *et al.*, 2021, 2022; PEREIRA *et al.*, 2022b). Therefore, this work aimed to understand the microbial ecology and metabolites formed

during standard (24 h) and extended (48 and 72 h) fermentation in the bottom, middle, and top layers of a static fermentation tank. Finally, sensory analysis was performed to determine the impact of processing time on the quality of the final beverage.

3.3 Materials and Methods

3.3.1 Coffee fermentation process

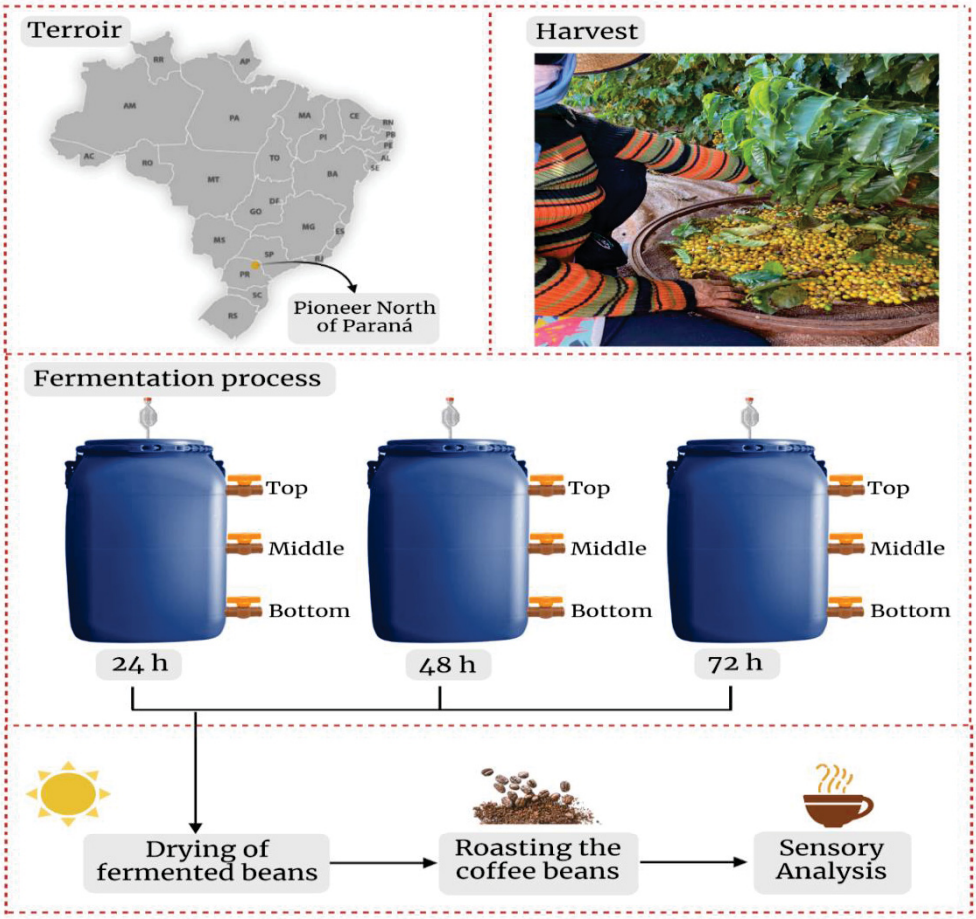
Fermentations were conducted at California farm (23°08′58.42″ S 50°01′39.33″ W; 650 m above sea level) located in Pioneer North of Paraná (Jacarezinho, Paraná, Brazil). Coffee cherries (*Coffea arabica* var. Catuaí Amarelo IAC 86) were harvested in July 2021 and mechanically pulped. About 175 kg of the pulped coffee beans were transferred to polypropylene fermentation tanks containing 30 L of water previously decontaminated with ozone. These fermentation tanks were cylindrical, 82 cm in height, and 47 cm in diameter. In addition, the fermentation tank contained three taps to perform the sample collection in the bottom, middle, and top layers (i.e., 15, 44, and 72 cm from the base of the fermentation tank, respectively), as shown in (Figure 1). The bioreactors were closed with a screw cap containing an airlock to allow the CO_2 generated during fermentation to escape and to prevent air from entering the bioreactor (self-induced anaerobioses fermentation—SIAF). Then, a standard fermentation of 24 h and two extended fermentations of 48 and 72 h were performed. The experiments were conducted in triplicate and about 50 mL of the fermentation liquid fraction was collected at 0, 6, 12, 18, 24, 30, 36, 48, 60, and 72 h for microbiological and physicochemical analyses. After the fermentations, coffee beans were washed and dispersed in a raised beds for drying until they reached a moisture content of approximately 12%.

3.3.2 Determination of microbial diversity by high-throughput sequencing

DNA was extracted from samples collected at 0, 12, 24, 36, 48, and 72 h from the bottom, middle and top layers using Power Soil Kit (Qiagen). DNA was quantified with a Nanodrop spectrophotometer (Thermo Scientific). The variable regions V3-V4 of the 16S rRNA gene of bacteria and the ITS region of fungi were amplified from the extracted total DNA using primers 341F/805R and 3F/805R, respectively, and both barcoded with Nextera indexes, according to manufacturer instructions (Illumina). The amplicons were quantified with Qubit DNA HS kit (Thermo) and sequenced with MiSeq Reagent 500v2 kit (Illumina), in paired 2x250b. The fastq files were filtered and demultiplexed with bcl2fastq (Illumina). Taxonomic identification was performed with QIIME2. Shannon,

Simpson, and Chao index were calculated and used to determine the alpha and beta diversity of the microbial communities

Figure 1: Flowchart showing the steps performed from harvest to sensory analysis.



Source: Author (2022)

3.3.3 Analysis of sugar consumption and organic acid production in the liquid fermentation fraction

High-Performance Liquid Chromatography (HPLC) was used to determine the concentration of sugars (glucose and fructose) and organic acids (lactic, acetic, malic, succinic, citric, and propionic acids) from the bottom, middle, and top layers of fermentation tanks. Aliquots of 2 mL were centrifuged at 12,000× rpm for 10 min and filtered through a 0.22 µm pore size filter (Millipore Corp., Billerica, MA, USA). The samples were analyzed in an HPLC (Agilent Technologies, Waldbronn, Germany) coupled to a diode matrix (DAD) and refraction index (RID). The separation of the compounds was obtained using a Hiplex-H column (300 × 7.7 mm) (Bio-Rad, Richmond, CA, USA) with an isocratic mobile phase composed of 4.0 mM H2SO4, with a flow rate of 0.5 mL min^{-1} for 30 min. The temperatures of the column and RID detector used during the entire race were 70 and 50 °C, respectively. The quantification of organic acids was

performed in DAD at 210 nm, while reducing sugars were determined in RID (PREGOLINI *et al.*, 2021).

3.3.4 GC/MS analysis of the fermentation liquid fraction

The volatile organic compounds generated during fermentation were identified by Gas Chromatography Coupled to Mass Spectrophotometry (GC/MS). For sample preparation, aliquots (3 mL) collected from the bottom, middle, and top layers at 0, 12, 24, 48, and 72 h were disposed of in hermetically sealed vials (20 mL). The samples were analyzed by Solid Phase Microextraction (SPME), using a DVB/CAR/PDMS Fiber (Supelco Co., Bellefonte, PA, USA). The SPME fiber was exposed for 30 min at 60 °C. The compounds were thermally desorbed at 260 °C and directly introduced into the gas chromatograph. The GC was equipped with a capillary column (model SH-Rtx-5MS; 30 m × 0.25 mm × 0.25 μm). The temperature within the GC was as follows: column oven at 60 °C, injection at 260 °C, and detector at 250 °C. Helium was the carrier gas used, at a flow rate of 1 mL/min, column press of 57.4 kPa, and split ratio of 1:20. The mass spectrophotometry range was 30–250 (*m*/*z*), at an ion source temperature of 250 °C. Volatiles were identified by comparing each mass spectrum either with the spectra from authentic compounds or with spectra in reference libraries. The relative abundance of each volatile compound present in the headspace was shown as peak area times 10^5 (JUNQUEIRA *et al.*, 2019).

3.3.5 GC/MS analysis of green and roasted coffee beans

The volatile profile of green coffee beans (i.e., fermented and dried coffee beans) processed for 24, 48, and 72 h was also determined by GC/MS. Briefly, beans with approximately 12% moisture were ground and the particles were standardized with a 0.35 mm sieve. Then, 3 g of the ground coffee was transferred into a hermetically sealed vial (20 mL).

To perform the analysis of the roasted beans, a roasting curve was developed to preserve the cellular structure of the beans using a Probat Leogap equipment model Probatino (Curtiba, Brazil). A lower initial temperature (130 °C) was used for this process, followed by a gradual increase in temperature, allowing for a longer roast (10:30 min) and a 17.4% development period (1:50 min after the first crack). A Javalytics handheld digital colorimeter model JAV-RDA-H was used 2 h after roasting to check the roast level according to the Specialty Coffee Association (SCA) protocol of 63 Agtron

(https://sca.coffee/research/coffee-standards, accessed on 10 November 2021). Then, the roasted beans were also ground, and the particles were standardized to 0.35 mm and transferred to the vial. The injection parameters used for the green and roasted beans followed the procedures described in the previous topic.

3.3.6 Sensory Analysis

To determine the effect of time on final beverage quality, roasted beans processed for 24, 48, and 72 h were prepared according to SCA recommendations (https://www.scaa.org/PDF/resources/cupping-protocols.pdf, accessed on 15 November 2021). Five cups containing 8.25 g of coffee and 150 mL of water were prepared for each sample. Then two certified Q-Graders were asked to describe and score the attributes aroma, flavor, aftertaste, acidity, body, balance, uniformity, clean cup, sweetness, and overall quality on a scale of 6 to 10 in intervals of 0.25. To facilitate the visualization of the sensory profile of each coffee, a sensory wheel was built in the Tastify® application (https://www.tastify.com/, accessed on 20 July 2022).

3.3.7 Statistical Analysis

The data obtained from the analyses of sugar consumption and production of volatile organic compounds were analyzed by post-hoc comparison of means by Tukey's test. Analyses were performed using the Statistica program, version 10.0 (Statsoft Inc., Tulsa, OK, USA). The level of significance was established using a two-sided *p*-value (<0.05). Pearson's correlation coefficient was used to calculate the correlations between the dominant microbial groups with the main metabolites generated in fermentation. A principal component analysis (PCoA) based on weighted UniFrac Distances was constructed using the relative percentage peak area data of roasted coffee beans obtained on GC/MS.

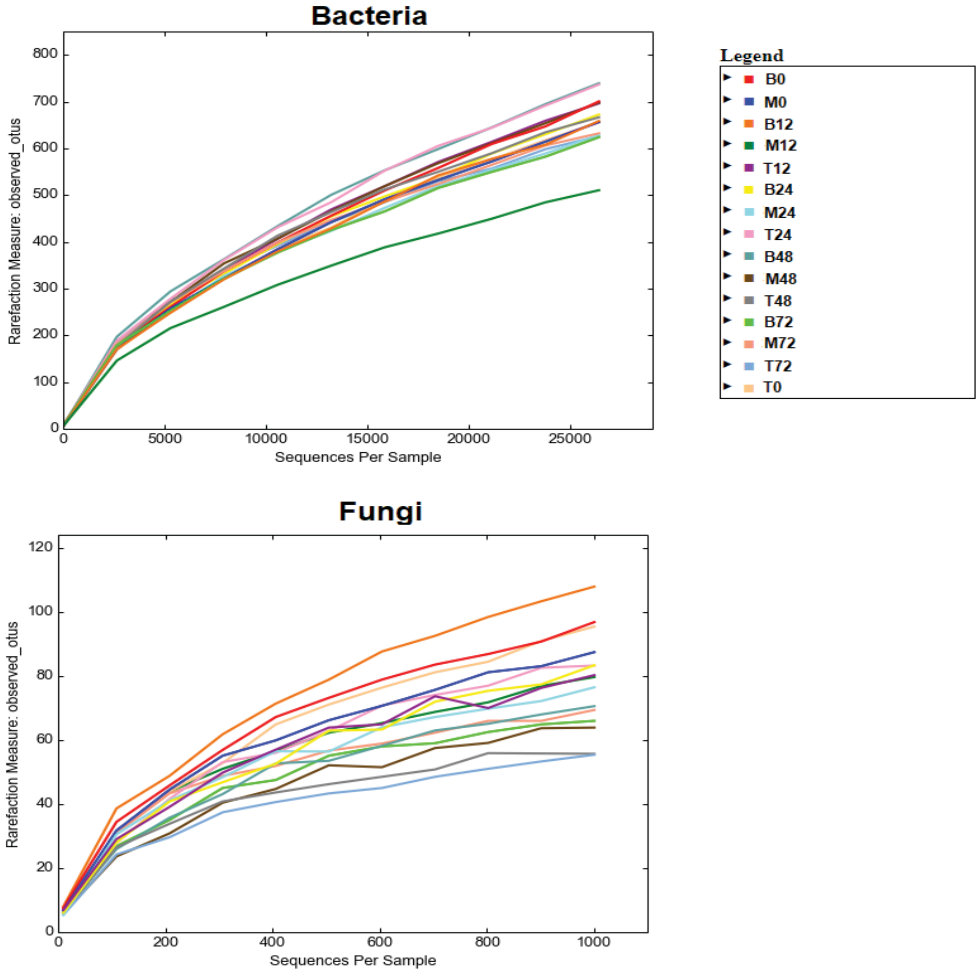
3.4 Results

3.4.1 Alpha and beta diversity analysis

A total of 800,118 and 80,740 sequences were obtained from the 16S and ITS genes, respectively. The alpha rarefaction curves from each analysis suggest that most of the microbial population was sampled (Figure 2). The microbiome involved in coffee fermentation is known to have a significant effect on the quality of the final beverage. Thus, there has been a major effort to elucidate the structure of the microbial community and the mechanisms of action behind this niche. Therefore, Shannon and Simpson indices

were calculated to estimate the diversity of each sample (collected from different layers of the fermentation tank and processes conducted at different times), while richness was determined by the Chao index. The indices showed non-significant differences in microbial diversity and richness between the bottom, middle, and top layers of the fermentation tank (Table 1). However, fermentation periods longer than 24 h showed a reduction in sample richness due to a decrease in the number of subdominant species, since the Chao estimator gives more weight to microbial groups with low frequency (KIM *et al.*, 2017). On the other hand, bacterial diversity increased throughout fermentation while fungal diversity decreased. Diversity depends not only on richness but also on equity, i.e., homogeneity of species distribution in a community. Therefore, the increase in Shannon and Simpson values may be associated with the uniformity of the bacterial community due to the gradual increase in the number of sequences related to the Lactobacillaceae family (Appendix 3)

Figure 2: Alpha rarefaction curves of the OTUs (operational taxonomic units) observed from the fermentation liquid fraction samples. (A) Bacterial analysis, (B) Fungal analysis.



Source: Author (2022)

Table 1: Microbial community richness and diversity in different layers of a static coffee beans fermentation tank conducted at different times (24, 48, and 72h).

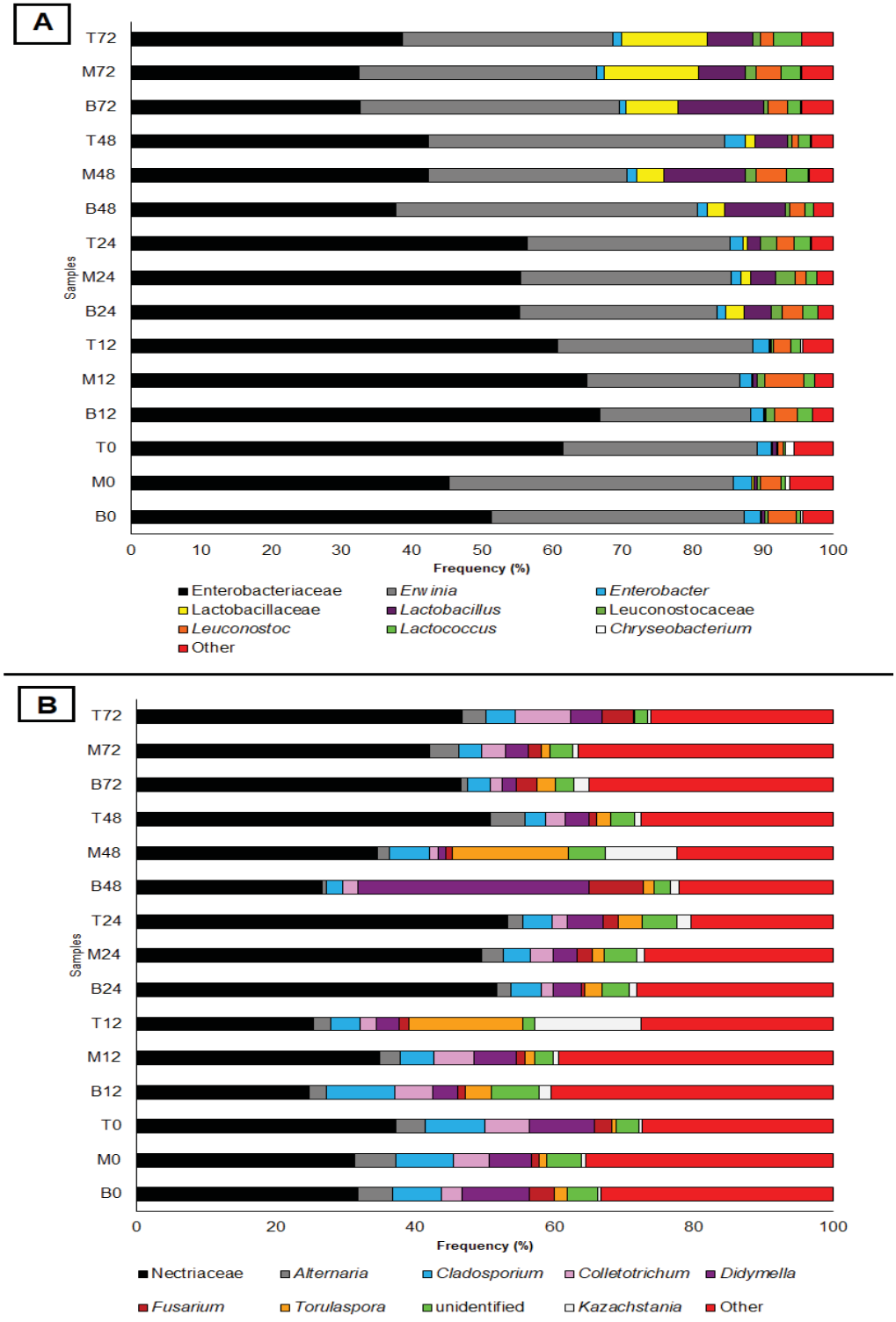
Sample	Bacteria indices			Fungi indices		
	Chao	Shannon	Simpson	Chao	Shannon	Simpson
B0	1686.7	1.56	0.68	149.4	2.79	0.87
M0	1391	1.65	0.69	143.9	2.73	0.87
T0	1526.9	1.52	0.64	179.5	2.59	0.83
B12	1740.6	1.44	0.63	179.5	2.98	0.90
M12	1114.1	1.40	0.61	110.2	2.67	0.85
T12	1637.9	1.53	0.66	108.6	2.64	0.87
B24	1424.5	1.56	0.67	137.4	2.19	0.71
M24	1358.3	1.53	0.67	117.8	2.26	0.73
T24	1774.6	1.59	0.68	111.5	2.19	0.70
B48	1628	1.61	0.69	102.4	2.24	0.80
M48	1444.5	1.89	0.77	83.8	2.40	0.83
T48	1358.3	1.62	0.70	74.3	2.16	0.72
B72	1142.7	1.84	0.76	85.2	2.00	0.75
M72	1216	1.94	0.78	88.9	2.52	0.80
T72	1293.3	1.93	0.78	89	2.19	0.75

B= sample taken at the bottom of the fermentation tank; M= sample taken at the middle of the fermentation tank; T = sample taken at the top of the fermentation tank. 0, 12, 24, 48, 72 h of fermentation time.

3.4.2 Microbial Diversity and Dynamics of Fermentation

Next-generation sequencing (NGS) was used to assess the spatial distribution (i.e., bottom, middle, and top layer) of bacteria and fungi in the static-state fermentation system conducted at different times (24, 48, and 72 h). A total of 247 and 92 bacterial and fungal groups were identified, respectively (Appendix 3). Temporal analysis of the fermentation showed that Enterobacteriaceae family, *Erwinia*, and *Enterobacter* represented about 85% of the bacterial composition of the bottom, middle, and top layers of the fermentation tank in the first 24 h (Figure 3A). The marked presence of the Enterobacteriaceae group was also observed at the beginning of the coffee fermentation conducted in Colombia, Ecuador, Australia, and Brazil (ELHALIS; COX; ZHAO, 2020; JUNQUEIRA *et al.*, 2019; PREGOLINI *et al.*, 2021; ZHANG *et al.*, 2019). VALE et al., (2021) and PREGOLINI et al., (2021) showed that these bacteria are associated with coffee fruits collected from the coffee tree, fruits before pulping, and pulped fruits.

Figure 3: Composition of bacteria (A) and fungi (B) of samples collected in the bottom (B), middle (M), and top (T) layers of spontaneous coffee fermentation. Only bacteria and fungi with prevalence greater than 1% and 5% are shown, respectively. The number after each letter represents the fermentation time (e.g., B0= bottom layer at 0 h). The complete list of minor microbial groups is reported in the Supporting Information (Table S1**).**

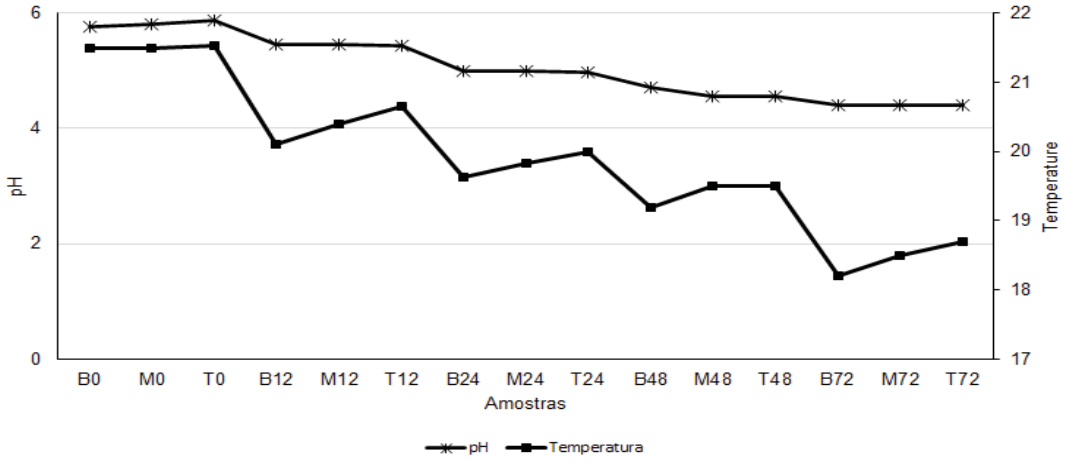


Source: Author (2022)

Lactic acid bacteria (LAB) present at the beginning of fermentation were mainly represented by Leuconostocaceae family (especially *Leuconostoc* genus) and accounted

for 3.94%, 2.81%, and 0.85% of the operational taxonomic unit (OTUs) identified in the bottom, middle, and top layers, respectively (Figure 3A). However, this population was not able to dominate the fermentation and remained stable throughout the fermentation process. On the other hand, a significant increase was observed in the frequency of the Lactobacillaceae family (represented mainly by *Lactobacillus*) in fermentations conducted at 48 and 72 h (Figure 3A). Moreover, a higher frequency was observed in the lower and middle layers at 24 and 48 h of fermentation compared to the top of the fermentation tank (Figure 3A). By 72 h, the relative abundance of Lactobacillaceae was approximately 20% in all three layers. This stabilization may be associated with the reduction of pH from 5.8 to 4.4 (Figure 4) since *Lactobacillus* are classified as acid-tolerant bacteria (POTHAKOS *et al.,* 2020). Furthermore, the acidification of the fermentation medium may also be related to the gradual reduction of enterobacteria (Figure 3A).

Figure 4: Temperature and pH analysis of the bottom (B), middle (M), and top (T) layers during spontaneous fermentation. The number after each letter represents the fermentation time (e.g., B0= bottom layer at 0 h).



Source: Author (2022)

Although Lactobacillaceae showed an increase after 48 h of fermentation, the total LAB population reached low proportions when compared to traditional fermentative processes performed in open tanks (CARVALHO NETO *et al.*, 2018a; POTHAKOS *et al.*, 2020b; ZHANG *et al.*, 2019, 2022). For example, the spontaneous fermentation of Colombian coffee beans performed by JUNQUEIRA et al., (2019) showed that, after 48 h, LAB showed a dominance ≥90%, while in our work, the total frequency was less than

29%. LAB are historically defined as a group of microaerophilic microorganisms (MAKAROVA *et al.*, 2006). Therefore, the anaerobiosis conditions generated by SIAF may not be favorable for LAB growth, since other studies using this processing method also showed low populations of this bacterial group (MARTINEZ *et al.*, 2021, 2022; PEREIRA *et al.*, 2022b).

Filamentous fungi belonging to the Nectriaceae family (e.g., *Fusarium*) and the genera *Cladosporium*, *Alternaria*, *Didymella*, and *Colletotrichum* showed high populations at the beginning of the fermentative process (Figure 3B). These fungal groups have been identified as part of the microbiota of coffee leaves and fruits (PREGOLINI *et al.*, 2021; VALE *et al.*, 2021a). The marked presence of the Nectriaceae family and the *Fusarium* genus has also been observed during coffee fermentation from Australia and Honduras, but the role of these microorganisms has not been reported (ELHALIS; COX; ZHAO, 2020; VALE *et al.*, 2021a). However, ELHALIS; COX; ZHAO, (2020) suggested that the presence of these fungi is due to their ability to produce hydrolytic enzymes (e.g., cellulases and pectinases) supporting their growth during fermentation, as well as contributing to the process of mucilage removal from coffee beans. On the other hand, VALE et al., (2021) associated the high presence of molds in coffee fermentation with a mechanism of fungus–rhizobacteria interaction, where bacteria are attached to fungal hyphae through fibrillar-like structures, reducing the hydrophobicity of the hyphae and promoting fungal growth in liquid medium. However, further studies are still needed to confirm any of these hypotheses.

Torulaspora was the only yeast with a frequency ≥1%, detected in the bottom and middle layers at the beginning of fermentation, while other genera, such as *Pichia, Candida, Saccharomyces, Wickerhamomyces, Kazachstania,* and *Kluyveromyces*, were part of the subdominant population (Appendix 3). The limited yeast growth during fermentation may be associated with the low oxygen availability present in the fermentation system adopted. A study conducted by SALMON; FORNAIRON; BARRE, (1998) showed that, even under anaerobic conditions, yeasts require small amounts of dissolved oxygen to perform the biosynthesis of sterols and unsaturated fatty acids since these compounds are essential for maintaining cell viability. Some studies have also suggested that there is a positive interaction between yeast and LAB. The complex nature of these interactions is highlighted by: (i) yeast autolysis early in the fermentative process provides amino acids, polysaccharides, riboflavin, and other nutrients for LAB growth; (ii) the increase in the LAB population leads to acidification of the medium, making the

environment prone to yeast development (HERVÉ ALEXANDRE; MICHÈLE GUILLOUX-BENATIER, 2006; JUNQUEIRA *et al.*, 2019; VALE *et al.*, 2019). Therefore, inefficient LAB growth and slow acidification of the medium may be associated with the low yeast population throughout the fermentative process.

3.4.3 Profile of sugars consumption, organic acids production, and volatile compounds

At the beginning of the process, a higher concentration of glucose and fructose was observed in the bottom and middle layers compared to the top of the fermentation tank (Table 2). This difference may be associated with partial precipitation of the pulp remaining in the coffee beans after the addition of water to the fermentation tank. In addition, the fructose concentration at the beginning of the fermentative process was about twice as high as glucose, which is a typical carbohydrate profile of the yellow Catuaí coffee variety (MARTINEZ *et al.*, 2022; MOTA *et al.*, 2022).

Table 2: Sugar consumption and organic acids production (g l^{-1}) during spontaneous fermentation.

Time (h)	Glucose			Fructose			Lactic acid			Acetic acid		
	Bottom	**Middle**	**Top**	**Bottom**	**Middle**	**Top**	**Bottom**	**Middle**	**Top**	**Bottom**	**Middle**	**Top**
0	9.12±0.42	8.52±1.05	3.06±0.92	20.65±0.90	16.23±0.77	9.59±0.72	ND	ND	ND	ND	ND	ND
6	8.03±1.16	5.43±1.31	4.4 ±0.82	20.37±0.92	18.14±0.98	13.41±0.25	0.63±0.09	0.41±0.05	0.28±0.08	ND	ND	ND
12	7.66±0.87	5.57±0.87	3.93±0.77	20.61±1.88	15.59±0.41	10.92±1.68	0.91±0.19	0.78±0.23	0.43±0.02	ND	ND	ND
18	6.88±0.23	4.49±1.33	3.53±0.27	19.25±0.59	14.46±1.91	10.98±0.35	2.06±0.38	1.60±0.44	1.10±0.07	0.25±0.03	0.24±0.06	0.14±0.01
24	4.74±0.94	3.63±0.32	3.06±0.61	16.36±0.57	12.37±0.70	8.52±0.14	2.11±0.23	1.66±0.05	1.00±0.02	0.38±0.11	0.36±0.03	0.19±0.00
30	4.56±0.25	2.77±0.32	2.43±0.03	16.10±0.94	10.63±1.04	8.98±0.55	2.36±0.22	2.15±0.02	1.36±0.16	0.54±0.11	0.48±0.06	0.24±0.05
36	5.07±0.70	2.58±0.30	2.29±0.28	17.96±2.00	11.28±1.12	8.19±1.30	2.77±0.28	2.26±0.23	1.39±0.25	0.59±0.06	0.51±0.03	0.26±0.05
48	4.31±0.51	2.04±0.23	1.91±0.14	16.23±1.55	10.62±0.66	9.23±0.39	3.18±0.27	2.83±0.22	2.13±013	0.67±0.00	0.63±0.06	0.50±0.02
60	4.36±0.06	1.23±0.07	1.06±0.04	17.76±0.49	9.56±0.20	5.67±0.13	4.31±0.23	3.24±0.06	2.03±0.02	0.88±0.06	0.71±0.01	0.40±0.04
72	4.47±0.04	1.04±0.12	0.76±0.01	18.67±0.80	11.09±0.46	5.34±0.07	5.06±0.02	4.70±0.02	2.18±0.01	1.00±0.11	0.94±0.00	0.40±0.04

ND = not detected.

Surprisingly, there was no significant consumption of sugars in the top layer in the first 48 h of fermentation. It can be speculated that microorganisms present in this layer needed more time to adapt to the anaerobic environment, as glucose only showed a significant reduction (3.06 ± 0.92 to 0.76 ± 0.01 g/L) at the end of fermentation, while fructose lasted 60 h (9.59 ± 0.72 to 5.67 ± 0.13 g/L) to be partially metabolized (Table 2). Furthermore, a marked consumption of glucose and fructose was observed in the middle layer, especially in the initial 24 h. On the other hand, there was no significant ($P < 0.05$) reduction of fructose in the bottom layer during the whole fermentative process, while glucose was metabolized only in the first 24 h, followed by a stabilization in

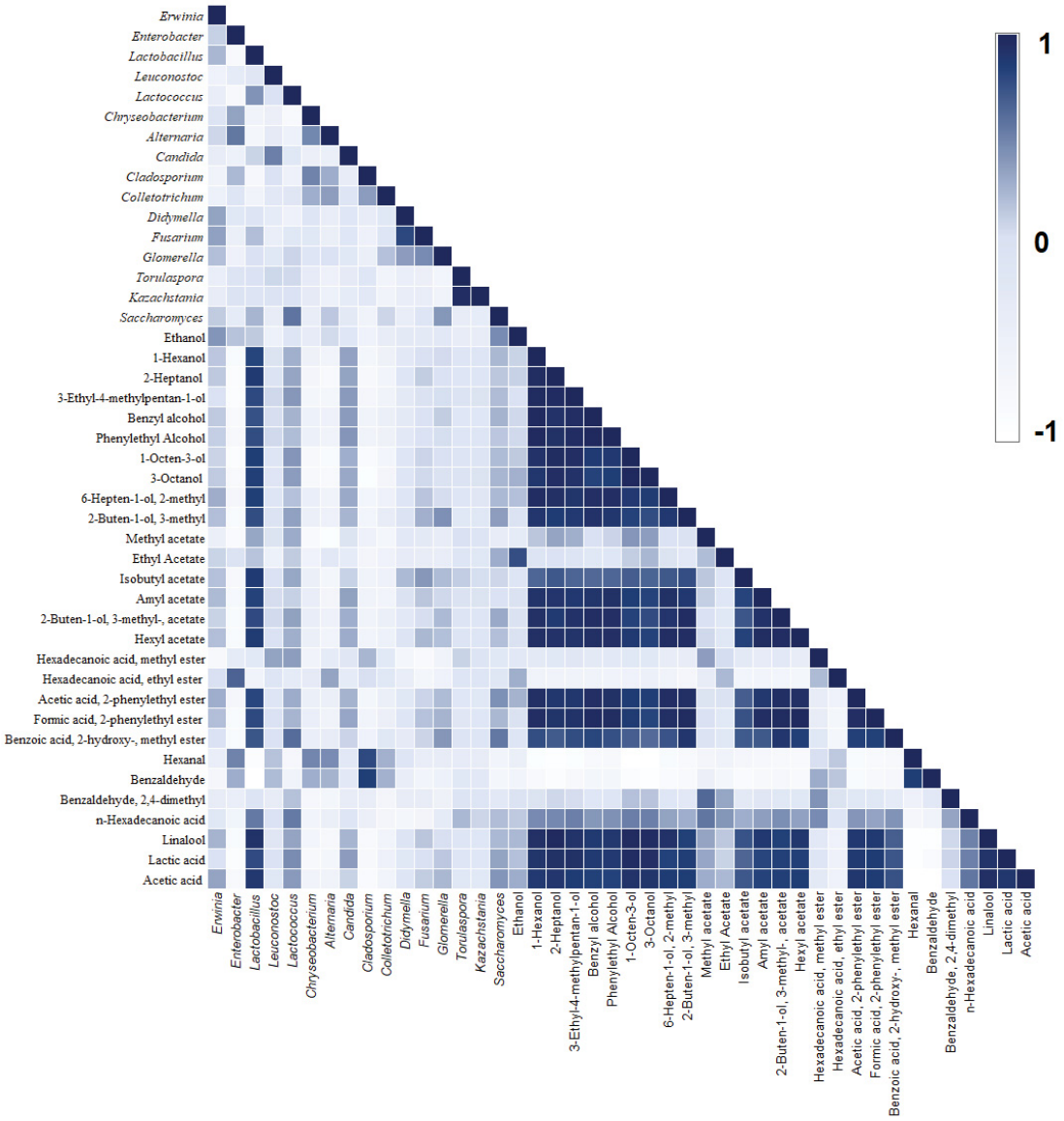
concentration until the end of fermentation. After 72 h, the residual concentration of glucose and fructose in the fermentation tank was 6.27 and 35.10 g/L, respectively. The presence of residual sugars is commonly reported in spontaneous coffee fermentations conducted in different countries (Brazil, Australia, Colombia, and Ecuador) and are usually associated with short fermentation cycles, i.e., between 24 and 36 h (DE BRUYN *et al.*, 2017; ELHALIS; COX; ZHAO, 2020; JUNQUEIRA *et al.*, 2019; PEREIRA *et al.*, 2015; VALE *et al.*, 2019). However, the results showed that only increasing the fermentation time does not guarantee that the indigenous microbiota can consume all the sugars in the mucilage, especially fructose. Thus, an alternative to optimizing fructose consumption is the use of starter cultures that exhibit a fructophilic phenotype, as recently suggested by JUNQUEIRA et al., (2022).

Lactic and acetic acids were the main organic acids produced during fermentation. Lactic acid showed a significant increase ($p < 0.05$), reaching a final concentration of 5.06 ± 0.02, 4.70 ± 0.02, and 2.18 ± 0.01 g/L in the bottom, middle, and top layers, respectively (Table 2); it showed a positive correlation (≥0.45) with microorganisms belonging to the genera *Lactobacillus*, *Lactococcus*, *Candida*, and *Saccharomyces* (Figure5). However, most of the lactic acid was probably produced by *Lactobacillus*, as its population gradually increased from the bottom to the top layer during fermentation. In addition, the lower concentration of lactic acid observed at the top of the fermentation tank may be associated with slower glucose metabolism, as discussed earlier. The production of this organic acid is of great importance for fermentation because it helps in the process of acidification of the medium without interfering with the quality of the final beverage, besides inhibiting the growth of undesirable microorganisms (PEREIRA *et al.*, 2020b).

Acetic acid was detected after 18 h of fermentation and its production gradually increased during the fermentative process (Table 2). However, production was not related to the presence of AAB since this microbial group showed less 1% of read sequences. Furthermore, AAB is known to be strictly aerobic, being able to oxidize alcohols, aldehydes, sugars, or sugar alcohols only in the presence of oxygen (GOMES *et al.*, 2018; LYNCH *et al.*, 2019). Therefore, it is speculated that acetic acid production is associated with the ability of some *Lactobacillus* strains to degrade lactic acid into acetic acid under anaerobic conditions and in acidic environments (OUDE ELFERINK *et al.*, 2001). Although the exact function of this pathway is unclear, some results suggest that it may be associated with the maintenance of cell viability (OUDE ELFERINK *et al.*, 2001).

Furthermore, a positive correlation (0.90) of bacteria belonging to the genus *Lactobacillus* with acetic acid was observed (Figure 5).

Figure 5: Persson correlation matrix of the main microbial groups and volatile compounds identified



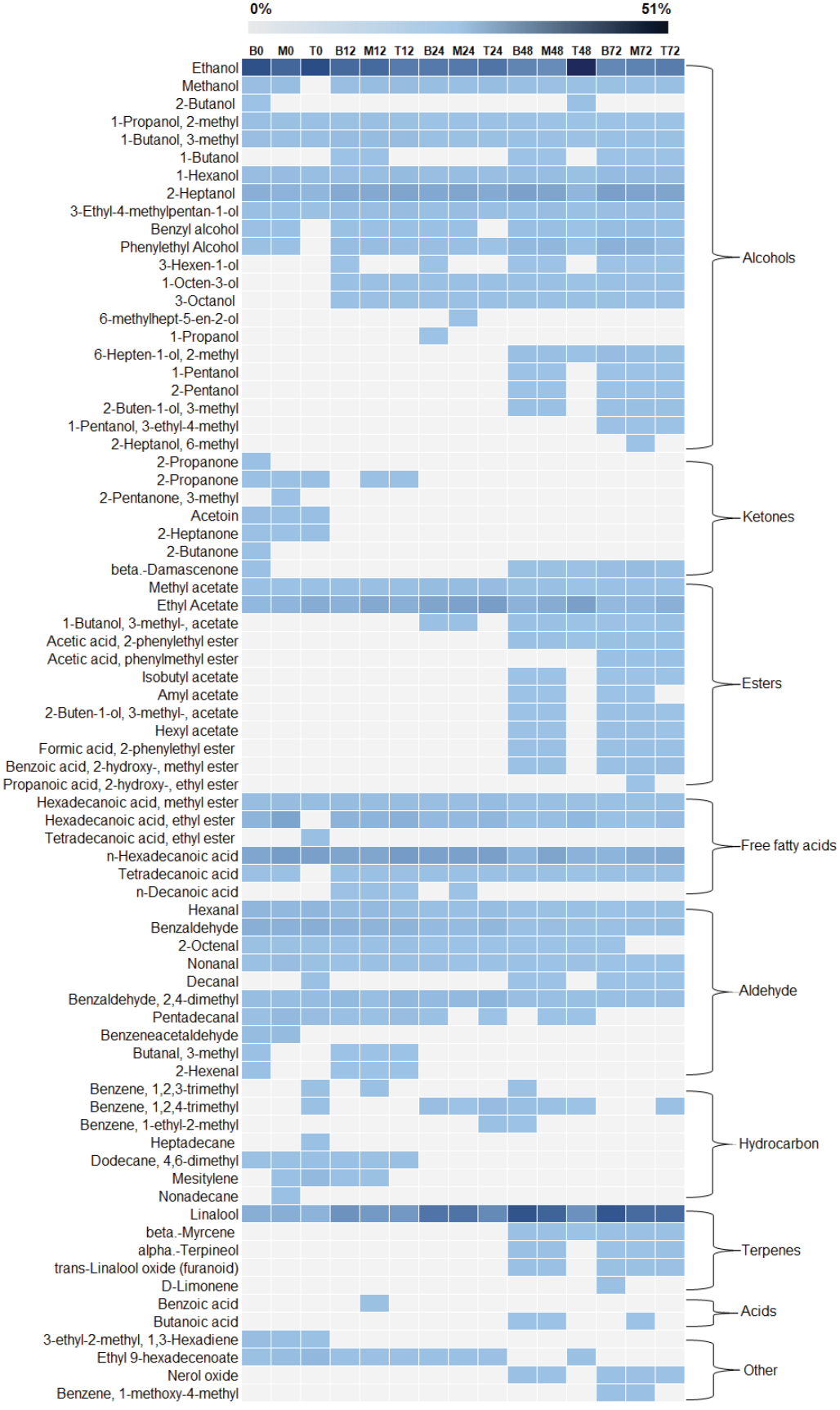
Source: Author (2022)

A total of 76 volatile compounds were identified by HS-SPME/GC in the fermentation liquid fraction. These compounds were grouped into nine chemical groups: alcohols (22), ketones (7), esters (13), free fatty acids (6), aldehydes (10), hydrocarbons (7), terpenes (5), acids (2), and others (4) are shown in (Figure 6 and Appendix 4). Alcohols were the chemical group with the highest relative percentage of peak areas (38.5–59.5%) and, although the intensity of this group varied throughout the

fermentation, only the top layer at 48 h showed a significant difference from the other samples (Figure 7). This difference is mainly associated with a peak in ethanol production, which can be attributed to a discrete increase in the frequency of *Saccharomyces* yeast at the top layer at 48 h. Figure 5 shows the positive correlation between *Saccharomyces* and ethanol. The fermentation time was an important variable in the production of molecules belonging to alcohols since, at the beginning of the fermentation process, only 10 volatile compounds were identified and, after 72 h, 22 molecules were produced (e.g., 1-Octen-3-ol; 3-Octanol; 6-Hepten-1-ol; 2-methyl, 2-Buten-1-ol, and 3-methyl) as shown in Figure 6. Moreover, long fermentation periods also allowed the accumulation of several alcohols (e.g., 1-Hexanol, 2-Heptanol, Benzyl alcohol, and Phenylethyl alcohol) in the liquid fraction of the fermentation and, at the end of the process, the rate of these compounds was also higher in the bottom and middle layers compared to the top of the fermentation tank. However, the relationship between alcohols and coffee quality has not yet been well established, but this chemical group is known for its high sensory threshold. Thus, it is speculated that to modulate the quality of the final beverage, a high concentration of these compounds and an intense diffusion process into coffee beans are required (DZIALO *et al.*, 2017; PEREIRA *et al.*, 2019).

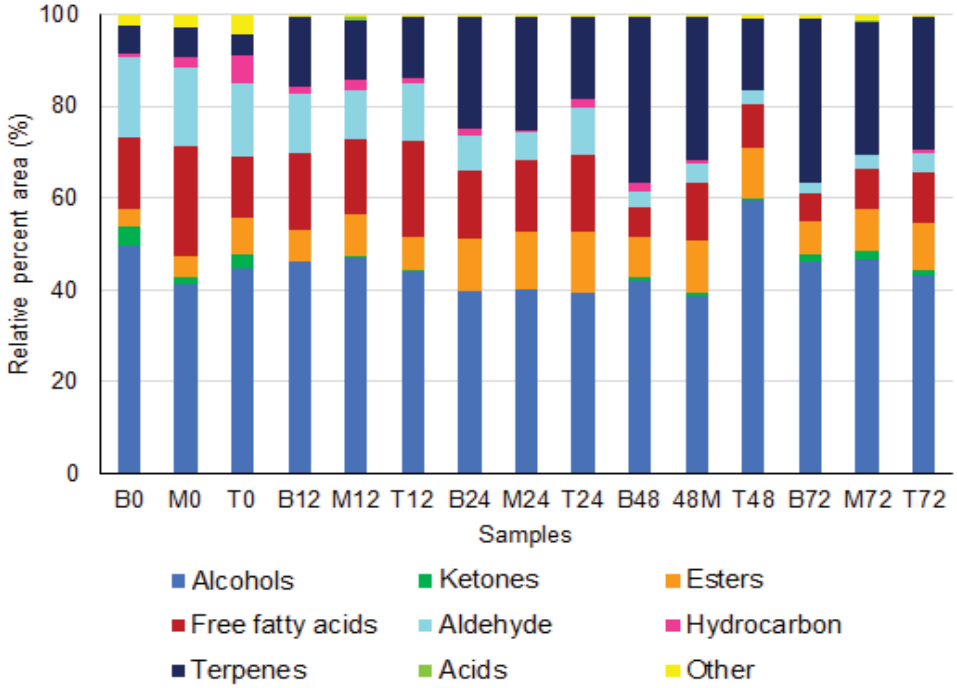
Esters also showed a relatively higher abundance when compared to other coffee fermentation studies (JUNQUEIRA *et al.*, 2019; PREGOLINI *et al.*, 2021; VALE *et al.*, 2019, 2021a). At the beginning of fermentation, this chemical group was mainly represented by methyl acetate and ethyl acetate. Fermentation time was also an important variable for ester production, as, after 48 h, 10 more compounds were produced (e.g., isobutyl acetate, amyl acetate, 2-buten-1-ol, 3-methyl-acetate, hexyl acetate, among others) that remained until the end of fermentation (Figure 6 and Appendix 4). Interestingly, the production of these molecules also showed a high correlation with *Lactobacillus* and alcohols, which was expected, since the generation of esters by *Lactobacillus* occurs through an esterification reaction between fatty acids and an alcohol molecule (PEREIRA *et al.*, 2020b). The accumulation of esters is highly desired, as they can impart floral, fruity, and buttery perceptions in the coffee beverage, even if they are in low concentrations (PEREIRA *et al.*, 2019).

Figure 6: Heatmap of the volatile compounds identified in the bottom (B), middle (M), and top (T) layers. The number after each letter represents the fermentation time (e.g., B0= bottom layer at 0 h).



Source: Author (2022)

Figure 7: Chemical groups identified in the bottom (B), middle (M), and top (T) layers in the fermentation liquid fraction. The number after each letter represents the fermentation time (e.g., B0= bottom layer at 0 h).



Source: Author (2022)

The terpenes were mainly represented by linalool, which showed a significant increase ($p < 0.05$) during the fermentation process. However, like the alcohols and esters, new terpenoids (beta-Myrcene, D-Limonene, trans-Linalool oxide (furanoid), and alpha-Terpineol) were produced in fermentations conducted for longer periods (48 and 72 h). Generally, these compounds originate from the activity of β-glycosidases that release monoterpenes from glycosidically bound precursors (MENDES-FERREIRA *et al.*, 2009; RIVAS *et al.*, 2013) In addition, it is speculated that some yeast species may also produce these molecules via the mevalonic acid pathway (CARRAU *et al.*, 2005; POTHAKOS *et al.*, 2020b). However, the formation of these compounds during coffee fermentation and the impact on the coffee quality is still unclear. On the other hand, no accumulation or production of new compounds belonging to aldehydes and ketones, which are frequently identified in the liquid fraction of spontaneous coffee fermentations, was observed. However, a gradual reduction of some aldehydes (e.g., hexanal, benzaldehyde) is noted throughout the fermentation, while ketones were only detected in the initial 12 (Figure 6).

We speculate that this reduction may be associated with a diffusion process into the coffee beans or that these molecules were precursors for the formation of other volatile

compounds (MARTINEZ *et al.*, 2022; SALEM *et al.*, 2020). Finally, the results showed that, in general, the consumption of sugars and the production of organic acids and volatile compounds showed significant differences among the bottom, middle, and top layers throughout the fermentation, suggesting that although the bacterial and fungal diversity did not show great variations, the metabolism of these microorganisms can change drastically among the layers. Furthermore, the use of a closed fermentation tank can decrease the loss of specific volatile compounds to the environment and increase the diffusion rate of these molecules into the coffee beans; however, this process can be optimized if there is an agitation/revolving process, as recently demonstrated in other works (CARVALHO NETO *et al.*, 2018b, 2020).

3.4.4 Volatile composition of green and roasted coffee beans

3.4.5 Green coffee beans

A total of 29 compounds were identified in the green coffee beans. These volatiles were grouped according to chemical class (Table 3). Ethanol and 1-Hexanol were the main compounds detected. The ethanol content in coffee beans fermented for 72 h was about six times higher than in the beans fermented for 24 h. This accumulation of ethanol may be associated with the induction of a fermentative metabolism due to the hypoxic conditions imposed by the fermentative process (JOËT *et al.*, 2010). Interestingly, some alcohols such as 2-Propanol, 1-methoxy; 2-Hexanol, 5-methyl, 3-Furanmethanol, were only identified in coffee beans fermented for 24 h, while 1-Propanol, 2 methyl was unique to the beans fermented for 72 h (Table 3). The increase in fermentation time resulted in the gradual decrease of some compounds belonging to the aldehydes, for instance: hexanal, nonanal, butanal, 3-methyl, and heptanal. A recent work by SALEM et al., (2020) evaluated the diffusion process of ester, alcohol, and aldehyde into coffee beans and showed that butanal was the compound that showed the fastest decrease among the molecules evaluated. The authors suggest that this reduction is due to a degradation mechanism of this compound inside the coffee beans.

Among the esters, only ethyl acetate and pentatonic acid ethyl ester were detected in coffee beans fermented for 72 h. Although ethyl acetate was the main ester detected in the liquid fraction of the fermentation, the presence of this compound was not observed in the green beans fermented for 24 and 48 h. Thus, it is speculated that the diffusion process of ethyl acetate may be relatively slow. Furthermore, the results indicate that other compounds (e.g., 1-Hexanol, 1-pentanol, 1-Butanol, 3-methyl, 1-Propanol, 2-methyl, and

2-pentanone, 3-methyl) detected in the green coffee beans may also come from microbial metabolism.

Table 3: Volatile compounds (area * 10^5) identified in fermented green coffee beans.

Compounds	Green beans		
	24 h	**48 h**	**72 h**
Alcohols			
Ethanol	0.73 ± 0.03^{A}	2.30 ± 0.27^{B}	6.69 ± 0.34^{C}
2-Propanol, 1-methoxy	0.10 ± 0.01	ND	ND
1-Butanol, 3-methyl	0.20 ± 0.02^{A}	0.34 ± 0.01^{B}	0.21 ± 0.02^{A}
1-Pentanol	0.28 ± 0.03^{AB}	0.26 ± 0.02^{A}	0.35 ± 0.02^{B}
2-Hexanol, 5-methyl	0.30 ± 0.28	ND	ND
2,3-Butanediol	0.24 ± 0.01^{AB}	0.20 ± 0.01^{A}	0.25 ± 0.01^{B}
3-Furanmethanol	0.55 ± 0.02	ND	ND
1-Hexanol	1.30 ± 0.02^{A}	1.15 ± 0.09^{B}	0.81 ± 0.02^{C}
1-Propanol, 2 methyl	ND	ND	0.03 ± 0.00
Aldehydes			
Butanal, 3-methyl	0.19 ± 0.03^{A}	0.16 ± 0.01^{A}	0.15 ± 0.01^{A}
Butanal, 2-methyl	0.11 ± 0.01^{A}	0.10 ± 0.01^{A}	0.07 ± 0.00^{B}
Pentanal	0.31 ± 0.03^{A}	0.27 ± 0.03^{AB}	0.21 ± 0.03^{A}
Hexanal	1.51 ± 0.16^{A}	1.19 ± 0.28^{AB}	0.92 ± 0.06^{B}
Heptanal	0.16 ± 0.07^{A}	0.12 ± 0.02^{A}	0.09 ± 0.00^{A}
Nonanal	0.53 ± 0.02^{A}	0.46 ± 0.10^{AB}	0.33 ± 0.05^{B}
Decanal	0.16 ± 0.01^{A}	0.15 ± 0.03^{A}	ND
Furans			
Furan, 2-methyl	0.12 ± 0.02^{A}	0.10 ± 0.00^{A}	0.22 ± 0.03^{B}
Furan, 2-pentyl	0.16 ± 0.01^{A}	0.22 ± 0.01^{A}	0.19 ± 0.04^{A}
Esters			
Ethyl Acetate	ND	ND	0.21 ± 0.01
Pentanoic acid, ethyl ester	ND	ND	0.17 ± 0.02
Acids			
Butanoic acid, 3-methyl	0.21 ± 0.02^{A}	0.16 ± 0.01^{A}	0.18 ± 0.03^{A}
Acetic acid	0.20 ± 0.00^{A}	0.62 ± 0.24^{B}	0.47 ± 0.06^{AB}
Ketones			
2-Propanone, 1-hydroxy	0.10 ± 0.00	ND	ND
5-Hepten-2-one, 6-methyl	0.08 ± 0.00^{A}	0.04 ± 0.00^{B}	0.11 ± 0.00^{C}
2-Propanone	ND	0.85 ± 0.11	ND
2-Pentanone, 3-methyl	ND	0.04 ± 0.00^{A}	0.03 ± 0.01^{A}
Pyrazines			
2-Isobutyl-3-methoxypyrazine	0.93 ± 0.01^{A}	1.03 ± 0.12^{A}	0.72 ± 0.05^{B}
Terpenes			
D-Limonene	ND	0.13 ± 0.02	ND
Hydrocarbon			
Nonane, 3-methyl-5-propyl	0.12 ± 0.01^{A}	0.11 ± 0.00^{A}	0.07 ± 0.00^{B}

ND= not detected

Means of triplicate in each row bearing the same letters (A, B, and C) are not significantly different ($p > 0.05$) from one another using Tukey's test (mean standard variation).

3.4.6 Roasted beans

A total of 70 compounds were identified among the roasted coffee beans. The most abundant chemical groups (i.e., largest relative peak area) were furans, alcohols, ketones, pyrazines, and acids. In general, there was not much variation in the number of volatile compounds identified among the coffee beans fermented by 24, 48, and 72 h. However, a significant increase in the content of several compounds (e.g., 2-furancarboxaldehyde 5-methyl; furfural; ethanol; 2-propanone, 1-hydroxy; 2,3-Pentanedione; 4-hydroxy-3-methylacetophenone; 2-furancarboxylic acid, methyl ester; butanal, 3-methyl; acetic acid among others) is noted in the coffee beans fermented for long periods (48 and 72 h), as shown in (Table 4). These compounds are formed by numerous biochemical reactions (e.g., Maillard reactions, pyrolysis, and Strecker degradation) that occur during the roasting process. For example, furans are heterocyclic compounds that exhibit high volatility and are generated during the Maillard reaction, with sucrose, glucose, and linoleic acid being their potential precursors (ANESE; SUMAN, 2013; CASSIMIRO *et al.*, 2022).

The alcohols were the second most abundant chemical group. This high content is mainly due to the accumulation of 3-furanomethanol, and its production can be partially attributed to the Cannizzaro reaction, in which furfural acts as a reactant (BI *et al.*, 2021; PFAMHAUSER, 1993). However, the impact of this chemical compound on the final beverage is not yet known. On the other hand, ketones (e.g., 2,3-Pentanedione, acetoin, 2-propanone) and pyrazines (e.g., pyrazine, 2,3-dimethyl, pyrazine, 2-ethyl-6-methyl), detected in roasted beans, can impart a buttery, creamy, sweet, nutty, and fruity flavor in the final coffee beverage (PEREIRA *et al.*, 2019).

Acetic acid was the main organic acid detected in roasted coffee beans. However, the impact of acetic acid on coffee quality is still unclear (KWON *et al.*, 2015; MOTA *et al.*, 2022). For example, (CHINDAPAN; SOYDOK; DEVAHASTIN, 2019) speculated that acetic acid and formic acid may contribute to coffee acidity, as well as impart a fruit-like flavor when they are at low concentrations, but an unpleasant taste at higher concentrations. However, the authors did not determine this threshold for roasted coffee beans. On the other hand, LIU et al., (2019a) showed that pre-treating green coffee beans with acetic acid were able to significantly increase the quality of the final product. Interestingly, roasted beans fermented for 48 h showed a higher rate of ester and aldehydes, as shown in (Table 4). Like ketones and pyrazines, these chemical groups can also attribute a fruity, floral, and buttery aroma; however, there should be a balance

between the compounds present in coffee beans, since the overall flavor of the coffee depends not only on the concentration but also on the detection threshold of each compound and its interactions with other volatile and non-volatile molecules (PEREIRA *et al.*, 2019).

Table 4: Volatile compounds (area * 10^5) identified in roasted beans

Compounds	Roasted beans		
	24 h	**48 h**	**72 h**
Furans			
Furan, 2-methyl	0.37 ± 0.06^{A}	1.46 ± 0.24^{B}	1.00 ± 0.11^{C}
Furan, 2,5-dimethyl	0.06 ± 0.01^{A}	0.12 ± 0.01^{B}	0.12 ± 0.01^{B}
2-Vinylfuran	0.23 ± 0.07^{A}	0.26 ± 0.02^{A}	0.22 ± 0.04^{A}
3(2H)-Furanone, dihydro-2-methyl	1.87 ± 0.03^{A}	2.72 ± 0.12^{B}	2.63 ± 0.25^{B}
Furfuryl formate	2.44 ± 0.12^{A}	2.90 ± 0.02^{B}	2.69 ± 0.12^{AB}
2,5-Dimethylfuran-3,4(2H,5H)-dione	2.39 ± 0.16^{A}	3.24 ± 0.21^{B}	3.93 ± 0.08^{C}
5-Ethylfurfural	ND	ND	0.30 ± 0.01
Furfural	77.06 ± 2.54^{A}	101.21 ± 1.78^{B}	110.10 ± 2.09^{C}
2-Furancarboxaldehyde, 5-methyl	75.75 ± 2.74^{A}	87.10 ± 3.56^{B}	82.83 ± 3.49^{AB}
Alcohols			
Ethanol	ND	0.22 ± 0.02^{A}	1.12 ± 0.05^{B}
3-Furanmethanol	84.08 ± 3.09^{A}	96.17 ± 3.65^{B}	96.76 ± 1.58^{B}
Propanol, 2 methyl	ND	0.63 ± 0.05	ND
Ketones			
2-Propanone, 1-hydroxy	17.44 ± 0.53^{A}	25.24 ± 2.32^{B}	28.24 ± 2.26^{B}
2,3-Pentanedione	2.08 ± 0.12^{A}	2.99 ± 0.16^{B}	3.03 ± 0.23^{B}
Acetoin	0.67 ± 0.03^{A}	1.31 ± 0.05^{B}	1.28 ± 0.04^{B}
2-Propanone	1.64 ± 0.21^{A}	1.73 ± 0.13^{A}	1.64 ± 0.05^{A}
1-Hydroxy-2-butanone	1.02 ± 0.04^{A}	1.47 ± 0.12^{B}	1.59 ± 0.12^{B}
3-Pentanone, 2-methyl	0.69 ± 0.02	ND	ND
Ketone, 2-furyl methyl	12.77 ± 0.49^{A}	15.92 ± 0.43^{B}	15.33 ± 0.32^{B}
2,5-Hexanedione	0.43 ± 0.02^{A}	0.55 ± 0.01^{A}	0.46 ± 0.09^{A}
2-Butanone, 1-hydroxy-, acetate	1.43 ± 0.15^{A}	1.59 ± 0.05^{A}	2.26 ± 1.34^{A}
1-Propanone, 1-(2-furanyl)	0.55 ± 0.06^{A}	0.79 ± 0.03^{A}	0.68 ± 0.04^{A}
3-Ethyl-2-hydroxy-2-cyclopenten-1-one	0.90 ± 0.06^{A}	1.07 ± 0.03^{A}	0.97 ± 0.03^{A}
2-Acetyl-3-methylpyrazine	8.47 ± 0.43^{A}	9.15 ± 0.60^{A}	10.83 ± 0.32^{A}
2-Propanone, 1-hydroxy-, acetate	8.68 ± 0.35^{A}	10.03 ± 0.33^{A}	8.82 ± 0.30^{A}
2-Acetyl-5-methylfuran	4.38 ± 0.54^{A}	5.16 ± 0.39^{A}	4.77 ± 0.07^{A}
Resorcinol, 2-acetyl	3.42 ± 0.56^{A}	4.95 ± 0.50^{A}	4.36 ± 0.04^{A}
4-Hydroxy-3-methylacetophenone	21.92 ± 4.30^{A}	27.58 ± 2.84^{A}	29.02 ± 0.20^{A}
Pyrazines			
1,3-Diazine	1.71 ± 0.13^{A}	2.07 ± 0.04^{AB}	2.27 ± 0.21^{B}
Pyrazine, methyl	24.12 ± 0.28^{A}	29.88 ± 0.74^{B}	34.60 ± 1.45^{C}
Pyrazine, 2,6-dimethyl	34.55 ± 1.30^{A}	38.35 ± 0.14^{A}	43.27 ± 2.48^{B}
Pyrazine, ethyl	2.91 ± 0.19^{A}	3.31 ± 0.08^{AB}	3.75 ± 0.33^{B}
Pyrazine, 2,3-dimethyl	0.99 ± 0.08^{A}	1.13 ± 0.05^{AB}	1.26 ± 0.10^{B}

Pyrazine, ethenyl	0.84 ± 0.13^{A}	1.10 ± 0.11^{B}	1.20 ± 0.00^{B}
Pyrazine, 2-ethyl-6-methyl	5.67 ± 0.27^{AB}	4.54 ± 0.18^{A}	6.02 ± 0.75^{B}
Pyrazine, trimethyl	6.27 ± 0.17^{A}	6.74 ± 0.15^{A}	9.59 ± 0.52^{B}
Pyrazine, 3-ethyl-2,5-dimethyl	6.35 ± 0.28^{A}	5.40 ± 0.16^{B}	6.70 ± 0.31^{A}
1-(6-Methyl-2-pyrazinyl)-1-ethanone	1.81 ± 0.17^{A}	1.77 ± 0.07^{A}	2.26 ± 0.03^{B}
Pyrazine, 2-methyl-5-(1-propenyl)-, (E)	0.99 ± 0.17^{A}	1.16 ± 0.11^{A}	1.14 ± 0.04^{A}
Pyrazine, 2-methyl-6-propenyl	ND	ND	1.23 ± 0.02
Pyrazine, 2,3-diethyl-5-methyl	ND	ND	0.23 ± 0.00
Acids			
Butanoic acid, 3-methyl	1.06 ± 0.17^{A}	1.95 ± 0.09^{B}	1.96 ± 0.31^{B}
Butanoic acid, 2-methyl	ND	ND	0.18 ± 0.04
Acetic acid	42.44 ± 4.45^{A}	53.49 ± 3.10^{B}	58.60 ± 0.73^{B}
Formic acid	0.62 ± 0.13^{A}	1.14 ± 0.05^{B}	1.24 ± 0.08^{B}
Propanoic acid	0.92 ± 0.07^{A}	1.24 ± 0.05^{B}	1.22 ± 0.08^{B}
2-Butenoic acid, 3-methyl	0.58 ± 0.02^{A}	1.76 ± 0.07^{B}	2.05 ± 0.09^{C}
Pyrroles			
1H-Pyrrole-2-carboxaldehyde	6.35 ± 0.58^{A}	7.67 ± 0.45^{B}	6.65 ± 0.26^{AB}
Ketone, methyl pyrrol-2-yl	8.34 ± 0.61^{A}	9.91 ± 0.71^{B}	9.71 ± 0.17^{AB}
Ethanone, 1-(1-methyl-1H-pyrrol-2-yl)	1.51 ± 0.13^{A}	1.57 ± 0.03^{A}	1.43 ± 0.27^{A}
1H-Pyrrole-2-carboxaldehyde, 1-methyl	8.90 ± 0.07^{A}	9.09 ± 0.71^{A}	ND
1H-Pyrrole, 1-(2-furanylmethyl)	1.80 ± 0.13^{A}	1.86 ± 0.06^{A}	1.76 ± 0.04^{A}
Pyrrole	ND	ND	1.93 ± 0.14
Esters			
Methyl acetate	0.31 ± 0.07^{A}	0.29 ± 0.01^{A}	0.27 ± 0.02^{A}
Propanoic acid, 2-oxo-, methyl ester	0.20 ± 0.03^{A}	0.20 ± 0.03^{A}	0.21 ± 0.03^{A}
Acetic acid, 4-methylphenyl ester	0.38 ± 0.06^{A}	1.90 ± 0.08^{B}	ND
Furfuryl acetate	7.97 ± 0.26^{A}	8.77 ± 0.12^{B}	7.38 ± 0.27^{C}
2-Furancarboxylic acid, methyl ester	1.56 ± 0.17^{A}	2.04 ± 0.20^{B}	2.04 ± 0.04^{B}
Pyrans			
4H-Pyran-4-one, 3-hydroxy-2-methyl	5.20 ± 0.86^{A}	6.84 ± 0.58^{B}	5.77 ± 0.17^{AB}
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	1.05 ± 0.08^{A}	1.88 ± 0.15^{B}	3.46 ± 0.18^{C}
Pyridines			
Pyridine	4.91 ± 0.34^{A}	8.63 ± 0.45^{B}	2.37 ± 0.03^{C}
Pyridine, 2-methyl	0.17 ± 0.07^{AB}	0.17 ± 0.01^{A}	0.28 ± 0.00^{B}
Aldehydes			
Propanal, 2-methyl	0.46 ± 0.03^{A}	0.63 ± 0.05^{B}	0.75 ± 0.05^{B}
Butanal, 3-methyl	0.40 ± 0.04^{A}	0.64 ± 0.01^{B}	0.72 ± 0.03^{B}
Benzeneacetaldehyde	1.88 ± 0.06^{A}	2.34 ± 0.07^{B}	2.18 ± 0.04^{C}
Other			
Cyclopent-4-ene-1,3-dione	1.30 ± 0.09^{A}	1.74 ± 0.07^{B}	1.72 ± 0.22^{B}
1,2-Cyclopentanedione, 3-methyl	2.93 ± 0.93^{A}	2.23 ± 0.10^{A}	1.95 ± 0.08^{A}
4-Methylthiazole	ND	ND	0.15 ± 0.03
2-Cyclopenten-1-one, 3-ethyl-2-hydroxy	ND	ND	0.90 ± 0.04
Terpenes			
Pulegone	ND	ND	3.37 ± 0.09

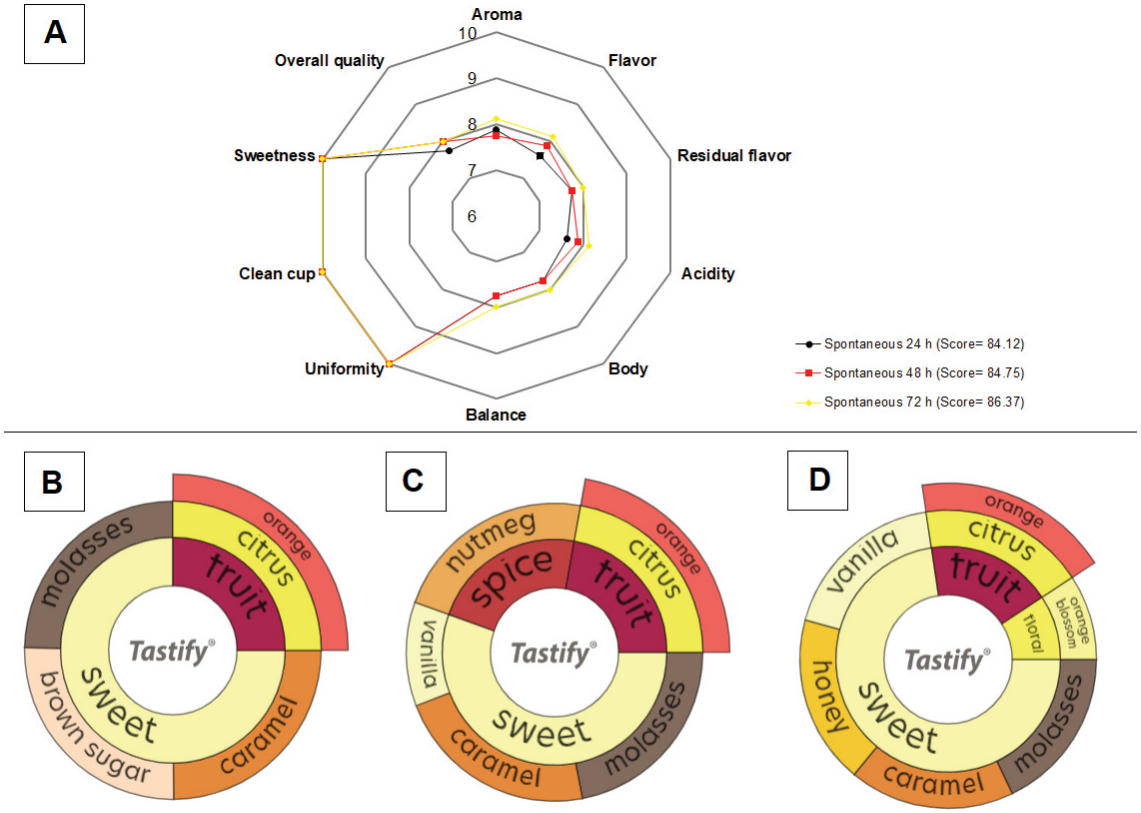
ND= not detected

Means of triplicate in each row bearing the same letters (A, B, and C) are not significantly different ($p > 0.05$) from one another using Tukey's test (mean standard variation).

3.4.7 Sensory analysis

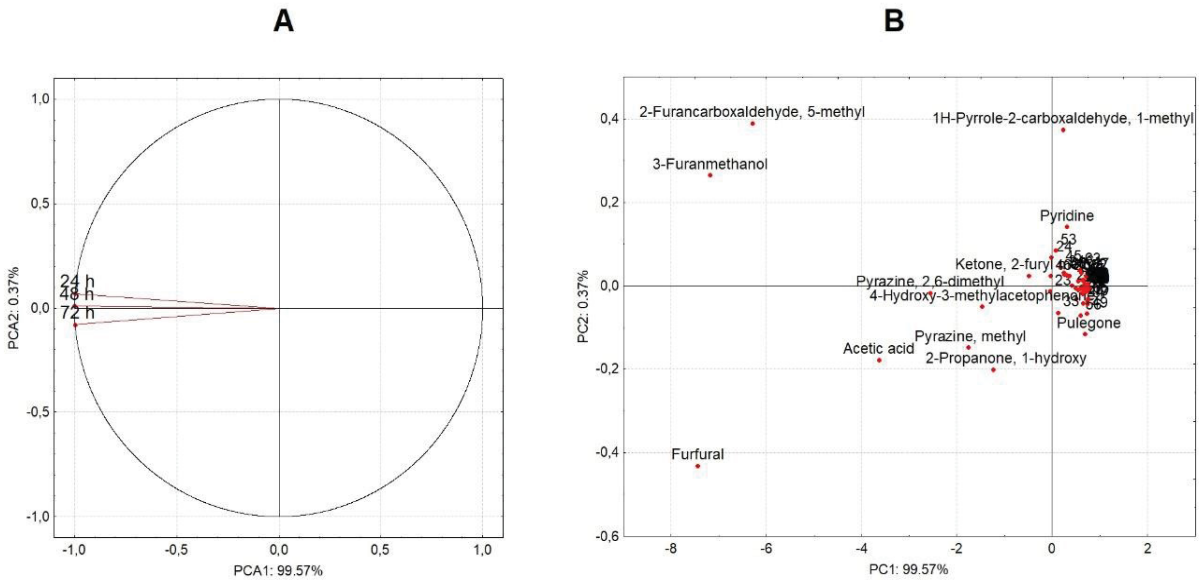
Coffee beverages produced from coffee beans fermented for 24, 48, and 72 h were evaluated by certified Q-Graders according to the SCA methodology All the coffee beverages scored ≥ 84 and were classified as specialty coffees (Figure 8). In general, the increase in fermentation time impacted the perception of aroma, flavor, acidity, body, overall quality, and balance attributes, while sweetness, clean cup, and uniformity showed no difference between treatments. Thus, coffee beans fermented for 48 and 72 h resulted in beverages with greater complexity and intensity compared for 24 h (Figure 8 Intensity is related to acidity, and it is noted that this sensory attribute is highly influenced by processing time. In addition, the presence of acetic acid and formic acid in roasted beans may be associated with the fruity perception detected by the Q-Graders in all treatments (CHINDAPAN; SOYDOK; DEVAHASTIN, 2019; NGUYEN; BYUN, 2013). The body and aftertaste of the beverages produced from the beans processed for 24 and 48 h were described by the Q-Graders as “medium pleasant” and “medium creamy,” while the coffee beans fermented for 72 h were “velvety/dense” and “long silky” (data not shown). To explain the differences observed in the sensory profiles of coffees processed at different times, PCA analysis was constructed based on the relative percentage of volatile compounds identified in the roasted coffee beans (Table 4 and Figure 9). In general, most of the volatiles are part of a single group (Figure 9B). Although the beverage flavor is shaped by a series of interactions between all constituents of the coffee beans, it can be speculated that the differences in the percentage of some compounds (e.g., acetic acid, furfural, pyrazine, methyl, 2-Propanone, 1-hydroxy among others) may be related to the different sensory profiles obtained in this study (Figure 8 and Figure 9). Finally, these results show a positive influence of time on the quality of the final coffee beverage.

Figure 8: Sensorial evaluation of beverages produced with roasted coffee beans. Final score of the processed coffees for different times (A) and description of the sensorial attributes of the fermented coffees for 24 h (B), 48 h (C) and 72 h (D).



Source: Author (2022)

Figure 7: PCA analysis of volatiles detected in roasted coffee beans processed for 24, 48 and 72 h (A) and dispersion of these compounds in the three treatments (B).



Source: Author (2022)

3.5 Conclusion

The SIAF method showed high prevalence of Enterobacteriaceae and filamentous fungi and low prevalence of LAB and yeast. The anaerobic conditions may be the main factor that influenced the growth of LAB and yeast, but further studies should be investigated to prove this hypothesis. Significant variations were observed in sugar consumption and metabolite formation between the different layers of the sampled tank. Agitation of the fermentation tank can be used as a potential way to homogenize microbial metabolism during SIAF, as observed in several other industrial processes. However, further studies are needed to assess the influence of a dedicated turning device for a coffee fermentation tank and coffee beverage quality. Finally, variations in fermentation time showed be a potential tool for delivering coffee beverages with novel and desirable flavor profiles.

3.6 References

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APPENDIX 1

APPENDIX 1: Relative abundance (%) of bacteria and fungi at the genus level detected in environmental samples collected from Honduran coffee plantations. Bacteria reported for the first-time soil, surface of coffee leaves and fruits, and water are highlighted in red asterisk (*)

	Family	Genera	Soil	Ground leaf	Ground fruit	Surface of tree cherry	Tree leaf	Over-ripe fruits	Depulped fruit	Water
Bacteria										
	Leuconostocaceae	*Leuconostoc*	0.05	<0.01	1.22	0.72	1.70	28.17	17.05	1.91
		Oenococcus	-	-	-	-	-	-	0.01	-
		Weissella	-	-	-	-	-	<0.01	-	-
	Acetobacteraceae	*Gluconobacter*	0.05	0.01	8.59	23.60	1.00	10.01	36.11	20.76
		Acetobacter	0.02	<0.01	0.70	3.19	0.61	7.69	22.54	9.52
		Gluconacetobacter	-	<0.01	0.20	0.20	-	0.91	3.12	2.62
		Roseomonas	-	-	0.03	-	0.02	<0.01	-	<0.01
		Roseococcus	-	0.01	-	-	-	-	-	-
		Acidiphilium	<0.01	-	-	-	-	-	-	-
	Enterobacteriaceae	*Klebsiella*	<0.01	0.06	0.06	0.07	<0.01	0.04	0.01	0.03
		Enterobacter	<0.01	0.03	0.15	0.07	0.03	0.82	0.36	0.07
		Pantoea	-	<0.01	<0.01	-	<0.01	<0.01	<0.01	-
		Erwinia	0.01	0.42	0.46	0.37	0.09	0.48	0.05	0.33
		Trabulsiella	-	0.01	0.03	0.01	-	0.07	0.01	0.01
		Citrobacter	-	<0.01	<0.01	-	-	<0.01	-	-
		Providencia	-	-	-	-	-	<0.01	-	<0.01
		Serratia	-	-	-	-	-	-	-	<0.01
		Salmonella	-	-	-	-	-	<0.01	-	-
	Streptococcaceae	*Lactococcus*	0.22	<0.01	-	<0.01	-	0.03	<0.01	6.00
		Streptococcus	0.01	<0.01	-	0.01	-	<0.01	-	-
	Lactobacillaceae	*Lactobacillus*	0.08	-	-	0.06	-	0.08	0.02	0.03
		Pediococcus	0.01	-	-	0.07	0.20	-	<0.01	0.03
		*Sharpea**	<0.01	-	-	<0.01	-	<0.01	-	-
	Pseudomonadaceae	*Pseudomonas*	0.23	4.83	7.40	1.19	2.66	10.10	2.45	11.87
		Methylobacterium	0.11	0.11	0.36	9.54	25.14	0.03	0.02	0.31

	*Balneimonas**	0.09	<0.01		-	-	-	-	-
Sphingomonadaceae	*Sphingomonas*	0.08	0.93	0.14	3.29	8.85	0.05	0.02	0.09
	Novosphingobium	-	-	-	-	-	-	0.02	-
	Sphingobium	0.20	0.47	0.23	0.06	0.28	0.02	<0.01	0.05
	Kaistobacter	1.55	-	-	-	-	-	-	-
	Sphingopyxis	-	0.05	-	-	-	-	-	-
Cytophagaceae	*Dyadobacter*	<0.01	0.36	0.05	<0.01	0.03	0.10	<0.01	0.04
	*Sporocytophaga**	0.30	-	-	-	-	-	-	-
	*Adhaeribacter**	0.21	-	-	-	-	-	-	-
	Spirosoma	-	0.01	-	0.37	1.47	<0.01	<0.01	0.04
	Leadbetterella	-	0.06	0.01	-	-	-	-	0.02
	*Emticicia**	-	0.04	<0.01	-	-	-	-	-
	*Flectobacillus**	-		<0.01	-			-	
	Larkinella	-	0.01	<0.01	-	0.02	-	-	-
	Rudanella	-	<0.01	-	<0.01	0.06	-	-	-
Sphingobacteriaceae	*Pedobacter*	0.10	3.53	0.80	-	<0.01	0.35	0.10	0.53
	Mucilaginibacter	-	-	-	-	-	-	-	-
	Sphingobacterium	0.09	4.11	0.08	-	0.12	1.94	0.11	1.03
Microbacteriaceae	*Curtobacterium*	-	0.02	0.02	<0.01	0.03	0.02	<0.01	<0.01
	*Agromyces**	<0.01	-	-	<0.01	-	-	-	-
	Cryocola	-	0.02	-	<0.01	0.02	<0.01	<0.01	-
	Microbacterium	-	0.01	-	-	-	-	-	-
	Salinibacterium	-	<0.01	<0.01	<0.01	-	<0.01	-	-
Weeksellaceae	*Chryseobacterium*	0.09	2.01	1.15	0.06	0.15	2.12	0.11	1.93
Nitrospiraceae	*Nitrospira*	0.91	-	-	-	-	-	-	-
Rhodobiaceae	*Afifella**	0.23	-	-	-	-	-	-	-
Thermoactinomycetaceae	*Shimazuella**	<0.01	-	-	-	-	-	-	-
Leptospiraceae	*Turneriella**	<0.01	-	-	-	-	-	-	-
Solibacteraceae	*Solibacter*	0.10	-	-	-	-	-	-	-
Paenibacillaceae	*Saccharibacillus*	0.01	0.84	0.06	<0.01	0.01	0.47	0.04	0.28
Planococcaceae	*Lysinibacillus**	0.10	-	-	-	-	-	-	-

	Planococcus	0.02	-	-	-	-	-	-	-
	*Rummeliibacillus**	0.04	-	-	-	-	-	-	-
	Paenibacillus	0.58	0.03	0.02	-	0.01	0.03	0.01	-
	*Cohnella**	<0.01	<0.01	-	-	-	-	-	-
Comamonadaceae	*Acidovorax**	<0.01	0.04	0.01	-	<0.01	<0.01	<0.01	0.03
	*Methylibium**	0.19	-	-	0.01	-	-	-	-
	Comamonas	<0.01	0.17	0.01	-	-	<0.01	<0.01	0.05
	Hylemonella	0.12	0.22	0.02	0.01	-	0.05	<0.01	0.08
	Delftia	0.01	0.03	0.01	-	-	<0.01	<0.01	0.06
	*Limnohabitans**	<0.01	<0.01	-	<0.01	-	<0.01	<0.01	<0.01
	Variovorax	-	<0.01	-	-	-	<0.01	-	-
	*Ramlibacter**	<0.01	0.01	-	-	-	<0.01	-	-
Caulobacteraceae	*Brevundimonas*	-	<0.01	-	-	-	-	-	-
	*Arthrospira**	0.03	-	-	-	-	-	-	-
	Phenylobacterium	0.14	0.13	<0.01	-	-	-	-	-
	*Asticcacaulis**	-	0.03	0.01	-	0.01	-	-	-
	Caulobacter	-	<0.01	-	-	-	-	-	-
Xanthomonadaceae	*Stenotrophomonas*	0.03	3.99	0.20	0.10	0.15	2.55	0.12	3.46
	*Dokdonella**	0.02	-	-	-	-	-	-	-
	Luteimonas	0.05	0.36	-	-	-	0.08	<0.01	<0.01
	*Lysobacter**	0.05	-	-	-	-	-	-	-
	Luteibacter	<0.01	<0.01	0.26	0.29	0.02	0.02	0.04	0.15
	*Pseudoxanthomonas**	0.01	0.11	-	-	-		-	
Streptomycetaceae	*Streptomyces*	0.19	-	-	-	-	-	-	-
Geodermatophilaceae	*Modestobacter**	0.02	-	-	-	-	-	-	-
	Geodermatophilus	0.01	-	-	-	-	-	-	-
Geobacteraceae	*Geobacter*	0.03	-	-	-	-	-	-	-
Koribacteraceae	*Koribacter**	0.12	-	-	-	-	-	-	-
Coxiellaceae	*Rickettsiella**	0.49			-			-	
	*Aquicella**	0.40	-	-	-	-	-	-	-
Pirellulaceae	*Pirellula**	0.58	-	-	-	-	-	-	-
	*A17**	0.06	-	-	-	-	-	-	-

	*Niastella**	0.03	-	-	-	-	-	-	-
	*Flavihumibacter**	0.29	-	-	-	-	-	-	-
	Niabella	-	0.39	0.01	-	-	-	-	-
	Flavisolibacter	0.61	-	-	-	-	-	-	-
	Chitinophaga	<0.01	0.28	0.01	-	-	-	-	-
	Sediminibacterium	<.01	<0.01	-	-	0.02	-	-	-
	Agrobacterium	0.14	11.06	0.28	0.51	0.56	0.51	0.01	0.70
	Rhizobium	0.17	0.01	0.03	-	-	<0.01	-	<0.01
	*Shinella**	-	<0.01	<0.01	-	-	-	<0.01	-
Beutenbergiaceae	*Salana*	-	0.01	-	-	0.01	<0.01	-	-
Flavobacteriaceae	*Flavobacterium*	1.50	4.85	0.59	0.01	-	0.16	0.14	0.39
	Wautersiella	<0.01	0.14	<0.01	0.09	0.06	2.18	0.06	0.96
	Hymenobacter	-	0.01	0.01	0.19	3.78	-	<0.01	-
	*Cloacibacterium**	<0.01	-	0.24	-	-	-	-	-
	Coprococcus	<0.01	-	0.04	<0.01	0.01	<0.01	0.02	0.021
	Blautia	0.01	-	-	0.01	-	<0.01	-	-
	Dorea	<0.01	<0.01	-	<0.01	-	<0.01	-	-
	*Shuttleworthia**	-	-	-	<0.01	-	<0.01	-	-
	Micrococcus	<0.01	-	-	-	-	-	-	-
	*Renibacterium**	<0.01	-	-	-	-	-	-	-
Bacillaceae	*Bacillus*	22.52	<0.01	-	0.02	-	<0.01	<0.01	<0.01
	*Anoxybacillus**	0.01	-	-	-	-	-	-	-
	*Ralstonia**	-	-	-	<0.01	-	-	-	-
	*Cupriavidus**	0.82	-	-	-	-	-	-	-
	Janthinobacterium	0.03	0.86	0.46	0.01	-	0.05	0.09	0.98
Cellulomonadaceae	*Cellulomonas*	0.04	0.06	-	-	-	<0.01	-	-
	*Actinotalea**	-	0.43	-	-	<0.01	-	-	-
Enterococcaceae	*Enterococcus*	0.01	0.07	-	-	<0.01	0.08	<0.01	0.09

	*Vagococcus**	-	-	-	-	-	0.04	-	<0.01
Phyllobacteriaceae	*Mesorhizobium**	0.08	0.05	-	<0.01	-	<0.01	-	-
	Aminobacter	-	0.06	-	-	<0.01	-	-	-
Brucellaceae	*Ochrobactrum*	0.25	0.34	0.03	0.01	-	0.02	<0.01	0.05
	Mycoplana	-	1.02	-	0.01	0.09	0.13	<0.01	0.06
Moraxellaceae	*Acinetobacter*	0.08	0.04	0.05	0.05	0.10	0.92	0.04	1.62
Nannocystaceae	*Plesiocystis**	0.07	-	-	-	-	-	-	-
	Nannocystis	<0.01	-	-	0.01	-	-	-	-
Burkholderiaceae	*Burkholderia*	0.48	<0.01	1.11	0.14	0.47	0.02	0.08	0.17
	*Limnobacter**	-	0.01	0.01	-	-	<0.01	-	-
Rhodospirillaceae	*Azospirillum*	<0.01	-	0.01	-	-	-	<0.01	0.09
	*Inquilinus**	0.01	-	-	-	-	-	-	-
	*Phaeospirillum**	-	0.01	-	-	-	-	-	-
Dysgonamonadaceae	*Dysgonomonas*	0.01	0.01	0.11	-	-	-	-	0.09
Alcaligenaceae	*Achromobacter**	0.06	0.03	>0.01	-	-	<0.01	<0.01	0.05
	Pigmentiphaga	-	0.47	-	-	-	0.06	<0.01	<0.01
Hyphomicrobiaceae	*Devosia**	0.03	0.87	0.01	0.01	-	0.03	<0.01	0.05
	Hyphomicrobium	0.01	-	-	-	-	-	-	-
	Rhodoplanes	5.72	0.01	<0.01	-	0.01	-	-	-
	Pedomicrobium	0.43	<0.01	-	-	-	<0.01	-	-
Cellvibrionaceae	*Cellvibrio*	0.01	0.27	0.01	-	-	-	0.01	0.05
Rhodobacteraceae	*Rhodobacter*	<0.01	2.34	<0.01	-	<0.01	0.03	<0.01	0.04
	*Paracoccus**	<0.01	0.01	-	-	-	-	-	-
	*Amaricoccus**	<0.01	<0.01	-	-	-	-	-	-
Clostridiaceae	*Clostridium*	0.10	-	0.03	0.04	-	0.03	<0.01	0.03
Fusobacteriaceae	*Cetobacterium**	<0.01	0.09	0.03	<0.01	0.26	<0.01	0.04	0.03
Pseudonocardiaceae	*Pseudonocardia*	<0.01	-	-	<0.01	-	-	<0.01	-

	Actinomycetospora	<0.01	-	-	0.39	-	-	-	-
Ruminococcaceae	*Faecalibacterium*	<0.01	-	-	-	-	-	-	-
	*Ruminococcus**	<0.01	-	-	<0.01	-	-	-	-
Rhabdochlamydiaceae	*Rhabdochlamydia**	0.03	-	-	-	-	-	-	-
Pectobacteriaceae	*Dickeya**	<0.01	<0.01	0.13	-	<0.01	0.02	<0.01	0.02
Myxococcaceae	*Anaeromyxobacter**	0.01	-	-	-	-	-	-	-
	*Myxococcus**	0.01	-	-	-	-	-	-	-
Blattabacteriaceae	*Blattabacterium*	-	-	-	-	-	<0.01	-	0.02
Beijerinckiaceae	*Beijerinckia*	-	0.01	<0.01	0.22	5.61	-	-	<0.01
Acidobacteriaceae	*Terriglobus**	-	<0.01	<0.01	0.23	1.53	-	-	<0.01
	*Edaphobacter**	0.02	-	-	-	0.01	-	-	-
Bacteroidales	*Bacteroides*	-	-	0.01	-	-	-	<0.01	<0.01
Bdellovibrionaceae	*Bdellovibrio*	0.25	0.16	0.02	0.06	0.09	<0.01	<0.01	<0.01
Bradyrhizobiaceae	*Bradyrhizobium**	0.02	-	-	-	<0.01	-	-	<0.01
Methylocystaceae	*Methylosinus*	<0.01	<0.01	<0.01	0.27	8.49	-	<0.01	<0.01
	*Pleomorphomonas**	0.6	-	0.01	-	-	-	-	-
	Methylopila	-	0.03	-	-	-	<0.01	-	-
Sporomusaceae	*Anaerospora**	-	<0.01	-	-	-	-	<0.01	<0.01
	*Pelosinus**	-	-	<0.01	-	-	-	-	-
Nocardiaceae	*Rhodococcus*	<0.01	<0.01	-	0.01	0.04	0.04	-	-
Verrucomicrobiaceae	*Prosthecobacter**	0.01	0.10	-	-	-	<0.01	-	-
Corynebacteriaceae	*Corynebacterium*	0.06	-	-	-	-	-	-	-
Prevotellaceae	*Prevotella*	0.01	-	-	<0.01	-	<0.01	-	-
Vibrionaceae	*Plesiomonas*	-	<0.01	<0.01	-	-	<0.01	-	-
Dermabacteraceae	*Brachybacterium*	-	-	-	-	-	<0.01	-	-
Erysipelotrichidae	*Catenibacterium**	<0.01	-	-	<0.01	-	<0.01	-	-
	*Erysipelothrix**	-	0.01	-	-	-	-	-	-

Erysipelotrichaceae	*Turicibacter*	<0.01	-	-	0.01	-	<0.01	-	-
Planococcaceae	*Planomicrobium**	0.02	-	-	-	-	-	-	-
Verrucomicrobiaceae	*Luteolibacter*	0.12	0.44	<0.01	-	-	<0.01	-	-
Bifidobacteriaceae	*Bifidobacterium*	<0.01	-	-	<0.01	-	<0.01	-	-
Coriobacteriaceae	*Collinsella**	<0.01	-	-	<0.01	-	<0.01	-	-
Brevibacteriaceae	*Brevibacterium*	-	-	-	-	-	<0.01	-	-
Desulfovibrionaceae	*Desulfovibrio**	<0.01	-	<0.01	-	-	<0.01	-	-
Nocardioidaceae	*Aeromicrobium**	-	-	-	-	-	<0.01	-	-
	*Kribbella**	0.03	-	-	-	-	-	-	-
	*Nocardioides**	0.08	-	-	-	-	-	-	-
Veillonellaceae	*Megasphaera**	<0.01	-	-	-	-	<0.01	-	-
	*Veillonella**	0.02	-	-	-	-	-	-	-
	*Dialister**	<0.01	-	-	<0.01	-	<0.01	-	-
Planctomycetaceae	*Gemmata*	0.84	0.03	<0.01	0.03	0.01	<0.01	-	-
	Planctomyces	0.22	<0.01	-	0.01	-	-	-	-
Sanguibacteraceae	*Sanguibacter**	-	0.14	-	-	-	-	-	-
Fimbriimonadaceae	*Fimbriimonas*	<0.01	0.06	-	0.12	0.09	-	-	-
Chthoniobacteraceae	*Chthoniobacter*	0.18	0.03	-	-	-	-	-	-
	*heteroC45_4W**	0.03	-	-	-	-	-	-	-
	Xiphinematobacter	0.26	-	-	-	-	-	-	-
	*OR-59**	0.47	-	-	-	-	-	-	-
	*DA101**	4.27	-	-	-	-	-	-	-
Legionellaceae	*Legionella*	0.02	0.03	<0.01	-	-	-	-	-
Methylophilaceae	*Methylotenera**	-	0.03	0.02	-	-	-	-	-
Isosphaeraceae	*Nostocoida**	0.03	-	-	-	-	-	-	-
Alicyclobacillaceae	*Alicyclobacillus*	0.03	-	-	-	-	-	-	-
Cryomorphaceae	*Fluviicola**	<0.01	0.01	<0.01	-	-	-	-	-

	Xanthobacteraceae	*Azorhizobium**	-	0.01	-	-	-	-	-	-
		Labrys	0.10	-	-	<0.01	-	-	-	-
	Opitutaceae	*Opitutus**	0.09	0.01	-	-	-	-	-	-
	Bacteroidaceae	*Cytophaga*	0.01	<0.01	<0.01	-	-	-	-	-
	Micromonosporaceae	*Actinoplanes*	<0.01	<0.01	-	-	-	-	-	-
		*Catellatospora**	0.02	-	-	-	-	-	-	-
		*Virgisporangium**	0.03	-	-	-	-	-	-	-
		*Dactylosporangium**	0.05	-	-	-	-	-	-	-
		*Pilimelia**	0.11	-	-	-	-	-	-	-
	Mycobacteriaceae	*Mycobacterium**	0.11	<0.01	-	0.01	0.04	-	<0.01	-
	Patulibacteraceae	*Patulibacter*	-	<0.01	-	-	-	-	-	-
	Porphyromonadaceae	*Parabacteroides*	-	<0.01	-	-	-	-	-	-
		Paludibacter	-	-	0.07	-	-	-	-	-
	Deinococcaceae	*Deinococcus*	-	-	-	-	0.29	-	-	-
	Rickettsiaceae	*Rickettsia**	-	-	-	-	0.06	-	-	-
	Nitrosomonadaceae	*Nitrosovibrio**	<0.01	-	-	-	-	-	-	-
	Staphylococcaceae	*Staphylococcus*	0.02	-	-	0.02	0.02	-	-	-
	Gemmatimonadaceae	*Gemmatimonas*	0.04	-	-	-	-	-	-	-
	Phormidiaceae	*Phormidium**	-	-	0.01	-	0.01	-	-	-
	Williamsiaceae	*Williamsia*	-	-	-	-	0.01	-	-	-
	Rhodocyclaceae	*Uliginosibacterium**	-	-	<0.01	-	-	-	-	-
		Dechloromonas*	<0.01	-	-	<0.01	-	-	-	-
	Sinobacteraceae	*Steroidobacter*	0.42	-	<0.01	-	-	-	-	-
Fungi										
	Saccharomycetaceae	*Pichia*	0.02	-	-	-	-	<0.01	-	-
		Candida	0.69	0.07	0.87	1.91	1.17	2.48	2.25	6.55
		Hanseniaspora	<0.01	<0.01	0.45	0.14	0.25	20.54	15.12	11.33

	Torulaspora	-	-	-	-	0.02	-	0.13	0.03
	*Lachancea**	0.24	-	-	-	-	-	0.04	0.03
	Saccharomyces	-	-	-	0.10	-	-	-	-
	Kazachstania	0.02	-	-	-	-	-	0.02	-
	Issatchenkia	-	-	-	0.74	-	0.017	-	0.09
	Zygosaccharomyces	<0.01	-	-	-	-	-	-	-
Necteriaceae	*Fusarium*	7.52	0.03	5.66	0.25	0.23	0.11	2.07	0.96
Debaryomicetaceae	*Meyerozyma*	0.10	0.06	0.03	0.18	0.03	0.79	0.18	0.69
Tremellaceae	*Cryptococcus*	0.59	0.10	0.50	2.59	1.60	0.35	0.77	0.58
Cystofilobasidiaceae	*Cystofilobasidium*	0.01	0.18	3.06	0.33	0.46	0.59	4.76	2.79
	*Occultifur**	0.01	-	-	-	-	-	0.04	-
Glomerellaceae	*Colletotrichum*	-	3.20	19.81	35.83	9.03	4.23	16.20	8.09
Mycosphaerellaceae	*Pseudocercospora*	<0.01	13.02	0.38	1.61	5.08	<0.01	0.35	0.11
	Neoceratosperma	-	-	-	-	0.08	-	-	-
	Lecanosticta	-	<0.01	-	0.09	-	-	-	-
Cladosporiaceae	*Cladosporium*	8.17	0.67	4.28	15.34	3.60	1.48	3.38	2.32
Saccharomycetales_Incertae	*Starmerella**	-	-	-	-	-	0.04	0.37	0.04
	*Cyberlindnera**	<0.01	-	-	-	-	1.17	0.07	-
Tremelalles_Incertae Sedis	*Hannaella*	<0.01	-	2.09	0.11	0.04	0.08	0.40	0.20
Cuniculitremaceae	*Kockovaella**	-	-	<0.01	0.28	0.98	-	0.08	0.05
Arthrodermataceae	*Microsporum**	-	-	-	-	-	0.03	-	-
Incertae Sedis	*Meira*	-	-	-	-	-	-	-	0.04
Didymellaceae	*Didymella**	0.06	0.04	0.15	0.18	1.62	0.26	0.17	0.20
Rhynchogastremaceae	*Rhynchogastrema**	-	-	-	-	-	-	0.15	-
Sporidiobolaceae	*Rhodotorula*	<0.01	-	<0.01	-	0.01	0.33	0.57	0.05
Trichocomaceae	*Aspergillus*	0.31	0.17	0.16	-	0.06	0.17	0.19	0.01
	Penicillium	8.00	-	0.06	0.02	0.06	6.82	1.23	0.66

	Talaromyces	0.06	-	-	-	-	-	-	-
	*Sagenomella**	<0.01	-	-	-	-	-	-	-
Chaetomiaceae	*Chaetomium*	0.01	-	-	-	-	-	-	-
	*Metarhizium**	0.08	-	-	-	-	-	-	-
Hypocreaceae	*Trichoderma*	-	0.01	0.04	-	0.10	-	-	0.03
	Acremonium	0.21	-	0.06	-	<0.01	0.01	-	-
	*Hypomyces**	0.01	-	-	-	-	-	0.01	-
Cordycipitaceae	*Lecanicillium**	-	<0.01	0.07	1.39	-	-	0.05	0.04
	*Simplicillium**	-	-	-	-	-	<0.01	-	-
	*Cordyceps**	0.01	-	-	-	-	-	-	-
	Beauveria	0.04	-	0.03	-	-	-	-	-
Strelitzianaceae	*Strelitziana*	<0.01	0.02	0.87	7.90	9.44	-	0.18	<0.01
	*Cladophialophora**	<0.01	-	-	-	0.08	-	-	-
	*Exophiala**	0.01	-	0.02	-	0.10	-	0.03	-
Wickerhamomycetaceae	*Wickerhamomyces**	-	-	0.01	-	-	-	0.02	0.03
Amphisphaeriaceae	*Pestalotiopsis*	0.09	-	0.01	-	0.47	-	-	-
Cerastobasidiaceae	*Ceratobasidium*	-	-	<0.01	<0.01	-	-	-	-
Hypocreales Incertae Sedis	*Geosmithia**	<0.01	-	0.03	0.08	0.04	<0.01	0.01	-
	Sarocladium	-	-	<0.01	-	-	-	-	-
	*Gliomastix**	0.03	-	-	-	-	-	-	-
	Myrothecium	4.88	0.16	0.04	-	-	-	-	-
	*Stachybotrys**	0.19	-	-	-	-	-	-	-
Stereaceae	*Stereum*	-	-	-	-	0.01	-	-	-
Symmetrosporaceae	*Symmetrospora**	-	-	-	<0.01	-	-	-	-
Lophiostomataceae	*Lophiostoma**	-	-	-	-	-	-	-	0.20
	*Sporothrix**	0.01	-	-	-	-	-	-	-
	*Acrocalymma**	0.05	0.03	0.09	-	0.07	-	0.04	-

Mortierellaceae	*Mortierella*	1.11	-	<0.01	-	-	-	-	-
Trichosporonaceae	*Trichosporon*	0.39	-	-	-	-	-	-	-
Xylariaceae	*Xylaria*	0.28	-	-	-	-	-	-	0.11
	Nemania	0.03	-	-	-	-	-	-	-
	*Muscodor**	<0.01	-	-	-	-	-	-	-
	Biscogniauxia	<0.01	-	-	-	-	-	-	-
	*Hypoxylon**	0.04	-	-	-	-	-	-	-
	Annulohypoxylon	0.02	-	-	-	-	-	-	-
Trichosporonaceae	*Apiotrichum**	0.12	-	-	-	-	-	-	-
Coronophorales Incertae sedis	*Pseudocatenomycopsis**	-	-	-	-	-	-	0.01	-
Spiculogloeaceae	*Phyllozyma**	-	-	-	0.01	0.34	-	-	-
Agaricaceae	*Cystolepiota**	0.01	-	-	-	-	-	-	-
	*Leucoagaricus**	<0.01	-	-	-	-	-	-	-
	*Lepiota**	0.26	-	<0.01	-	-	-	-	-
	*Melanophyllum**	<0.01	-	-	-	-	-	-	-
	*Agaricus**	0.04	-	-	-	0.08	-	-	-
	*Micropsalliota**	0.35	-	-	-	-	-	-	-
	*Calvatia**	0.05	-	-	-	-	-	-	-
	*Lycoperdon**	0.05	-	-	0.05	-	-	-	-
Diaporthaceae	*Diaporthe*	-	-	-	0.01	-	-	-	-
	Phomopsis	-	-	-	-	0.03	-	-	-
Botryosphaeriaceae	*Dothiorella**	-	<0.01	-	-	-	-	-	-
	*Alanphillipsia**	-	-	-	-	<0.01	-	-	-
	*Melanops**	-	-	-	-	0.18	-	-	-
	*Basidioascus**	<0.01	-	-	-	-	-	-	-
Sporodiobolales_Incertae	*Sporobolomyces*	-	-	-	-	0.01	-	-	-
Cyphellophoraceae	*Cyphellophora**	0.30	0.10	0.12	1.07	2.35	-	0.21	0.19

	*Mucor**	<0.01	-	-	-	-	-	-	0.02
Myxotrichaceae	*Oidiodendron**	0.01	-	-	-	-	-	-	-
Elsinoaceae	*Sphaceloma**	-	-	-	-	<0.01	-	-	-
Lindgomycetaceae	*Lindgomyces**	-	-	-	-	<0.01	-	-	-
Schizoporaceae	*Hyphodontia**	-	-	-	-	0.02	-	-	-
Ophiocordycipitaceae	*Purpureocillium*	0.99	<0.01	0.02	0.84	0.15	-	0.03	0.18
	*Haptocillium**	0.01	-	-	-	-	-	-	-
	*Sporormiella**	-	-	-	-	<0.01	-	-	-
Rhizopodaceae	*Rhizopus**	-	-	-	-	0.01	-	-	-
Kondoaceae	*Bensingtonia**	-	-		-	0.02	-	-	-
Hydnodontaceae	*Trechispora**	0.08	-	-	-	-	-	-	-
Pleosporaceae	*Alternaria*	-	-	-	-	0.08	-	-	-
	*Curvularia**	-	-	-	-	0.03	-	-	-
	Bipolaris	<0.01	-	-	-	0.20	-	-	-
Wallemiaceae	*Wallemia**	-		-	-	<0.01	-	-	0.07
Septobasidiaceae	*Septobasidium**	-	0.01	-	0.31	-	-	-	-
Trichosphaeriales	*Nigrospora**	1.08	0.26	<0.01	0.01	3.82	-	0.01	<0.01
Tephromelataceae	*Tephromela**	-	-	-	-	0.03	-	-	-
	*Polyporus**	<0.01	-	-	-	-	-	-	-
Bionectriaceae	*Mycoarachis**	0.6	-	-	-	-	-	-	-
	*Pyricularia**	-	-	-	-	-	-	0.03	-
	*Gaeumannomyces**	-	<0.01	-	<0.01	<0.01	-	0.01	-
Marasmiaceae	*Marasmius**	-	-	-	-	0.08	-	-	-
Hymenochaetales Incertae sedis	*Trichaptum**	-	-	-	-	0.08	-	-	-
Mycenaceae	*Mycena**	0.01	-	-	-	0.04	-	-	-
	*Hypogymnia**	-	-	-	-	<0.01	-	-	-
	*Sulcaria**	-	-	-	-	0.01	-	-	-

	*Parmelia**	-	-	-	-	0.01	-	-	-
	*Austroparmelina**	-	-	-	-	0.05	-	-	-
Ramalinaceae	*Bacidia**	-	-	-	-	0.13	-	-	-
	*Waynea**	-	-	-	-	0.01	-	-	-
	*Tylothallia**	-	-	-	-	0.08	-	-	-
	*Bacidina**	-	-	-	0.01	-	-	-	-
Sympoventuriaceae	*Ochroconis**	0.04	0.08	0.12	0.19	0.38	<0.01	0.03	<0.01
Filobasidiaceae	*Heterocephalacria**	-	-	-	-	<0.01	-	-	-
Bolbitiaceae	*Conocybe**	1.04	<0.01	<0.01	-	-	-	0.01	-
	*Galerella**	0.03	-	-	-	-	-	-	-
	*Bolbitius**	0.01	-	-	-	-	-	-	-
Ganodermacetaceae	*Ganoderma**	0.09	-	-	-	-	-	-	-
	*Coniothyrium**	-	-	0.04	-	0.14	-	-	-
Psathyrellaceae	*Coprinellus**	0.03	-	-	-	-	-	-	-
	*Psathyrella**	0.06	-	-	-	-	-	-	-
	*Coprinopsis**	<0.01	-	-	-	-	-	-	-
	*Parasola**	0.05	-	-	-	-	-	-	-
Nectriaceae	*Volutella**	1.11	0.45	2.16	-	<0.01	0.01	-	<0.01
	*Mariannaea**	<0.01	-	<0.01	-	-	-	-	-
	Nectria	0.14	-	<0.01	-	-	-	-	-
	*Thelonectria**	0.14	-	0.07	-	-	-	-	-
Leotiomycetes Incertae sedis	*Leohumicola**	0.49	-	-	-	-	-	-	-
Pleosporales Incertae sedis	*Periconia**	0.44	-	-	-	<0.01	-	-	-
Sordariomycetes Incertae sedis	*Myrmecridium*	0.28	-	-	-	-	-	-	-
	*Pleurotheciella**	<0.01	-	-	-	-	-	-	-
Entolomataceae	*Entoloma**	0.25	-	-	-	-	-	-	-
	*Clitopilus**	0.10	-	-	-	-	-	-	-

Chaetosphaeriaceae	*Chaetosphaeria*	0.23	-	-	-	-	-	-	-
Geminibasidiaceae	*Geminibasidium**	0.17	-	-	-	-	-	-	-
Helotiales Incertae sedis	*Scytalidium**	0.13	-	-	-	-	-	-	-
	*Tiarosporella**	-	-	-	-	<0.01	-	-	-
	Cadophora	<0.01	-	-	-	-	-	-	-
	Dactylaria	-	-	-	0.02	0.13	-	-	-
Lyophyllaceae	*Lyophyllum**	0.13	-	-	-	-	-	-	-
Spizellomycetaceae	*Spizellomyces**	0.11	-	-	-	-	-	-	-
Saccharomycopsidaceae	*Saccharomycopsis*	-	-	-	-	0.13	0.38	0.78	1.34
Orbiliaceae	*Arthrobotrys**	0.09	-	-	-	-	-	-	-
	Dactylella	0.04	-	-	-	-	-	-	-
Geastraceae	*Geastrum**	0.08	-	-	-	-	-	-	-
Auriscalpiaceae	*Lentinellus**	-	-	-	-	0.06	-	-	-
Cortinariaceae	*Cortinarius**	0.07	-	-	-	-	-	-	-
Plectosphaerellaceae	*Lectera**	-	-	-	-	0.06	-	-	-
Tremellales	*Mingxiaea**	0.05	<0.01	-	0.89	-	-	-	-
	Fellomyces	0.04	-	-	-	-	-	-	-
Xylariales Incertae sedis	*Microdochium*	0.04	-	-	-	-	-	-	-
Pezizomycotina Incertae sedis	*Leptodiscella**	0.03	0.02	-	-	-	-	-	-
	*Liberomyces**	-	-	-	0.02	<0.01	-	-	-
	*Radulidium**	-	-	-	-	0.07	-	-	-
	Knufia	-	-	<0.01	<0.01	0.10	-	-	-
	*Wiesneriomyces**	-	-	0.71	-	-	-	-	-
Pyronemataceae	*Scutellinia**	0.03	-	-	-	-	-	-	-
Strophariaceae	*Psilocybe**	0.02	-	-	-	-	-	-	-
	*Pholiota**	<0.01	-	-	-	-	-	-	
	*Gymnopilus**	0.01	-	-	-	-	-	-	-

Umbelopsidaceae	*Umbelopsis**	0.02	-	-	-	-	-	-	-
Inocybaceae	*Crepidotus**	0.01	-	-	-	-	-	-	-
	*Simocybe**	<0.01	-	-	-	-	-	-	-
Pluteaceae	*Pluteus**	0.01	-	-	-	-	-	-	-
Hymenochaetaceae	*Phellinus**	0.01	-	-	-	-	-	-	-
	*Tropicoporus**	-	-	-	0.25	-	-	-	-
Claroideoglomeraceae	*Claroideoglomus*	<0.01	-	-	-	-	-	-	-
Clavariaceae	*Clavaria**	<0.01	-	-	-	-	-	-	-
Sclerodermataceae	*Scleroderma*	<0.01	-	-	-	-	-	-	-
Thelephoraceae	*Tomentella**	<0.01	-	-	-	-	-	-	-
Auriculariales Incertae sedis	*Heterochaete**	<0.01	-	-	-	-	-	-	-
Dothideomycetes Incertae sedis	*Biatriospora**	<0.01	-	-	-	-	-	0.04	-
Erythrobasidiales Incertae sedis	*Bannoa**	<0.01	-	-	-	0.04	-	-	-
Kickxellaceae	*Ramicandelaber**	<0.01	-	-	-	-	-	-	-
Omphalotaceae	*Gymnopus**	<0.01	-	-	<0.01	-	-	-	-
Stephanosporaceae	*Stephanospora**	<0.01	-	-	-	-	-	-	-
Malasseziaceae	*Malassezia**	-	0.02		-	0.11	<0.01	-	-
Dothioraceae	*Pringsheimia**	-	<0.01	-	-	-	-	-	-
Dacampiaceae	*Teichospora**	-	<0.01	-	-	-	-	-	-
Capnodiales Incertae sedis	*Toxicocladosporium**	-	-	-	-	0.04	-	-	-
Teloschistaceae	*Caloplaca**	-	<0.01	-	<0.01	0.05	-	-	-
Tetraplosphaeriaceae	*Tetraplosphaeria**	-	-	-	-	0.05	-	-	-
Teratosphaeriaceae	*Neodevriesia**	-	<0.01	-	0.02	0.01	-	-	-
	*Teratosphaeria**	-	-	-	-	0.70	-	-	-

APPENDIX 2

APPENDIX 2: Relative abundance (%) of bacteria and fungi at the genus level during spontaneous Honduran coffee fermentation process. Bacteria reported for the first time in coffee fermentation are highlighted in red asterisk (*)

	Family	Genera	Fermentation time (h)					
			0	12	16	18	20	24
Bacteria								
	Leuconostocaceae	*Leuconostoc*	52.47	0.78	3.78	6.89	24.26	69.92
		Oenococcus	-	-	-	0.05	-	-
	Acetobacteraceae	*Gluconobacter*	27.20	57.40	59.13	56.04	31.73	20.16
		Acetobacter	6.75	13.93	13.90	12.09	3.29	2.54
		Gluconacetobacter	0.03	0.29	0.12	0.12	0.06	-
		*Kosakia**	0.08	0.10	0.16	-	0.12	-
		Asaia	0.03	0.49	0.23	0.35	0.00	0.00
	Enterobacteriaceae	*Klebsiella*	0.03	0.03	0.20	0.09	-	-
		Enterobacter	0.37	1.18	1.01	0.55	0.32	0.09
		Kluyvera	2.19	3.35	3.69	2.22	1.41	0.69
		Pantoea	1.07	2.80	1.41	2.42	1.01	0.43
		Tatumella	0.69	1.21	0.43	0.35	0.20	-
		*Kosakonia**	2.91	4.90	5.11	3.26	4.87	2.22
		*Lelliottia**	0.84	1.24	1.15	0.98	0.87	0.37
		*Pectobacterium**	0.84	1.50	0.58	0.61	0.23	0.06
		*Yokenella**	-	0.43	-	0.17	0.23	-
		*Rahnella**	0.03	0.20	0.09	0.09	0.09	0.03
		*Buttiauxella**	0.02	0.05	-	0.04	-	-
		*Brenneria**	-	0.05	-	-	-	-
		*Cedecea**	-	-	-	0.05	-	-
		*Enterobacillus**	-	-	-	-	-	0.04
		Citrobacter	0.08	0.25	0.08	-	-	-
		*Hafnia_Obesumbacterium**	-	-	0.04	-	-	-
	Streptococcaceae	*Lactococcus*	0.52	0.03	0.75	6.69	28.35	0.17

Lactobacillaceae	*Lactobacillus*	-	0.10	-	0.05	-	0.12
Rhodanobacteraceae	*Frauteria**	0.89	2.28	2.80	1.21	0.37	0.52
Pseudomonadaceae	*Pseudomonas*	0.95	4.04	3.52	2.83	1.79	1.15
Methylobacteriaceae	*Methylobacterium_Methylorubrum*	1.28	1.19	0.77	0.55	0.21	0.15
Sphingomonadaceae	Plot4-2H12*	0.14	0.33	0.18	0.11	0.02	0.03
	Novosphingobium	0.09	0.17	0.23	0.14	0.00	0.06
Cytophagaceae	*Dyadobacter*	-	-	0.04	-	-	-
Sphingobacteriaceae	*Pedobacter*	0.09	0.23	0.17	0.20	0.12	-
	*Mucilaginibacter**	0.02	-	-	-	-	-
	Sphingobacterium	0.40	0.17	0.26	0.17	0.20	0.06
Microbacteriaceae	*Curtobacterium**	0.03	0.06	0.00	0.12	0.09	-
	*Parafrigoribacterium**	-	0.10	-	0.10	-	-
Weeksellaceae	*Chryseobacterium*	0.46	0.72	0.17	0.49	-	0.14
Flexibacteraceae	*Siphonobacter**	0.03	-	-	-	-	0.29
Paenibacillaceae	*Saccharibacillus*	0.14	0.69	0.26	0.49	0.17	0.12
	Paenibacillus	-	0.05	0.02	<0.01	<0.01	<0.01
Comamonadaceae	*Acidovorax**	0.04	0.05	-	0.10	-	-
	*Rhodoferax**	0.06	0.53	0.08	0.04	0.01	0.01
	Comamonas	-	0.05	-	0.05	-	-
Caulobacteraceae	*Brevundimonas*	0.14	0.03	-	0.03	-	0.09
Xanthomonadaceae	*Stenotrophomonas*	0.37	0.37	0.06	-	-	0.35
	Luteimonas	-	-	0.04	-	-	-
	Luteibacter	-	-	0.04	-	-	-
Chitinophagaceae	*Edaphobaculum**	-	-	-	-	0.06	-
Rhizobiaceae	*Allorhizobium_Rhizobium*	0.09	0.14	0.37	-	-	0.17
Flavobacteriaceae	*Flavobacterium*	0.03	0.14	0.00	0.17	0.00	0.09
	*Chishuiella**	0.06	0.23	0.03	0.09	0.06	-
Lachnospiraceae	*Lachnoclostridium**	-	-	0.04	-	-	-
Oxalobacteraceae	*Massilia**	0.02	-	0.08	-	-	-
	*Noviherbaspirillum**	-	-	-	-	0.06	-
	Oxalicibacterium	-	-	-	-	-	0.04

Polyangiaceae	*Pajaroellobacter**	-	-	0.04	-	-	-
Cellulomonadaceae	*Cellulomonas*	-	-	0.04	0.10	-	-
Enterococcaceae	*Enterococcus*	0.12	0.03	0.14	0.58	0.29	0.00
Phyllobacteriaceae	*Mesorhizobium*	0.02	-	-	0.02	-	<0.01
Brucellaceae	*Ochrobactrum*	-	0.05	-	-	-	-
Beijerinckiaceae	1174-901-12	-	0.05	-	-	-	-
Fungi							
Saccharomycetaceae	*Pichia*	21.66	32.53	15.31	16.32	12.07	11.59
	Candida	31.12	16.37	30.58	31.68	29.78	23.28
	Hanseniaspora	25.33	22.46	17.50	18.81	10.59	14.49
	Torulaspora	0.07	0.02	0.05	0.06	0.04	0.01
	*Lachancea**	0.05	0.10	0.12	0.06	0.13	0.02
	Saccharomyces	0.03	0.02	-	-	0.01	0.01
	Kazachstania	0.02	0.07	0.06	0.09	0.06	0.04
	Issatchenkia	0.02	-	-	-	-	-
	Debaryomyces	-	<0.01	-	-	-	-
	*Ogataea**	-	-	0.01	-	-	-
	*Saturnispora**	-	-	0.02	-	-	-
Necteriaceae	*Fusarium**	9.00	17.27	17.50	14.35	25.16	29.70
	*Bisifusarium**	-	-	-	-	0.01	-
Debaryomicetaceae	*Meyerozyma*	1.28	0.97	1.46	1.79	2.34	2.08
Tremellaceae	*Cryptococcus**	1.74	0.84	1.58	1.26	1.88	2.04
	*Tremella**	0.02	-	-	-	0.03	-
	*Dioszegia**	-	-	-	-	-	0.05
Cystofilobasidiaceae	*Cystofilobasidium**	0.51	0.66	1.72	2.56	2.05	1.66

	*Itersonilia**	-	0.01	-	-	-	-
	*Occultifur**	-	<0.01	-	0.01	-	-
Capnodiaceae	*Scorias**	-	-	<0.01	-	-	-
Glomerellaceae	*Colletotrichum**	0.38	0.30	0.23	0.21	0.44	0.30
Mycosphaerellaceae	*Pseudocercospora**	0.21	0.14	0.10	0.13	0.16	0.23
	*Cercospora**	-	0.01	<0.01	-	-	-
	*Zymoseptoria**	-	-	-	-	0.04	-
Cladosporiaceae	*Cladosporium*	0.27	0..54	0.34	0.26	0.41	0.81
	*Rachicladosporium**	-	-	0.01	-	-	-
Saccharomycetales_Incertae	*Starmerella*	0.21	0.17	0.05	0.06	0.12	0.08
	*Kodamaea**	0.12	-	-	-	-	-
Tremelalles_Incertae Sedis	*Hannaella**	0.21	0.35	0.59	0.44	0.61	0.84
Cuniculitremaceae	*Kockovaella**	0.18	0.01	0.07	0.07	0.04	0.15
Pleurotaceae	*Nematoctonus**	0.14	-	-	-	0.01	-
Incertae Sedis	*Meira**	0.08	0.03	<0.01	0.05	0.07	-
Didymellaceae	*Didymella**	0.07	0.07	0.17	0.21	0.26	-
	*Phoma**	-	0.01	-	-	-	-
Rhynchogastremaceae	*Rhynchogastrema**	0.06	-	0.04	-	-	0.02
Sporidiobolaceae	*Rhodotorula*	0.05	0.01	0.03	0.03	0.02	-
	*Rhodosporidiobolus**	0.01	0.01	0.02	0.02	0.03	0.02
Trichocomaceae	*Aspergillus*	0.04	0.03	0.02	0.05	0.13	0.05
	Penicillium	0.04	0.06	0.11	0.10	0.05	0.06
	*Phialosimplex**	-	-	0.01	<0.01	-	-
	*Talaromyces**	-	-	-	<0.01	0.19	-
Chaetomiaceae	*Chaetomium**	0.03	0.01	0.02	-	-	-
Clavicipitaceae	*Claviceps**	0.03	0.02	-	-	-	-
	*Metarhizium**	0.01	-	-	-	-	-

Hypocreaceae	*Trichoderma*	0.02	0.02	-	0.01	0.05	-
	*Acremonium**	0.01	-	0.01	0.02	0.01	0.02
Verrucariaceae	*Agonimia**	0.02	-	-	-	-	-
Amanitaceae	*Amanita**	0.02	0.04	0.02	0.05	0.06	0.04
Cordycipitaceae	*Lecanicillium**	0.02	-	-	-	0.03	0.13
Strelitzianaceae	*Strelitziana**	0.01	0.25	0.09	0.02	0.10	0.09
Herpotrichiellaceae	*Phialophora**	0.01	-	-	-	-	0.04
	*Capronia**	0.01	-	-	-	-	-
	*Exophiala**	-	-	-	-	0.01	-
Metschnikowiaceae	*Metschnikowia**	0.01	0.01	-	0.01	0.01	0.02
Didymosphaeriaceae	*Paraphaeosphaeria**	0.01	-	-	-	-	-
Wickerhamomycetaceae	*Wickerhamomyces*	0.01	0.03	0.02	0.02	0.02	-
Amphisphaeriaceae	*Pestalotiopsis**	0.01	-	-	<0.01	0.01	0.01
Cerastobasidiaceae	*Ceratobasidium**	0.01	-	-	-	-	-
Erythrobasidiaceae	*Erythrobasidium**	0.01	-	-	-	-	-
Hypocreales_Incertae Sedis	*Geosmithia**	0.01	-	0.01	0.01	0.01	-
Meruliaceae	*Bjerkandera**	0.01	-	-	-	0.01	-
Stereaceae	*Stereum**	0.01	-	-	0.01	-	-
Tubeufiaceae	*Acanthostigma**	0.01	-	-	-	-	-
Symmetrosporaceae	*Symmetrospora**	-	0.03	<0.01	0.01	0.03	-
Lophiostomataceae	*Lophiostoma**	-	0.02	-	-	-	-
	*Acrocalymma**	-	-	-	-	-	0.04
Microascaceae	*Microascus**	-	0.02	0.01	0.01	0.06	0.01
	*Scedosporium**	-	<0.01	-	-	-	-
	*Wardomycopsis**	-	-	<0.01	-	-	-
	*Scopulariopsis**	-	-	-	<0.01	-	-
Mortierellaceae	*Mortierella**	-	0.01	0.04	-	0.01	0.02

Patellariales_Incertae Sedis	*Rhytidhysteron**	-	0.01	-	-	-	-
Gymnoascaceae	*Gymnoascus**	-	0.01	-	-	-	-
Ajellomycetaceae	*Histoplasma**	-	0.01	-	-	-	-
Onygenaceae	*Auxarthron**	-	0.01	<0.01	-	0.01	0.02
	*Spiromastix**	-	-	-	-	0.01	-
Trichosporonaceae	*Trichosporon**	-	0.01	0.02	-	0.03	-
Xylariaceae	*Xylaria**	-	0.01	-	-	0.01	-
Trichosporonaceae	*Apiotrichum**	-	<0.01	<0.01	-	-	-
Agaricaceae	*Cystolepiota**	-	<0.01	-	-	-	-
	*Leucoagaricus**	-	-	-	0.03	0.05	-
	*Lepiota**	-	-	-	<0.01	-	-
	*Micropsalliota**	-	-	-	-	0.01	-
Diaporthaceae	*Diaporthe**	-	<0.01	-	-	-	-
Botryosphaeriaceae	*Dothiorella**	-	<0.01	-	-	0.01	-
Dipodascaceae	*Lipomyces**	-	<0.01	-	-	-	-
Sporodiobolales_Incertae	*Sporobolomyces**	-	-	0.02	0.02	0.02	-
Cyphellophoraceae	*Cyphellophora**	-	-	0.02	0.02	0.05	0.02
Auriculariaceae	*Auricularia**	-	-	<0.01	-	-	-
Mucoraceae	*Absidia**	-	-	<0.01	-	-	-
	*Mucor**	-	-	<0.01	0.10	-	-
Myxotrichaceae	*Oidiodendron**	-	-	<0.01	-	-	-
Ophiocordycipitaceae	*Purpureocillium**	-	-	<0.01	-	-	0.01
	*Tolypocladium**	-	-	<0.01	-	-	-
Sporormiaceae	*Preussia**	-	-	<0.01	-	-	-
Xylariaceae	*Annulohypoxylon**	-	-	<0.01	-	-	-
Trichomonascaceae	*Blastobotrys**	-	-	-	0.02	-	-
Eremomycetaceae	*Arthrographis**	-	-	-	0.02	0.01	-

Kondoaceae	*Bensingtonia*	-	-	-	0.01	-	-
Hydnodontaceae	*Trechispora**	-	-	-	<0.01	0.14	0.01
Pleosporaceae	*Alternaria**	-	-	-	<0.01	-	-
Wallemiaceae	*Wallemia**	-	-	-	<0.01	0.02	0.01
Septobasidiaceae	*Septobasidium**	-	-	-	-	0.03	-
Trichosphaeriales	*Nigrospora**	-	-	-	-	0.03	-
Diatrypaceae	*Diatrypella**	-	-	-	-	0.02	-
Polyporaceae	*Funalia**	-	-	-	-	0.02	-
Bionectriaceae	*Mycoarachis**	-	-	-	-	0.01	-
Magnaporthaceae	*Mycoleptodiscus**	-	-	-	-	0.01	-
Marasmiaceae	*Marasmius**	-	-	-	-	0.01	-
Microbotryomycetes_Incertae	*Pseudohyphozyma**	-	-	-	-	0.01	-
Mycenaceae	*Mycena**	-	-	-	-	0.01	-
Parmeliaceae	*Usnea**	-	-	-	-	0.01	-
Peniophoraceae	*Peniophora**	-	-	-	-	0.01	-
Ramalinaceae	*Bacidia**	-	-	-	-	0.01	-
Sympoventuriaceae	*Ochroconis**	-	-	-	-	-	0.05
Filobasidiaceae	*Heterocephalacria**	-	-	-	-	-	0.04
Incertae Sedis	*Acaromyces**	-	-	-	-	-	0.04
Bolbitiaceae	*Conocybe**	-	-	-	-	-	0.01
Ganodermacetaceae	*Ganoderma**	-	-	-	-	-	0.01
Leptosphaeriaceae	*Leptosphaeria**	-	-	-	-	-	0.01
Fuscostagonosporaceae	*Fuscostagonospora**	-	-	-	-	-	0.01
Psathyrellaceae	*Coprinellus**	-	-	-	-	-	0.01
Castanediellaceae	*Castanediella**	-	-	-	-	-	0.01

APPENDIX 3

Appendix 3: Relative abundance (%) of bacteria and fungi identified during fermentation of Brazilian coffee beans. The number after each letter represents the fermentation time (e.g., B0= bottom layer at 0 h).

Microorganisms	Bacteria														
	B0	M0	T0	B12	M12	T12	B24	M24	T24	B48	M48	T48	B72	M72	T72
Other	0,957625673	0,924161035	0,992397687	1,011105586	1,008736828	1,246301299	0,736855571	0,648057157	0,875672928	0,549213513	0,7913124	0,724453943	0,478236986	0,482854879	0,639705085
Enterobacteriaceae	51,28779941	45,36475481	61,54231347	66,86971656	64,92763772	60,7246237	55,39209415	55,4832541	56,51671634	37,77626601	42,33942251	42,34071069	32,59766698	32,48330756	38,71842134
Erwinia	36,01695948	40,50135736	27,65967133	21,45905851	21,77537682	27,92358163	28,07470897	30,06560251	28,82558166	42,96807593	28,3862278	42,21755352	36,95996381	33,85265382	30,00975821
Enterobacter	2,395282536	2,645410963	1,973414667	1,786010277	1,824062959	2,343046443	1,322758091	1,304082229	1,839144197	1,431937347	1,393214917	2,894193502	1,040488577	1,063789656	1,134121219
Klebsiella	0,00731012	0	0,009104566	0,004143875	0,020008003	0,003216261	0,005117053	0,007967916	0,046209653	0,006637021	0,004209109	0,007244539	0	0,003772304	0,008673967
Providencia	0	0	0	0	0	0	0	0	0,004620965	0	0	0,007244539	0	0	0
Salmonella	0	0	0,002276141	0,002071938	0	0	0	0	0	0	0	0,00362227	0	0,003772304	0
Serratia	0,645727235	1,08011321	0,915008877	0,354301343	0,313458717	0,678631159	0,058846105	0,079679159	0,145560408	0,04977766	0,143109689	0,12315717	0,048469965	0,007544607	0,11926705
Lactobacillaceae	0,173006165	0,213712239	0,252651705	0,120172385	0,123382686	0,06914962	2,5303825	1,508592069	0,732423003	2,404260968	3,830288745	1,467019234	7,286651372	13,4935305	12,30835954
Lactobacillus	0,399619874	0,537168602	0,546273956	0,306646776	0,526877418	0,209056992	3,960598695	3,561658389	1,707446686	8,614853654	11,53295732	4,625638425	12,31137105	6,70715606	6,488127507
Pediococcus	0	0	0	0	0	0,001608131	0,002558526	0	0	0,003318511	0	0	0	0	0
Weissella	0,095031555	0,080864091	0,020485273	0,225841207	0,181739362	0,032162614	0,376103364	0,20450984	0,136318477	0,214043937	0,387237983	0,094179013	0,475005655	0,671470067	0,236365608
Leuconostocaceae	0,494651429	0,508288569	0,186643602	1,085695342	1,205482193	0,34092371	1,565818089	2,71971528	2,395970518	0,721776067	1,633134102	0,583185424	0,594564901	1,433475423	1,097256858
Leuconostoc	3,949901313	2,818691157	0,849000774	3,356539035	5,540549553	2,373600926	2,834847128	1,543119705	2,451422102	2,05913586	4,310127115	1,021480059	2,814489288	3,579916255	1,867071452
Enterococcaceae	0,002436707	0	0	0	0	0	0,017909684	0,005311944	0,004620965	0,001659255	0	0	0	0,003772304	0,002168492
Enterococcus	0,065791077	0,075088084	0,07738881	0,031079065	0,050020008	0,020905699	0,094665473	0,058431383	0,057762066	0,122784894	0,105227713	0,076067664	0,313439106	0,24142744	0,219017673
Lactococcus	0,650600648	0,693120776	0,3072791	2,109232554	1,433906896	1,473047729	2,169630293	1,532495817	2,370555209	1,309152452	3,131576732	1,658999529	1,783694704	2,870723151	3,883768839
Acetobacteraceae	0,384999634	0,635360712	0,901352028	0,070445881	0,156729358	0,358613148	0,061404631	0,084991102	0,140939442	0,53262096	0,416701743	0,326004274	1,689986105	0,720510015	0,379486067
Acetobacter	0,031677185	0,028880032	0,034142122	0	0,005002001	0,020905699	0,005117053	0,007967916	0,004620965	0,004977766	0,042091085	0,021733618	0,029081979	0,022633822	0,006505475
Pseudomonadaceae	0,004873413	0,011552013	0,006828424	0,006215813	0,003334667	0,012865046	0,010234105	0,007967916	0,011552413	0,014933298	0,004209109	0,014489079	0,03231331	0,018861519	0,008673967
Pseudomonas	0,155949219	0,462080518	0,375563345	0,159539201	0,10670935	0,246043998	0,117692209	0,148734429	0,145560408	0,232295746	0,218873643	0,144890789	0,252043817	0,505488702	0,338284723

Chryseobacterium	0,404493287	0,629584705	1,101652479	0,033151003	0,10170735	0,2042326	0,028143789	0,018591804	0,080866893	0,038162873	0,109436821	0,159379867	0,109865253	0,109396809	0,097582132
Aurantimonadaceae	0,024367065	0,005776006	0,011380707	0,024863252	0,003334667	0,025730091	0,012792631	0,002655972	0,016173379	0,011614787	0,008418217	0,014489079	0,009693993	0,015089215	0,052043804
Comamonadaceae	0,004873413	0,057760065	0,066008103	0,010359688	0,015006002	0,038595137	0,007675579	0,007967916	0,018483861	0,011614787	0,021045543	0,021733618	0,006462662	0,022633822	0,073728722
Oxalobacteraceae	0,021930359	0,017328019	0,059179679	0,022791314	0,021675337	0,041811398	0,010234105	0,015935832	0,025415309	0,013274043	0,016836434	0,00362227	0,029081979	0,06035686	0,030358885
Sphingomonadaceae	0,060917664	0,167504188	0,068284245	0,002071938	0,01834067	0,072365882	0,02046821	0,010623888	0,016173379	0,008296277	0,037881977	0,025355888	0,019387986	0,015089215	0,04770682
Acinetobacter	0,036550598	0,127072142	0,036418264	0,047654567	0,040016006	0,059500836	0,023026737	0,039839579	0,018483861	0,011614787	0,021045543	0,083312203	0,016156655	0,026406126	0,021684918
Agrobacterium	0,026803772	0,011552013	0,043246688	0,008287751	0,006669334	0,017689438	0,002558526	0,007967916	0,006931448	0,008296277	0,02946376	0,014489079	0,006462662	0,007544607	0,019516426
Bacteroides	0,065791077	0,063536071	0,081941093	0,020719377	0,01834067	0,028946353	0,010234105	0,037183607	0,10397172	0,031525851	0,037881977	0,036222697	0,016156655	0,045267645	0,117098558
[Barnesiellaceae]	0,00731012	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0319-6A21	0	0	0	0	0	0,001608131	0,002558526	0	0	0	0	0	0	0	0
Aeromonadaceae	0,009746826	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alcaligenaceae	0,009746826	0,005776006	0	0	0,003334667	0,009648784	0,005117053	0,002655972	0,002310483	0,003318511	0	0	0,006462662	0,003772304	0
Alicyclobacillaceae	0	0	0	0,002071938	0	0	0	0,002655972	0	0	0	0	0	0	0
Bacillaceae	0	0	0	0	0	0	0	0	0,004620965	0	0	0	0	0	0
Bacteriovoracaceae	0	0	0,002276141	0	0	0,001608131	0	0,01327986	0	0	0	0	0	0,007544607	0,006505475
Beijerinckiaceae	0,004873413	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bifidobacteriaceae	0	0	0	0	0	0	0	0	0	0	0	0	0,006462662	0	0
Bradyrhizobiaceae	0	0	0,002276141	0	0	0	0	0	0	0	0	0	0,006462662	0	0,002168492
Brucellaceae	0	0	0	0	0,001667334	0	0	0	0	0	0	0,007244539	0	0	0
Caulobacteraceae	0,019493652	0,011552013	0	0,004143875	0,005002001	0,009648784	0	0,002655972	0,002310483	0,001659255	0,008418217	0,018111349	0,029081979	0	0,013010951
Cellulomonadaceae	0	0	0	0	0	0,001608131	0,002558526	0	0	0	0	0,00362227	0	0,003772304	0
Chitinophagaceae	0,014620239	0,034656039	0,047798971	0,006215813	0,013338669	0,006432523	0,012792631	0,002655972	0,002310483	0	0	0	0,003231331	0,011316911	0
Christensenellaceae	0,002436707	0	0	0,002071938	0	0	0	0,002655972	0	0	0	0	0	0	0,002168492
Clostridiaceae	0,021930359	0,023104026	0,004552283	0,004143875	0,005002001	0	0,010234105	0,007967916	0,004620965	0,003318511	0,004209109	0	0	0,011316911	0,002168492
Coxiellaceae	0	0	0	0	0	0	0	0	0,004620965	0	0	0	0	0	0
Cytophagaceae	0	0	0	0	0	0,003216261	0	0	0	0	0,004209109	0	0	0	0
D2	0	0	0	0,004143875	0	0	0	0	0	0	0	0	0	0	0

Dermabacteraceae	0	0	0	0	0	0	0	0	0	0,003318511	0	0	0	0,026406126	0
Ellin6075	0,017056946	0	0,013656849	0,002071938	0,006669334	0,009648784	0,017909684	0,010623888	0,016173379	0,003318511	0,008418217	0,007244539	0	0,003772304	0,032527377
Erysipelotrichaceae	0	0	0,002276141	0	0	0	0	0,002655972	0	0	0	0	0	0	0
Erythrobacteraceae	0,002436707	0,005776006	0,002276141	0	0,001667334	0,004824392	0	0	0,006931448	0,001659255	0	0	0	0	0,006505475
FFCH4570	0	0	0	0	0	0	0	0	0	0	0,008418217	0	0	0	0
Flavobacteriaceae	0	0	0,018209132	0	0	0,003216261	0	0	0	0	0	0	0	0	0
Frankiaceae	0	0	0	0	0,001667334	0	0	0	0	0	0	0	0	0	0,002168492
Gaiellaceae	0	0	0	0	0	0	0	0,002655972	0	0	0	0	0,003231331	0,007544607	0
Gemellaceae	0	0	0	0	0	0	0	0	0	0,004977766	0	0	0	0	0
Gemmataceae	0	0	0	0	0	0	0,005117053	0	0	0	0	0	0	0	0
Geodermatophilaceae	0	0	0	0	0	0,004824392	0	0	0,002310483	0	0	0	0	0	0
Helicobacteraceae	0	0	0	0	0	0	0,002558526	0	0,002310483	0,003318511	0	0	0	0	0
Hyphomonadaceae	0	0	0	0	0	0,001608131	0	0	0	0	0,004209109	0	0	0	0
Isosphaeraceae	0,004873413	0	0,006828424	0	0	0,003216261	0	0	0	0	0	0,00362227	0	0	0
Koribacteraceae	0	0	0	0	0,001667334	0,001608131	0,002558526	0	0	0	0	0	0	0	0,004336984
Lachnospiraceae	0,14132898	0,023104026	0,029589839	0,014503564	0,008336668	0,012865046	0,005117053	0,007967916	0,032346757	0,013274043	0,021045543	0	0,012925324	0,026406126	0,054212295
mb2424	0	0	0	0	0,001667334	0	0,007675579	0	0	0	0,004209109	0	0	0	0
Methylobacteriaceae	0,00731012	0,011552013	0,01593299	0,002071938	0,001667334	0,064325228	0	0,005311944	0,013862896	0,001659255	0,046300194	0,00362227	0,006462662	0	0,043369836
Methylocystaceae	0	0	0	0	0	0,030554483	0,002558526	0	0,002310483	0	0	0	0	0	0,015179443
Methylophilaceae	0	0	0	0	0,001667334	0	0	0	0,006931448	0	0	0	0	0	0
Microbacteriaceae	0,002436707	0	0,002276141	0,004143875	0,001667334	0,001608131	0,002558526	0,002655972	0	0,001659255	0,004209109	0,00362227	0,003231331	0	0,019516426
Micrococcaceae	0	0	0	0	0	0,003216261	0	0,002655972	0	0	0,004209109	0	0	0	0
Micromonosporaceae	0	0	0	0	0	0,006432523	0	0	0,002310483	0	0,004209109	0	0	0	0
Moraxellaceae	0,002436707	0,005776006	0	0	0	0,001608131	0	0	0	0	0	0	0	0	0
Nocardioidaceae	0	0	0,002276141	0,002071938	0	0,011256915	0,002558526	0	0,002310483	0,001659255	0	0,00362227	0,006462662	0,015089215	0,04770682
Paenibacillaceae	0	0	0,006828424	0	0	0,006432523	0,002558526	0	0,002310483	0	0	0	0,009693993	0,041495341	0,006505475
Phyllobacteriaceae	0	0,011552013	0	0	0	0,001608131	0	0	0	0	0	0	0	0	0

Pirellulaceae	0	0	0,020485273	0	0	0	0	0	0	0	0	0	0	0	0
Piscirickettsiaceae	0	0	0	0	0,001667334	0	0,007675579	0	0	0	0,004209109	0	0	0	0
Porphyromonadaceae	0,017056946	0,005776006	0,018209132	0,014503564	0,005002001	0,016081307	0	0,061087355	0,013862896	0,001659255	0,008418217	0,010866809	0,012925324	0,033950734	0,034695869
Procabacteriaceae	0	0	0	0	0	0	0	0	0	0	0	0	0,012925324	0	0
PRR-10	0	0	0	0	0	0,003216261	0,005117053	0	0	0	0	0	0	0	0
Rhizobiaceae	0,002436707	0	0	0	0	0	0	0	0	0,001659255	0	0	0,003231331	0	0,002168492
Rhodobacteraceae	0,002436707	0	0,004552283	0	0	0	0	0	0	0	0	0	0	0	0
Rhodocyclaceae	0	0	0,002276141	0,002071938	0	0	0,005117053	0,002655972	0	0	0,004209109	0	0	0,011316911	0
Rhodospirillaceae	0	0	0	0	0	0,001608131	0,007675579	0,002655972	0	0	0,008418217	0	0	0	0,002168492
Ruminococcaceae	0,026803772	0,005776006	0,009104566	0	0	0	0	0	0,009241931	0	0	0,007244539	0	0	0
S24-7	0,053607544	0,011552013	0,011380707	0	0	0	0	0,002655972	0,004620965	0	0	0	0	0	0,021684918
Sinobacteraceae	0	0	0,004552283	0	0	0,008040654	0	0	0,002310483	0	0	0	0	0,007544607	0
Solibacteraceae	0	0	0,002276141	0,002071938	0	0	0	0	0	0	0	0	0	0	0
Sphingobacteriaceae	0,00731012	0,011552013	0,047798971	0,018647439	0,003334667	0,014473176	0,005117053	0,007967916	0,006931448	0,013274043	0,016836434	0,014489079	0,003231331	0	0,067223246
Syntrophobacteraceae	0	0,005776006	0	0	0	0	0,002558526	0	0	0	0,004209109	0	0	0,003772304	0
Veillonellaceae	0,012183533	0,005776006	0,009104566	0,014503564	0,010004002	0,009648784	0,005117053	0	0,011552413	0,008296277	0,025254651	0,00362227	0,016156655	0	0,002168492
Xanthomonadaceae	0,00731012	0	0	0,002071938	0,001667334	0,017689438	0,002558526	0,007967916	0,002310483	0,011614787	0,012627326	0,00362227	0,071089282	0,026406126	0
[Prevotella]	0	0	0	0	0	0	0	0	0	0,003318511	0	0	0	0	0
[Ruminococcus]	0,002436707	0	0	0	0	0	0	0	0	0	0	0	0	0	0,002168492
A17	0	0	0	0	0	0	0	0,002655972	0,006931448	0	0	0	0	0	0
Achromobacter	0	0	0	0	0	0,006432523	0	0,002655972	0	0,003318511	0	0	0	0	0
Acidaminobacter	0,004873413	0	0	0,002071938	0	0	0	0	0,002310483	0	0	0	0	0	0
Acidovorax	0,002436707	0	0,006828424	0	0	0,003216261	0	0	0,006931448	0	0,016836434	0,00362227	0	0	0
Actinomyces	0	0	0	0	0	0	0	0	0	0,003318511	0	0	0	0	0
Actinomycetospora	0,00731012	0,005776006	0,013656849	0,006215813	0	0,003216261	0	0,002655972	0	0	0,008418217	0	0,003231331	0	0
Aerococcus	0	0	0	0	0	0	0	0	0	0,001659255	0	0	0,003231331	0,007544607	0,006505475
Aeromicrobium	0	0	0	0	0	0	0,007675579	0	0	0	0	0	0	0,007544607	0

Aggregatibacter	0	0	0	0	0	0	0	0	0	0,003318511	0	0	0	0	0
Akkermansia	0,004873413	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Allochromatium	0	0	0	0	0	0,001608131	0	0	0	0	0	0	0	0	0,004336984
Aminobacter	0	0,011552013	0,002276141	0	0	0,001608131	0	0	0	0	0	0	0	0	0
Ammoniphilus	0	0	0	0	0	0,001608131	0	0	0	0	0	0	0	0	0,002168492
Amycolatopsis	0	0	0	0	0	0	0	0	0	0	0,008418217	0	0	0	0
Anaerofilum	0	0	0,009104566	0	0	0	0	0	0	0	0	0	0	0	0
Anaerolinea	0	0	0	0	0	0,001608131	0	0	0	0	0,004209109	0	0	0	0
Anaeromusa	0	0	0	0	0	0,003216261	0	0,002655972	0	0,003318511	0	0	0	0	0
Anoxybacillus	0	0	0	0	0	0	0	0	0,004620965	0	0	0	0	0	0
Aquicella	0	0	0,006828424	0	0	0	0	0	0	0	0	0	0	0	0
Arcobacter	0,00731012	0,028880032	0,027313698	0	0,010004002	0,020905699	0,012792631	0,007967916	0,013862896	0,048118404	0,004209109	0,018111349	0,012925324	0,056584556	0
Arsenicicoccus	0	0	0	0	0	0,006432523	0	0	0	0	0	0	0	0	0
Arthrobacter	0	0	0	0	0	0,003216261	0	0	0	0,001659255	0	0,00362227	0	0	0
Aurantimonas	0,00731012	0	0,009104566	0	0	0,008040654	0	0	0	0	0	0,00362227	0	0,026406126	0,032527377
Azospira	0,002436707	0	0	0	0,001667334	0	0	0	0	0,001659255	0	0	0	0	0
Bacillus	0	0	0	0	0,003334667	0	0	0,002655972	0,030036275	0,028207341	0,004209109	0	0,009693993	0	0
Bdellovibrio	0,004873413	0	0,002276141	0	0,001667334	0,009648784	0	0	0	0	0,004209109	0,007244539	0	0	0
Bifidobacterium	0,009746826	0,005776006	0	0	0	0	0	0	0,002310483	0	0	0	0	0	0
Blastomonas	0	0	0,002276141	0	0	0,001608131	0,005117053	0	0	0	0	0	0	0	0,006505475
Blvii28	0	0	0	0	0	0	0,005117053	0	0	0	0	0	0	0,018861519	0
Brachybacterium	0	0	0,006828424	0	0	0	0	0	0,002310483	0,004977766	0,004209109	0,00362227	0,003231331	0,026406126	0,021684918
Brevibacterium	0	0	0	0	0	0	0	0	0	0	0	0	0,006462662	0	0
Brevundimonas	0,029240479	0,011552013	0,002276141	0	0	0,001608131	0	0	0	0	0	0	0	0	0
Burkholderia	0,014620239	0,017328019	0,004552283	0,002071938	0,001667334	0,008040654	0,002558526	0	0,004620965	0,003318511	0,025254651	0,010866809	0	0,052812252	0,026021902
Candidatus Koribacter	0	0	0,004552283	0	0	0,001608131	0	0	0	0	0	0	0	0	0,008673967
Candidatus Portiera	0,002436707	0,005776006	0,011380707	0	0,003334667	0,001608131	0,002558526	0	0	0,009955532	0	0,00362227	0,019387986	0,037723037	0,006505475

Candidatus Solibacter	0	0	0	0	0	0	0,002558526	0	0,002310483	0	0	0	0	0	0,002168492
Carnobacterium	0	0	0	0	0	0	0	0	0	0,001659255	0	0,00362227	0	0,052812252	0
Cellvibrio	0	0	0	0	0	0,003216261	0	0	0	0	0	0	0	0	0
Chthoniobacter	0	0	0,004552283	0	0	0	0	0	0	0	0	0	0	0	0
Citrobacter	0,051170837	0,034656039	0,029589839	0,043510691	0,06502601	0,049852052	0,017909684	0,021247776	0,057762066	0,028207341	0,042091085	0,028978158	0,06462662	0,018861519	0,026021902
Cloacibacterium	0,004873413	0	0,018209132	0,012431626	0,006669334	0,001608131	0,005117053	0,055775411	0,060072549	0	0,008418217	0,010866809	0	0	0
Clostridium	0,121835327	0,716224802	0,072836528	0,05387038	0,025010004	0,020905699	0,023026737	0,01327986	0,050830619	0,016592553	0,008418217	0,014489079	0,012925324	0,022633822	0,075897213
Collimonas	0	0	0	0	0	0	0,002558526	0	0	0,003318511	0	0	0,003231331	0	0
Collinsella	0,009746826	0	0	0	0	0	0,002558526	0	0,002310483	0	0	0	0	0	0
Comamonas	0,112088501	0,103968116	0,034142122	0,018647439	0,025010004	0,043419529	0,033260842	0,069055271	0,023104827	0,036503617	0,021045543	0,224580722	0,061395289	0,022633822	0,039032853
Coprococcus	0,031677185	0,017328019	0,011380707	0	0,005002001	0,003216261	0,010234105	0,005311944	0,013862896	0,006637021	0,016836434	0,014489079	0,03231331	0,022633822	0,010842459
Curtobacterium	0,012183533	0	0,011380707	0	0,003334667	0,008040654	0	0	0	0,008296277	0,012627326	0	0,016156655	0,003772304	0,032527377
Cytophaga	0	0	0	0,008287751	0	0	0	0	0	0	0	0	0	0	0
DA101	0	0	0	0	0	0	0,005117053	0	0	0	0	0	0	0	0
Deinococcus	0	0	0,006828424	0	0	0	0	0	0	0	0	0	0	0	0
Delftia	0,009746826	0,017328019	0,013656849	0,03522294	0,005002001	0,004824392	0	0,005311944	0	0,006637021	0	0,010866809	0,003231331	0,018861519	0,054212295
Desulfovibrio	0	0	0	0	0,001667334	0	0	0,002655972	0,004620965	0,001659255	0	0	0	0	0
Devosia	0	0	0,004552283	0	0,001667334	0,003216261	0	0	0	0	0	0,00362227	0	0,003772304	0
Dorea	0	0	0	0	0,011671335	0	0	0	0	0	0	0	0	0	0
Dyadobacter	0,00731012	0	0	0	0	0	0	0	0	0	0	0,00362227	0	0	0
Dysgonomonas	0,041424011	0,051984058	0,100150225	0,002071938	0,011671335	0,04502766	0,002558526	0,015935832	0,023104827	0,031525851	0,067345736	0,115912631	0,025850648	0,026406126	0,004336984
Ellin506	0	0	0	0	0	0,004824392	0	0	0	0	0	0	0	0	0
Enhydrobacter	0,029240479	0,075088084	0,050075113	0,070445881	0,02334267	0,083622797	0,010234105	0,002655972	0,023104827	0,014933298	0,046300194	0,025355888	0	0,003772304	0,026021902
Epulopiscium	0	0	0	0	0	0,003216261	0	0	0	0	0	0	0	0	0
Erysipelothrix	0,004873413	0,017328019	0,006828424	0	0	0,003216261	0,002558526	0,002655972	0	0	0,008418217	0,00362227	0,025850648	0	0,028190394
Exiguobacterium	0	0	0	0	0	0	0	0	0	0,014933298	0	0	0,006462662	0,045267645	0,008673967
Faecalibacterium	0,041424011	0,005776006	0	0	0	0	0	0	0,004620965	0	0	0	0	0	0,002168492

Fimbriimonas	0	0,005776006	0,002276141	0	0	0	0,005117053	0	0,002310483	0	0	0	0	0,007544607	0,019516426
Flavisolibacter	0	0	0	0	0	0,003216261	0	0	0	0	0	0	0	0	0
Flavobacterium	0,004873413	0,057760065	0,01593299	0,002071938	0,005002001	0,004824392	0,007675579	0,018591804	0,009241931	0,004977766	0	0,025355888	0	0,011316911	0
Fluviicola	0	0	0,004552283	0,008287751	0	0,001608131	0,015351158	0	0	0,001659255	0	0,010866809	0	0,011316911	0,058549279
Friedmanniella	0	0	0	0	0	0,006432523	0	0	0	0	0	0	0	0	0
Fusibacter	0,00731012	0	0	0	0	0	0	0,002655972	0,002310483	0	0	0	0	0	0
Gallionella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,017347935
Gemmata	0	0	0	0	0	0,003216261	0	0,002655972	0	0	0	0	0,003231331	0	0
Geobacter	0	0	0	0	0,003334667	0	0	0	0	0	0	0	0	0	0
Geodermatophilus	0	0	0,002276141	0	0	0,001608131	0	0	0	0	0	0	0	0	0
Giesbergeria	0,002436707	0,011552013	0,006828424	0,008287751	0	0	0,002558526	0,002655972	0	0	0	0	0	0,011316911	0
Granulicatella	0	0	0	0	0	0	0	0	0	0,008296277	0	0	0	0	0
Haemophilus	0	0	0	0	0	0	0	0	0	0,003318511	0	0	0	0	0
Hymenobacter	0,009746826	0,011552013	0,059179679	0,004143875	0,008336668	0,014473176	0,005117053	0,010623888	0,048520136	0,031525851	0,02946376	0,050711776	0,03231331	0,03017843	0,164805378
Janibacter	0	0	0	0	0	0	0	0	0	0,001659255	0,004209109	0,00362227	0,019387986	0,007544607	0
Janthinobacterium	0,00731012	0	0,013656849	0,002071938	0	0,004824392	0,002558526	0,002655972	0	0,001659255	0	0,00362227	0,003231331	0,011316911	0
K82	0	0	0	0	0	0	0	0	0,004620965	0	0	0	0	0	0,002168492
Kaistobacter	0,00731012	0,017328019	0,038694405	0	0,005002001	0,033770745	0,023026737	0,005311944	0,006931448	0,008296277	0	0,010866809	0,003231331	0,018861519	0,006505475
KD1-23	0	0	0,004552283	0	0	0	0	0,002655972	0	0	0	0	0	0	0
Kineococcus	0	0	0	0	0,003334667	0	0	0	0	0	0	0	0	0	0
Kocuria	0	0	0	0	0,001667334	0	0	0	0,020794344	0	0	0	0	0	0
Lachnospira	0,00731012	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lampropedia	0	0	0,009104566	0	0	0	0	0	0	0	0	0	0	0	0
Legionella	0	0	0	0	0	0,003216261	0	0	0	0	0	0	0	0	0
Listeria	0	0	0	0	0	0	0	0	0	0	0	0	0	0,011316911	0
Luteibacter	0	0,005776006	0	0	0	0,011256915	0	0,002655972	0,009241931	0,001659255	0,012627326	0,00362227	0	0	0
Luteimonas	0	0,005776006	0,002276141	0	0	0	0	0	0	0,001659255	0	0,007244539	0	0	0

Lysobacter	0	0,005776006	0	0	0	0,006432523	0,012792631	0	0,002310483	0	0	0	0	0	0
Magnetospirillum	0	0	0	0	0	0	0,005117053	0	0,002310483	0,013274043	0,004209109	0	0,003231331	0	0
Megasphaera	0	0	0,002276141	0	0,003334667	0	0	0	0	0	0	0	0	0,003772304	0,002168492
Methylibium	0	0	0,004552283	0	0	0,001608131	0	0	0	0	0	0	0	0	0
Methylobacterium	0,073101196	0,121296136	0,152501479	0,076661694	0,026677338	0,192975685	0,030702315	0,031871663	0,067003997	0,044799894	0,109436821	0,057956315	0,061395289	0,067901467	0,201669739
Microbacterium	0,004873413	0	0	0	0	0,003216261	0	0	0	0,001659255	0,004209109	0	0	0	0
Mitsuaria	0	0	0	0	0	0,004824392	0	0	0	0	0	0	0	0	0
Mycobacterium	0	0	0	0	0	0,006432523	0	0	0	0	0,004209109	0	0	0,007544607	0
Neisseria	0	0	0	0	0	0	0	0	0	0,003318511	0	0	0	0	0
Nitrospira	0	0	0	0	0	0,004824392	0,002558526	0	0	0	0,004209109	0	0	0	0
Nocardioides	0	0	0	0	0	0	0	0	0	0	0	0	0	0,011316911	0
Novispirillum	0,002436707	0	0	0	0,001667334	0	0	0	0	0	0	0	0,003231331	0	0
Novosphingobium	0,00731012	0,017328019	0,013656849	0	0,010004002	0,019297569	0,002558526	0,005311944	0,002310483	0,008296277	0,025254651	0	0,003231331	0	0,013010951
Ochrobactrum	0,012183533	0,034656039	0,006828424	0	0,001667334	0,012865046	0	0,002655972	0	0	0,025254651	0,018111349	0,019387986	0,033950734	0,019516426
Opitutus	0	0	0	0	0	0,001608131	0,002558526	0	0	0	0	0	0	0	0
Oscillospira	0,004873413	0	0	0,002071938	0	0	0	0	0,004620965	0	0	0	0,003231331	0	0,002168492
Paenibacillus	0,00731012	0	0,01593299	0	0,001667334	0	0,002558526	0,002655972	0	0	0	0	0	0,018861519	0,002168492
Paludibacter	0	0,011552013	0	0,006215813	0,001667334	0	0,005117053	0,007967916	0,006931448	0,003318511	0	0,010866809	0	0	0
Pantoea	0,021930359	0,034656039	0,025037556	0,022791314	0,021675337	0,024121961	0,030702315	0,021247776	0,048520136	0,033185107	0,02946376	0,014489079	0,029081979	0,052812252	0,017347935
Paracoccus	0,00731012	0	0	0	0,001667334	0	0	0,002655972	0,006931448	0	0	0	0,006462662	0	0,013010951
Pedobacter	0,00731012	0,017328019	0,031865981	0,002071938	0	0,004824392	0,002558526	0,002655972	0,006931448	0,003318511	0,012627326	0,054334046	0,016156655	0,045267645	0,02385341
Perlucidibaca	0	0	0	0	0	0,036987006	0	0	0	0	0,025254651	0	0	0	0
Pigmentiphaga	0	0	0	0,002071938	0	0,001608131	0	0	0	0	0	0	0	0	0
Planctomyces	0	0	0	0	0	0	0,005117053	0	0	0	0	0	0	0	0
Plesiocystis	0	0	0	0	0	0	0,002558526	0	0	0	0	0	0	0,003772304	0
Prauseria	0	0	0	0	0,001667334	0	0	0	0	0	0	0	0	0	0,002168492
Prevotella	0,124272034	0,306128343	0,289069969	0,15125145	0,056689342	0,109352888	0,030702315	0,021247776	0,067003997	0,09955532	0,046300194	0,032600427	0,180954535	0,207476706	0,140951968

Propionibacterium	0	0	0	0	0	0	0	0	0,002310483	0,001659255	0	0	0	0	0
Proteus	0	0	0	0	0	0,003216261	0,002558526	0	0	0,001659255	0	0,00362227	0,003231331	0	0,002168492
Pseudoclavibacter	0	0	0,002276141	0	0	0	0	0	0	0	0	0	0	0	0,002168492
Pseudonocardia	0	0	0	0	0	0	0	0,002655972	0	0	0,008418217	0,010866809	0	0	0
Rhodobacter	0	0	0,006828424	0	0	0	0	0	0	0	0	0	0	0	0
Rhodococcus	0	0	0	0	0	0,006432523	0	0	0	0	0	0	0	0	0
Ruminococcus	0,034113892	0	0	0	0,001667334	0	0	0	0,013862896	0	0	0	0	0	0,002168492
Sedimentibacter	0	0	0	0	0	0	0	0,005311944	0	0	0	0	0	0	0
Segetibacter	0,009746826	0	0	0,010359688	0	0	0	0	0	0	0	0	0	0	0
Selenomonas	0	0	0	0	0	0	0	0	0	0,003318511	0	0	0	0	0
Simplicispira	0,002436707	0	0,013656849	0	0	0,001608131	0	0	0,002310483	0,003318511	0	0	0	0	0
SMB53	0,004873413	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sphingobacterium	0,146202393	0,09819211	0,109254791	0,037294878	0,005002001	0,02251383	0,017909684	0,268253167	0,016173379	0,014933298	0,058927519	0,217336183	0,045238634	0,064129164	0,028190394
Sphingobium	0,012183533	0	0,009104566	0	0,008336668	0,009648784	0,002558526	0	0	0,019911064	0	0	0,006462662	0,026406126	0,028190394
Sphingomonas	0,06335437	0,103968116	0,157053762	0,122244323	0,02334267	0,119001672	0,017909684	0,02655972	0,067003997	0,044799894	0,037881977	0,119534901	0,077551944	0,101852201	0,286240919
Spirosoma	0	0,011552013	0,070560386	0	0,001667334	0,001608131	0	0	0,013862896	0,003318511	0,021045543	0,00362227	0,006462662	0,007544607	0,032527377
Sporolactobacillus	0,009746826	0,132848149	0,011380707	0,012431626	0,006669334	0,054676444	0,012792631	0,002655972	0,009241931	0,013274043	0,016836434	0	0	0,003772304	0,002168492
Sporotomaculum	0	0	0	0,004143875	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus	0	0	0	0	0	0	0	0	0	0	0	0	0	0,007544607	0
Stenotrophomonas	0,00731012	0,005776006	0,004552283	0,010359688	0,001667334	0,024121961	0	0	0,009241931	0,02488883	0,008418217	0,018111349	0,006462662	0,015089215	0,086739673
Steroidobacter	0,002436707	0	0	0	0	0,003216261	0,002558526	0	0	0	0	0	0	0	0
Streptococcus	0,004873413	0,005776006	0	0	0	0	0	0	0	0,044799894	0	0	0	0	0
Sulfurospirillum	0	0	0,006828424	0	0	0	0	0,010623888	0,013862896	0,006637021	0,004209109	0,00362227	0,006462662	0,011316911	0,036864361
Sutterella	0,014620239	0	0	0	0	0	0	0,002655972	0	0	0	0	0	0	0
Swaminathania	0	0	0,002276141	0	0	0,001608131	0	0	0	0	0	0	0	0	0
Symbiobacterium	0	0	0	0,008287751	0	0	0	0,002655972	0	0	0	0	0	0	0
Thermomonas	0	0	0	0	0	0	0	0	0	0	0,008418217	0	0	0	0

Thiobacillus	0	0	0	0	0	0,003216261	0,002558526	0	0	0	0,008418217	0	0	0,003772304	0
Trabulsiella	0,019493652	0	0,020485273	0,002071938	0,011671335	0,017689438	0,010234105	0,005311944	0,011552413	0,001659255	0,008418217	0,00362227	0,009693993	0	0,010842459
Trichococcus	0	0	0	0	0	0	0	0	0	0,003318511	0	0	0	0	0
Turicibacter	0,004873413	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vadinHB04	0	0	0	0	0	0,004824392	0	0	0	0	0,008418217	0	0	0	0
Variovorax	0	0	0,004552283	0	0	0,003216261	0	0	0	0	0	0	0	0	0
Veillonella	0,002436707	0	0	0	0	0	0	0	0	0,013274043	0	0	0	0	0
Wautersiella	0	0,005776006	0	0,018647439	0,071695345	0	0	0,042495551	0,32115709	0,004977766	0,004209109	0	0	0	0,019516426
Xenorhabdus	0,002436707	0,005776006	0	0,002071938	0	0	0,002558526	0	0	0	0	0	0	0	0
Zoogloea	0	0	0	0	0,001667334	0	0	0	0	0	0	0	0,006462662	0	0,015179443
Microorganisms	**Fungi**														
	B0	**M0**	**T0**	**B12**	**M12**	**T12**	**B24**	**M24**	**T24**	**B48**	**M48**	**T48**	**B72**	**M72**	**T72**
Nectriaceae	31,79723502	31,35634419	37,21428571	24,73246136	34,89604566	25,43815088	51,78303866	49,57898958	53,28366987	26,61115134	34,61683654	50,84637496	46,67303407	42,11145997	46,68794892
Mycosphaerellaceae	2,534562212	2,430724356	1,142857143	1,902497027	1,182225846	1,066802134	0,689241834	1,623897354	1,50950794	0,615496017	0,20385051	0,862344299	0,270614454	2,276295133	2,075019952
Trichosporonaceae	1,900921659	1,263976665	1,5	1,783590963	2,609050143	0,96520193	0,71920887	0,741780273	1,274259949	0,579290369	0,792751982	0,606834877	0,668576886	4,081632653	1,356743815
Tremellales	0,806451613	3,354399611	2,642857143	2,615933413	1,182225846	1,371602743	1,798022176	1,002405774	0,960595962	1,013758146	1,834654587	1,149792399	0,620821394	1,628728414	0,03990423
Alternaria	5,011520737	5,930967428	4,285714286	2,615933413	2,935181411	2,489204978	1,947857357	3,047313553	2,117231915	0,687907314	1,67610419	4,982433727	0,891435848	4,238618524	3,471667997
Aureobasidium	0,460829493	0,923675255	0,571428571	1,426872771	0,224215247	0,292100584	0,569373689	0,300721732	0,470495981	0,108616944	0,279350698	0,127754711	0,397962432	0,412087912	0,359138069
Candida	0,921658986	0,777831794	0,428571429	1,664684899	4,443538524	0,571501143	0,149835181	1,864474739	0,352871986	1,593048516	1,381653454	0,606834877	3,311047437	2,06043956	0,399042298
Chaetomella	1,900921659	0,097228974	0	0	0,020383204	0	0	0	0	0,072411296	0,007550019	0	0,015918497	0	0
Cladosporium	7,02764977	8,26446281	8,5	9,750297265	4,891969018	4,152908306	4,465088403	3,869286287	4,332483827	2,353367125	5,790864477	2,906419674	3,215536453	3,198587127	4,269752594
Colletotrichum	2,880184332	5,05590666	6,357142857	5,469678954	5,707297187	2,311404623	1,618219958	3,287890938	2,195647912	2,136133237	1,27595319	2,746726286	1,671442216	3,375196232	7,82122905
Didymella	9,735023041	6,174039864	9,357142857	3,567181926	6,15572768	3,314706629	4,075516931	3,408179631	5,077435797	33,20057929	0,989052473	3,481315874	2,148997135	3,296703297	4,588986433
Fusarium	3,571428571	0,972289742	2,571428571	1,070154578	1,161842642	1,371602743	0,389571471	2,205292702	2,195647912	7,784214337	1,019252548	1,117853721	2,849411016	1,962323391	4,509177973
Glomerella	1,728110599	2,139037433	1,928571429	0,951248514	1,691805952	0,93980188	1,798022176	1,06255012	1,195843952	3,548153512	0,792751982	1,181731076	0,986946832	4,297488226	3,431763767
Kazachstania	0,576036866	0,631988333	0,428571429	1,783590963	0,631879331	15,13843028	1,138747378	1,042502005	2,156439914	1,158580739	10,19252548	1,022037688	2,180834129	0,686813187	0,478850758
Kodamaea	0,17281106	0	0,928571429	0	2,833265389	0,787401575	0,599340725	0,501202887	0,019603999	0,18102824	0	0,031938678	0	0	0,997605746

Penicillium	0,633640553	1,409820126	0,428571429	0,237812128	1,121076233	0,139700279	0,179802218	0,641539695	0,294059988	0,687907314	0,015100038	0,095816033	0	0,35321821	0,159616919
Pestalotiopsis	0,230414747	0,194457948	0,214285714	0,118906064	0,67264574	0,228600457	0,389571471	0,521251002	0,274455989	0,615496017	0	0,542957522	0,206940465	1,766091052	0,678371907
Saccharomyces	0,057603687	0,097228974	0	0,118906064	0,040766408	0,038100076	0,059934073	0,140336808	0,156831994	0,072411296	0,249150623	0,894282977	0,238777459	0,941915228	1,037509976
Strelitziana	0,576036866	0,097228974	0,5	1,070154578	2,833265389	0,431800864	0,119868145	0,781876504	0,294059988	0,253439537	0,347300868	0,031938678	0,334288443	0,824175824	0,03990423
Torulaspora	1,843317972	1,215362178	0,642857143	3,68608799	1,386057888	16,39573279	2,637099191	1,764234162	3,352283866	1,556842867	16,69309173	2,044075375	2,626552053	1,157770801	0,199521149
unidentified	4,320276498	4,958677686	3,214285714	6,896551724	2,772115777	1,765303531	3,775846569	4,711307137	4,959811802	2,353367125	5,269913175	3,321622485	2,722063037	3,335949765	1,835594573
Wickerhamomyces	0,34562212	0,048614487	0,214285714	1,307966706	0	0,457200914	0,059934073	0,080192462	0,725347971	0,144822592	0,27180068	0	0,095510984	0,667189953	0
Other	16,30184332	19,6402528	13	21,52199762	18,263351	16,5481331	17,86035361	16,13873296	10,21368359	11,07892831	12,7595319	17,88565953	24,03693091	14,14835165	11,93136472
Agaricaceae	0	0	0	0	0	0	0	0	0	0	0	0,031938678	0	0	0
Amphisphaeriaceae	0	0	0,071428571	0	0	0	0	0	0	0	0	0	0	0	0
Bionectriaceae	0	0	0	0	0	0	0,059934073	0	0	0	0	0	0	0	0
Botryosphaeriaceae	0	0	0	0,237812128	0	0	0	0	0	0	0	0	0	0	0
Chaetomiaceae	0	0	0	0	0	0	0	0	0	0	0	0,063877355	0	0	0
Diaporthaceae	0	0	0,071428571	0	0	0	0	0	0	0	0	0	0	0	0
Dothioraceae	0	0	0	0	0	0	0	0	0	0	0	0	0,015918497	0	0
Glomerellaceae	0	0	0,071428571	0	0	0	0	0,080192462	0	0	0	0	0	0	0
Herpotrichiellaceae	0	0	0	0	0	0	0	0	0	0	0,007550019	0	0	0	0
Lophiostomataceae	0	0	0	0	0	0,012700025	0	0	0	0	0	0	0	0	0
Pezizomycotina	0	0	0	0	0	0	0,089901109	0	0	0	0	0	0	0,039246468	0
Pleosporales	0	0,097228974	0	0,118906064	0,244598451	0,050800102	0,059934073	0,100240577	0,039207998	0,108616944	0	0	0	0,019623234	0
Saccharomycetaceae	0	0	0	0	0	0,203200406	0	0	0,235247991	0	0,868252171	0,223570744	0,222858962	0	0
Saccharomycetales	0,057603687	0	0	0	0	0	0,179802218	0	0,235247991	0	0,113250283	0	0,015918497	0	0
Sporormiaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,03990423
Teratosphaeriaceae	0	0	0	0	0	0	0,029967036	0	0,019603999	0	0	0	0	0	0
Trichocomaceae	0,17281106	0	0,071428571	0,118906064	0,020383204	0	0,029967036	0,060144346	0	0,108616944	0	0	0	0,039246468	0
Valsaceae	0	0	0	0	0	0	0,149835181	0	0,235247991	0	0	0,766528266	0,620821394	0,078492936	0,758180367
Acremonium	0	0	0	0	0	0	0,179802218	0	0	0	0	0	0	0	0

Apiotrichum	0,288018433	0,097228974	0	0,356718193	0,061149613	0,063500127	0,029967036	0,040096231	0,117623995	0,036205648	0,052850132	0	0,143266476	0,274725275	0,159616919
Arnium	0	0	0,071428571	0	0	0	0	0	0	0	0	0	0	0	0
Aspergillus	0,115207373	0,194457948	0,214285714	0,475624257	0,285364859	0,076200152	0,539406653	0,020048115	0,411683984	0,072411296	0	0,734589588	0,875517351	0,058869702	0,319233839
Beauveria	0,057603687	0	0	0	0	0	0,029967036	0,100240577	0	0	0	0	0	0,039246468	0
Bipolaris	0,403225806	0,048614487	0	0,237812128	0,14268243	0,127000254	0,119868145	0,120288693	0	0,434467777	0	0	0,493473416	0,470957614	0,11971269
Buckleyzyma	0	0	0	0	0	0,088900178	0	0	0,058811998	0	0	0	0	0	0
Bullera	0	0,145843461	0,071428571	0,118906064	0	0	0	0	0	0	0,354850887	0	0	0	0
Cercospora	0	0	0	0	0	0,012700025	0	0	0	0	0	0	0	0,039246468	0
Chaetomium	0	0	0,142857143	0	0,020383204	0	0	0	0,019603999	0	0	0	0,015918497	0	0
Coprinellus	0	0	0,214285714	0	0	0	0	0	0	0	0	0	0	0	0,07980846
Curvularia	0,115207373	0	0	0	0	0	0	0	0	0	0	0	0	0,019623234	0
Cutaneotrichosporon	0	0	0,785714286	0,951248514	0	0,050800102	0,329637399	0	0,156831994	0,036205648	0,234050585	0	0	0	0,598563448
Diaporthe	0	0	0,071428571	0	0	0,012700025	0	0,300721732	0	0,108616944	0	0	0	0,058869702	0
Dioszegia	0	0	0	0	0	0,355600711	0	0	0	0	0,007550019	0	0	0	0
Diplodia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,03990423
Dothiorella	0	0	0	0,356718193	0	0	0	0	0	0	0	0	0	0	0
Endomelanconiopsis	0	0	0	0	0	0,012700025	0	0	0	0	0	0	0	0	0
Exophiala	0	0	0	0	0	0	0	0	0	0	0	0	0	0,196232339	0
Filobasidium	0	0	0	0	0	0	0	0	0,019603999	0	0	0	0	0	0
Geosmithia	0	0	0	0	0	0	0	0	0	0	0	0	0,015918497	0	0
Hannaella	0,576036866	0,437530384	0	0,118906064	0	0,063500127	0	0	0,019603999	0	0,302000755	0	0	0	0
Hanseniaspora	0,17281106	0,194457948	0	0,118906064	0,122299225	0,33020066	0,299670363	0,40096231	0,156831994	0,108616944	0,347300868	0,095816033	0,477554919	0,058869702	0,239425379
Heterogastridium	0	0	0	0	0	0	0	0	0	0	0,052850132	0	0	0	0
Isaria	0	0	0,071428571	0	0	0	0	0	0,117623995	0,036205648	0	0	0	0	0
Issatchenkia	0,8640553	0,048614487	0,857142857	0,237812128	0	0,177800356	0,119868145	0	0	0	0,226500566	0	0,191021968	0	0,159616919
Kluyveromyces	0	0	0	0	0	0	0	0,040096231	0	0	0	0	0	0	0
Kwoniella	0	0,145843461	0	0	0,264981655	0	0,179802218	0	0	0	0,030200076	0,191632066	0	0	0

Meira	0,288018433	0,777831794	0,071428571	0,356718193	0,346514472	0,419100838	0,149835181	0	0,215643991	0,217233888	0,143450359	0,063877355	0,366125438	0,431711146	0,798084597
Meyerozyma	0,17281106	0,194457948	0,357142857	0,951248514	0,101916021	0,381000762	0,059934073	0,100240577	0,235247991	0,217233888	0,27180068	0,127754711	0,095510984	0,706436421	0
Microdochium	0	0	0	0	0	0	0	0	0	0	0,007550019	0	0	0	0
Mortierella	0,230414747	0	0	0,237812128	0,020383204	0	0	0,020048115	0	0	0	0,031938678	0,015918497	0,019623234	0
Mucor	0	0	0	0	0	0,609601219	0	0,120288693	0	0	0,279350698	0	0	0	0
Nigrospora	0	0	0,071428571	0	0,163065634	0	0	0	0	0	0	0	0	0,019623234	0
Ochroconis	0,17281106	0,145843461	0,428571429	0	0,407664085	0,063500127	0,059934073	0,040096231	0,098019996	0	0	0	0,079592486	0	0
Periconia	0	0	0	0	0	0,203200406	0	0	0	0	0	0	0	0	0
Pichia	0	0,145843461	0	0,356718193	0	0	0	0	0,019603999	0	0,211400529	0	0	0	0
Purpureocillium	0	0	0	0	0	0,076200152	0	0	0,019603999	0	0	0	0	0	0
Rhizopus	0	0	0	0	0	0	0	0	0	0	0	0	0,015918497	0	0
Rhodosporidiobolus	0	0	0	0	0	0	0	0	0	0	0	0	0,015918497	0,412087912	0,319233839
Rhodotorula	0	0	0	0	0	0	0,029967036	0	0,019603999	0,072411296	0	0,127754711	0	0	0
Rhynchogastrema	0	0	0,214285714	0,237812128	0	0	0,089901109	0	0,137227995	0	0,015100038	0	0	0	0
Saccharomycopsis	0	0	0	0	0	0	0	0	0	0	0	0	0,143266476	0	0
Saturnispora	0	0	0	0	0	0,368300737	0	0	0	0	0	0	0	0	0
Sporobolomyces	0	0,048614487	0	0	0	0	0	0,040096231	0	0	0	0	0	0,196232339	0
Stagonosporopsis	0	0	0	0,118906064	0,122299225	0,025400051	0,029967036	0,100240577	0	0	0	0	0	0	0
Starmerella	0,921658986	0,194457948	0	0	0	0	0,329637399	0	0	0	0	1,053976365	0	0	0
Sterigmatomyces	0	0	0	0	0	0	0	0	0	0,036205648	0	0	0	0	0
Talaromyces	0,057603687	0	0	0	0	0	0	0	0	0	0,007550019	0	0	0	0
Trichoderma	0	0	0	0	0	0	0	0	0	0	0	0	0,015918497	0	0
Trichosporon	0	0,048614487	0	0	0,020383204	0	0	0	0	0	0,007550019	0	0	0	0

APPENDIX 4

Appendix 4: Statistical analysis of volatile compounds detected in the liquid fraction from fermentation of Brazilian coffee beans. The number after each letter represents the fermentation time (e.g., B0= bottom layer at 0 h).

Compounds	0 h			12 h			24 h			48 h			72 h		
	Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom
Higher alcohols															
Ethanol	15.62±3.97AB	15.16 ± 0.83 AB	18.25 ± 4.07 A	10.56 ± 1.05 B	16.68 ± 2.75 AB	20.10 ± 2.13 AC	16.10 ± 2.07 AB	16.76 ± 0.64 AB	20.53 ± 1.06 AC	41.05 ± 5.00 E	19.87 ± 1.12 A	21.44 ± 4.44 AC	17.62 ± 1.82 AB	27.02 ± 1.28 CD	33.85 ± 2.63 D
Methanol	-	0.98 ± 0.24 AB	0.73 ± 0.00 A	0.92 ± 0.14 AB	0.99 ± 0.32 AB	0.98 ± 0.42AB	1.16 ± 0.29AB	0.86 ± 0.04 AB	1.35 ± 0.09 AB	0.91 ± 0.21 AB	0.73 ± 0.16 A	1.09 ± 0.16 AB	0.88 ± 0.24 AB	0.96 ± 0.07 AB	1.54 ± 0.38 B
2-Butanol	-	-	0.12 ± 0.02 A	-	0.10 ± 0.01	-	-	-	-	0.88 ± 0.10 B	-	-	-	-	-
1-Propanol, 2-methyl	0.31±0.18ABC	0.42 ± 0.16 BCD	0.57 ± 0.19 CD	0.39 ± 0.03 ABCD	0.67 ± 0.09 D	0.66 ± 0.13 D	0.22 ± 0.03 AB	0.24 ± 0.02 AB	0.22 ± 0.05 AB	0.13 ± 0.04 A	0.28 ± 0.01 ABC	0.40 ± 0.04 ABCD	0.23 ± 0.05 AB	0.35 ± 0.01 ABC	0.56 ± 0.03 CD
1-Butanol, 3-methyl	0.40±0.09A	0.63 ± 0.12 AB	0.77 ± 0.17 ABC	0.91 ± 0.17 BCD	1.21 ± 0.20 CDE	1.38 ± 0.35 E	0.59 ± 0.16 AB	0.58 ± 0.06 AB	0.64 ± 0.01 AB	0.48 ± 0.10 AB	0.74 ± 0.06 AB	0.83 ± 0.14 ABCD	0.64 ± 0.11 AB	0.92 ± 0.08 BCDE	1.27 ± 0.12 DE
1-Butanol	-	-	-	-	0.20 ± 0.01 A	0.17 ± 0.11 AB	-	-	-	-	0.12 ± 0.02 B	0.13 ± 0.02 B	0.11 ± 0.00 B	0.17 ± 0.04 AB	0.21 ± 0.02 A
1-Hexanol	0.65±0.05 A	0.83 ± 0.11 AB	0.66 ± 0.18 A	1.08 ± 0.12 AB	0.91 ± 0.11 AB	1.04 ± 0.01 AB	0.87 ± 0.23 AB	1.28 ± 0.22 ABC	1.29 ± 0.16 ABC	1.26 ± 0.78 ABC	2.08 ± 0.04 D	1.87 ± 0.06 CD	1.57 ± 0.15 BCD	2.99 ± 0.12 E	4.20 ± 0.37 F
2-Heptanol	1.65±0.55A	2.48 ± 0.72 AB	3.13 ± 0.26 ABC	4.35 ± 0.63 BCD	4.70 ± 0.71 BCD	5.58 ± 0.61 CDE	5.10 ± 1.12 BCDE	6.46 ± 0.25 DE	7.49 ± 0.54 E	6.24 ± 1.51 DE	10.86 ± 0.24 F	11.49 ± 2.00 F	7.5 ± 0.64 E	12.71 ± 0.50 F	20.08 ± 0.91 G
3-Ethyl-4-methylpentan-1-ol	0.36±0.05A	0.49 ± 0.04 AB	0.56 ± 0.14 ABC	0.90 ± 0.10 BCD	0.84 ± 0.12 BCD	0.92 ± 0.03 BCD	0.79 ± 0.19 ABCD	0.94 ± 0.06 BCD	1.28 ± 0.07 CDE	0.80 ± 0.13 ABCD	1.47 ± 0.05 E	1.25 ± 0.39 DE	1.10 ± 0.25 DE	1.95 ± 0.09 F	2.47 ± 0.19 G
Benzyl alcohol	-	0.32 ± 0.01 A	0.32 ±0.06 A	0.32 ± 0.08 A	0.39 ± 0.02 A	0.54 ± 0.11 A	-	0.33 ± 0.00 A	0.47 ± 0.07 A	0.47 ± 0.06 A	1.34 ± 0.21 B	1.08 ± 0.12 B	1.00 ± 0.06 B	2.51 ± 0.20 C	3.45 ± 0.23 D
Phenylethyl Alcohol	-	0.38 ± 0.05 AB	0.48 ± 0.08 ABC	0.71 ± 0.07 ABC	1.03 ± 0.01 ABCD	1.22 ± 0.07 BCD	0.20 ± 0.05 A	0.67 ± 0.14 ABC	1.25 ± 0.06 CD	1.07 ± 0.16 BCD	4.12 ± 0.08 E	3.05 ± 0.60 F	1.58 ± 0.16 D	6.60 ± 0.27 G	9.88 ± 0.74 H
3-Hexen-1-ol	-	-	-	-	-	0.20 ± 0.02 AB	-	-	0.15 ± 0.01 A	-	0.19 ± 0.04 AB	0.25 ± 0.03 AB	0.23 ± 0.02 AB	0.31 ± 0.10 B	0.57 ± 0.04 C
1-Octen-3-ol	-	-	-	0.28 ± 0.02 A	0.24 ± 0.04 A	0.30 ± 0.05 A	0.35 ± 0.02 A	0.38 ± 0.08 A	0.48 ± 0.08 C	0.41 ± 0.04 AB	0.66 ± 0.01 BC	0.73 ± 0.15 C	0.44 ± 0.12 AB	0.85 ± 0.07 C	1.22 ± 0.13 D

3-Octanol	-	-	-	0.41 ± 0.05 AB	0.38 ± 0.02 A	0.35 ± 0.02 A	0.40 ± 0.14 A	0.50 ± 0.06 AB	0.56 ± 0.05 ABD	0.701 ± 0.04 BCD	0.81 ± 0.06 CD	0.91 ± 0.13 C	0.50 ± 0.08 AB	0.98 ± 0.08 C	1.45 ± 0.20 E
6-methylhept-5-en-2-ol	-	-	-	-	-	-	-	0.25 ± 0.02	-	-	-	-	-	-	-
1-Propanol	-	-	-	-	-	-	-	-	0.13 ± 0.01	-	-	-	-	-	-
6-Hepten-1-ol, 2-methyl	-	-	-	-	-	-	-	-	-	0.57 ± 0.07 A	1.11 ± 0.02 B	1.11 ± 0.11 B	0.64 ± 0.03 A	1.45 ± 0.26 B	2.90 ± 0.06 C
1-Pentanol	-	-	-	-	-	-	-	-	-	-	0.20 ± 0.00 A	0.24 ± 0.05 A	0.22 ± 0.05 A	0.33 ± 0.02 AB	0.41 ± 0.07 B
2-Pentanol	-	-	-	-	-	-	-	-	-	-	0.20 ± 0.02 AB	0.12 ± 0.02 A	0.17 ± 0.07 A	0.40 ± 0.10 B	0.36 ± 0.00 B
2-Buten-1-ol, 3-methyl	-	-	-	-	-	-	-	-	-	-	0.57 ± 0.10 A	0.77 ± 0.11 A	0.61 ± 0.06 A	1.38 ± 0.06 B	1.29 ± 0.07 B
1-Pentanol, 3-ethyl-4-methyl	-	-	-	-	-	-	-	-	-	-	-	-	0.12 ± 0.02 A	0.35 ± 0.09 B	0.59 ± 0.07 C
2-Heptanol, 6-methyl	-	-	-	-	-	-	-	-	-	-	-	-	-	0.33 ± 0.09	-
Ketones															
2-Propanone	-	-	0.23 ± 0.03	-	-	-	-	-	-	-	-	-	-	-	-
2,3-Butanedione	0.46±0.15A	0.21 ± 0.09 B	0.28 ± 0.04 Ab	0.25 ± 0.05 AB	0.28 ± 0.00 AB	-									
2-Pentanone, 3-methyl	-	0.16 ± 0.06	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetoin	0.42±0.04A	0.26 ± 0.17 A	0.73 ± 0.10 B	-	-	-	-	-	-	-	-	-	-	-	-
2-Heptanone	0.52±0.10A	0.19 ± 0.04 B	0.38 ± 0.02 A	-	-	-									
2-Butanone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
beta.-Damascenone	-	-	0.35 ± 0.05 A	-	-	-	-	-	-	0.50 ± 0.06 AB	0.98 ± 0.16 C	0.85 ± 0.14 BC	0.96 ± 0.02 BC	2.79 ± 0.27 D	2.78 ± 0.27 D
Esters															
Methyl acetate	0.27±0.01AB	0.18 ± 0.04 A	0.23 ± 0.02 A	0.51 ± 0.04 ABCD	0.69 ± 0.03 BCDE	0.84 ± 0.14 DE	1.05 ± 0.13 EFG	1.46 ± 0.20 GH	1.79 ± 0.27 H	0.39 ± 0.13 ABC	1.32 ± 0.33 FG	1.01 ± 0.08 EF	0.55 ± 0.02 ABCD	0.72 ± 0.02 CDE	1.00 ± 0.12 EF
Ethyl Acetate	3.04±1.64ABC	2.16 ± 0.21 AB	1.69 ± 0.28 A	2.94 ± 0.66 ABC	4.80 ± 0.84 BCD	3.86 ± 0.85 ABC	7.69 ± 0.29 EF	7.44 ± 0.85 DEF	8.19 ± 0.31 F	16.39 ± 2.03 G	8.16 ± 1.04 F	5.65 ± 0.95 CDEF	18.37 ± 1.45 BC	4.50 ± 0.25 BC	5.02 ± 0.22 CDE
1-Butanol, 3-methyl-, acetate	-	-	-	-	-	-	-	0.28 ± 0.06 A	0.35 ± 0.04 BC	0.39 ± 0.14 ABC	0.52 ± 0.07 BC	0.49 ± 0.11 ABC	0.33 ± 0.07 AB	0.41 ± 0.06 ABC	0.59 ± 0.47 C
Acetic acid, 2-phenylethyl ester	-	-	-	-	-	-	-	-	-	0.47 ± 0.25 A	0.65 ± 0.05 A	0.55 ± 0.16 A	0.45 ± 0.09 A	1.24 ± 0.10 B	1.45 ± 0.15 B

Acetic acid, phenylmethyl ester													0.33 ± 0.06 A	0.64 ± 0.03 B	0.79 ± 0.01 C
Capric acid, ethyl ester	-	-	-	-	-	-	-		-	0.38 ± 0.10	-	-			
Isobutyl acetate	-	-	-	-	-	-	-	-	-	-	0.19 ± 0.00 A	0.18 ± 0.02 AB	0.07 ± 0.00 C	0.10 ± 0.03 C	0.12 ± 0.01 BC
Amyl acetate	-	-	-	-	-	-	-	-	-	-	0.15 ± 0.01 A	0.14 ± 0.01 A	-	0.16 ± 0.01	0.25 ± 0.03 B
2-Buten-1-ol, 3-methyl-, acetate	-	-	-	-	-	-	-	-	-	-	0.41 ± 0.02 A	0.21 ± 0.04 B	0.21 ± 0.02 B	0.67 ± 0.11 C	0.71 ± 0.01 C
Hexyl acetate	-	-	-	-	-	-	-	-	-	-	0.41 ± 0.03 AB	0.38 ± 0.15 AB	0.22 ± 0.01 A	0.51 ± 0.06 B	0.75 ± 0.03 C
Formic acid, 2-phenylethyl ester	-	-	-	-	-	-	-	-	-	-	0.65 ± 0.06 A	0.69 ± 0.13 A	0.36 ± 0.00 B	1.26 ± 0.05 C	1.52 ± 0.09 D
Benzoic acid, 2-hydroxy-, methyl ester	-	-	-	-	-	-	-	-	-	-	1.00 ± 0.07 A	0.37 ± 0.02 C	1.12 ± 0.28 AB	1.48 ± 0.12 B	1.07 ± 0.12 AB
Propanoic acid, 2-hydroxy-, ethyl ester	-	-	-	-	-	-	-	-	-	-	-	-	-	0.17 ± 0.02	-
Free fatty acids															
Hexadecanoic acid, methyl ester	0.79±0.21AB	1.03 ± 0.24 ABCD	0.92 ± 0.28 ABC	0.87 ± 0.18 ABC	1.09 ± 0.25 ABCD	1.49 ± 0.08 D	1.23 ± 0.12 ABCD	1.04 ± 0.07 ABCD	1.26 ± 0.11 BCD	0.98 ± 0.19 ABCD	1.35 ± 0.08 CD	0.71 ± 0.06 A	0.98 ± 0.05ABCD	1.03 ± 0.05 ABCD	0.91 ± 0.15 ABC
Hexadecanoic acid, ethyl ester	-	5.02 ± 0.94 A	2.29 ± 0.70 B	2.81 ± 0.32 AB	2.75 ± 0.61 AB	3.56 ± 0.65 AB	2.54 ± 0.30 AB	2.13 ± 0.31 B	2.44 ± 0.14 B	5.10 ± 2.73 B	1.83 ± 0.09 B	1.15 ± 0.33 B	1.28 ± 0.34 B	1.09 ± 0.08 B	1.06 ± 0.06 B
Tetradecanoic acid, ethyl ester	0.40±0.04	-	-	-	-	-									
n-Hexadecanoic acid	4.58±1.49AB	6.23 ± 0.75 ABCDE	4.37 ± 1.11 A	5.78 ± 1.01 ABCD	5.37 ± 1.21 ABC	6.53 ± 1.43 ABCDE	6.99 ± 0.71 ABCDE	7.56 ± 1.44 ABCDE	9.21 ± 0.79 EF	7.77 ± 1.63 BCDEF	10.91 ± 0.20 AF	4.79 ± 0.27 AB	6.34 ± 0.77 ABCDE	8.95 ± 0.70 DEF	8.50 ± 1.54 CDEF
Tetradecanoic acid	-	0.37 ± 0.01 AB	0.33 ± 0.05 AB	0.30 ± 0.05 A	0.34 ± 0.02 AB	0.42 ± 0.07 AB	0.31 ± 0.05 AB	0.40 ± 0.07 AB	0.46 ± 0.07 ABC	0.47 ± 0.00 ABCD	0.68 ± 0.07 CDE	0.48 ± 0.05 ABCD	0.55 ± 0.19 BCDE	0.76 ± 0.13 E	0.71 ± 0.07 DE
n-Decanoic acid	-	-	-	0.14 ± 0.03 A	0.26 ± 0.04 B		-	0.17 ± 0.02 B	0.22 ± 0.02 BC						
Aldehyde															
Hexanal	1.69±0.30A	1.65 ± 0.30 A	1.96 ± 0.04 A	0.85 ± 0.03 CD	1.56 ± 0.30 AB	1.64 ± 0.04 A	1.16 ± 0.11 BC	0.72 ± 0.07 CDE	0.94 ± 0.13 CD	0.91 ± 0.08 CD	1.04 ± 0.03 CD	0.75 ± 0.05 CDE	0.67 ± 0.11 ED	0.76 ± 0.10 CDE	0.30 ± 0.05 E
Benzaldehyde	2.74±0.45ACD	2.93 ± 0.07 ABC	3.12 ± 0.07 AB	2.14 ± 0.02 DEF	2.68 ± 0.74 ACD	3.68 ±0.12 A	2.28 ± 0.18 CDE	1.62 ± 0.21 EFG	2.26 ± 0.13 CDE	1.10 ± 0.19 GH	1.58 ± 0.07 EFG	0.79 ± 0.05 H	1.41 ± 0.09 FGH	1.46 ± 0.07 FGH	1.15 ± 0.22 GH
2-Octenal	0.35±0.08ACD	0.39 ± 0.02 ABC	0.48 ± 0.08 AB	0.44 ± 0.08 AB	0.41 ± 0.07 AB	0.53 ± 0.09 B	0.18 ± 0.01 E	0.10 ± 0.00 E	0.24 ± 0.01 CDE	0.16 ± 0.01 E	0.19 ± 0.03 DE	0.13 ± 0.03 E	-	-	0.12 ± 0.02 E

Nonanal	0.37±0.08ACD	0.67 ± 0.19 B	0.34 ± 0.01 CDE	0.21 ± 0.06 DEF	0.33 ± 0.08 CDEF	0.23 ± 0.04 DEF	0.23 ± 0.02 DEF	0.12 ± 0.03 F	0.57 ± 0.00 AB	0.50 ± 0.05 ABC	0.32 ± 0.05 CDEF	0.26 ± 0.23 ACD	0.15 ± 0.00 EF	0.33 ± 0.04 CDE	0.52 ± 0.05 ABC
Decanal	0.19±0.03AB	-	-	-	-	-	-	-	-	-	0.21 ± 0.03 AB	0.15 ± 0.02 A	0.16 ± 0.07 A	0.30 ± 0.06 B	0.27 ± 0.03 AB
Benzaldehyde, 2,4-dimethyl	0.92±0.13A	0.88 ± 0.18 A	0.96 ± 0.27 A	1.49 ± 0.23 ABCD	0.91 ± 0.05 A	2.13 ± 1.05 CDE	2.84 ± 0.61 E	2.05 ± 0.25 BCDE	2.33 ± 0.46 DE	1.65 ± 0.16 ABCD	1.44 ± 0.03 ABCD	1.45 ± 0.19 ABCD	1.08 ± 0.12 ABC	1.43 ± 0.06 ABCD	1.79 ± 0.07 ABCDE
Pentadecanal	0.53±0.10ABC	1.44 ± 0.32 D	0.63 ± 0.13 BC	0.61 ± 0.03 ABC	0.32 ± 0.05 ABC	0.83 ± 0.40 C	0.11 ± 0.00 A	-	0.39 ± 0.09 ABC	0.27 ± 0.09 AB	0.18 ± 0.02 AB	-	-	-	-
Benzeneacetaldehyde	-	1.20 ± 0.09 A	1.12 ± 0.13 A	-	-	-	-	-	-	-	-	-	-	-	-
Butanal, 3-methyl	-	-	0.24 ± 0.04 A	0.08 ± 0.02 B	0.12 ± 0.02 B	0.14 ± 0.03 B	-	-	-	-	-	-	-	-	-
2-Hexenal	-	-	0.22 ± 0.03 A	0.08 ± 0.01 B	0.10 ± 0.01 BC	0.17 ± 0.05 AC	-	-	-	-	-	-	-	-	-
Hydrocarbon															
Benzene, 1,2,3-trimethyl	0.38±0.06A	-	-	0.26 ± 0.03 A	-	-	-	-	-	-	-	0.63 ± 0.08 B			
Benzene, 1,2,4-trimethyl	0.27±0.06A	-	-				1.04 ± 0.52 BC	0.17 ± 0.02 A	1.21 ± 0.00 B	0.36 ± 0.04 A	0.63 ± 0.09 AC	1.32 ± 0.00 B	0.37 ± 0.10 A	-	-
Benzene, 1-ethyl-2-methyl	-	-	-	-	-	-	0.25 ± 0.02 A	-	-	-	-	0.16 ± 0.02 B			
Heptadecane	0.19±0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dodecane, 4,6-dimethyl	0.41±0.09A	0.42 ± 0.09 A	0.45 ± 0.09 A	0.50 ± 0.03 A	0.55 ± 0.11 A	0.50 ± 0.08 A	-	-	-	-	-	-	-	-	-
Mesitylene	1.24±0.23A	-	-	-	0.53 ± 0.07 B	0.51 ± 0.20 B	-	-	-	-	-	-	-	-	-
Nonadecane	-	0.36 ± 0.03	-				-	-	-	-	-	-	-	-	-
Acids															
Benzoic acid	-	-	-	-	0.47 ± 0.18	-	-	-	-	-	-	-	-	-	-
Butanoic acid	-	-	-	-	-	-	-	-	-	-	0.35 ± 0.04 A	0.30 ± 0.10 A		0.54 ± 0.01 A	
Terpenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Linalool	2.04±0.32A	3.49 ± 0.43 A	3.23 ± 0.42 A	6.35 ± 1.23 AB	7.68 ± 1.37 AB	10.93 ± 0.39 B	11.60 ± 2.14 BC	18.14 ± 0.67 CD	21.89 ± 1.65 D	23.73 ± 4.31 D	35.05 ± 3.24 E	38.27 ± 5.41 E	22.42 ± 2.53 D	37.24 ± 1.02 E	63.06 ± 2.53 F
beta.-Myrcene	-	-	-	-	-	-	-	-	-	0.42 ± 0.07 A	0.51 ± 0.02 AB	0.67 ± 0.10 C	0.44 ± 0.03 AB	0.59 ± 0.03 BC	0.91 ± 0.02 D
D-Limonene	-	-	-	-	-	-	-	-	-	-					0.81 ± 0.07
alpha.-Terpineol	-	-	-	-	-	-	-	-	-	-	0.57 ± 0.06 A	0.50 ± 0.06 A	0.48 ± 0.09 A	0.24 ± 0.04 B	0.49 ± 0.09 A

trans-Linalool oxide (furanoid)	-	-	-	-	-	-	-	-	-	-	0.71 ± 0.07 A	0.73 ± 0.13 A	0.51 ± 0.07 A	1.06 ± 0.04 B	1.64 ± 0.17 C
Terpenes															
Linalool	2.04±0.32A	3.49 ± 0.43 A	3.23 ± 0.42 A	6.35 ± 1.23 AB	7.68 ± 1.37 AB	10.93 ± 0.39 B	11.60 ± 2.14 BC	18.14 ± 0.67 CD	21.89 ± 1.65 D	23.73 ± 4.31 D	35.05 ± 3.24 E	38.27 ± 5.41 E	22.42 ± 2.53 D	37.24 ± 1.02 E	63.06 ± 2.53 F
beta.-Myrcene	-	-	-	-	-	-	-	-	-	0.42 ± 0.07 A	0.51 ± 0.02 AB	0.67 ± 0.10 C	0.44 ± 0.03 AB	0.59 ± 0.03 BC	0.91 ± 0.02 D
D-Limonene	-	-	-	-	-	-	-	-	-	-					0.81 ± 0.07
alpha.-Terpineol	-	-	-	-	-	-	-	-	-	-	0.57 ± 0.06 A	0.50 ± 0.06 A	0.48 ± 0.09 A	0.24 ± 0.04 B	0.49 ± 0.09 A
trans-Linalool oxide (furanoid)	-	-	-	-	-	-	-	-	-	-	0.71 ± 0.07 A	0.73 ± 0.13 A	0.51 ± 0.07 A	1.06 ± 0.04 B	1.64 ± 0.17 C
Other															
3-ethyl-2-methyl, 1,3-Hexadiene	0.39±0.05A	0.53 ± 0.02 A	0.66 ± 0.23 A	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl 9-hexadecenoate	1.37±0.41A	0.96 ± 0.18 ABC	0.47 ± 0.07 BC	0.30 ± 0.02 C	0.37 ± 0.07 C	0.40 ± 0.08 C	0.42 ± 0.05 C	0.31 ± 0.03 C	0.50 ± 0.12 BC	1.21 ± 0.64 AB	-	-	-	-	-
Nerol oxide	-	-	-	-	-	-	-	-	-	-	0.39 ± 0.07 A	0.42 ± 0.00 A	0.27 ± 0.01 A	0.84 ± 0.11 B	0.93 ± 0.11 B
Benzene, 1-methoxy-4-methyl	-	-	-	-	-	-	-	-	-	-	-	-	-	0.99 ± 0.10 A	0.88 ± 0.18 A

Means of triplicate in each row bearing the same letters (A, B, and C) are not significantly different ($p > 0.05$) from one another using Tukey's test (mean standard variation).