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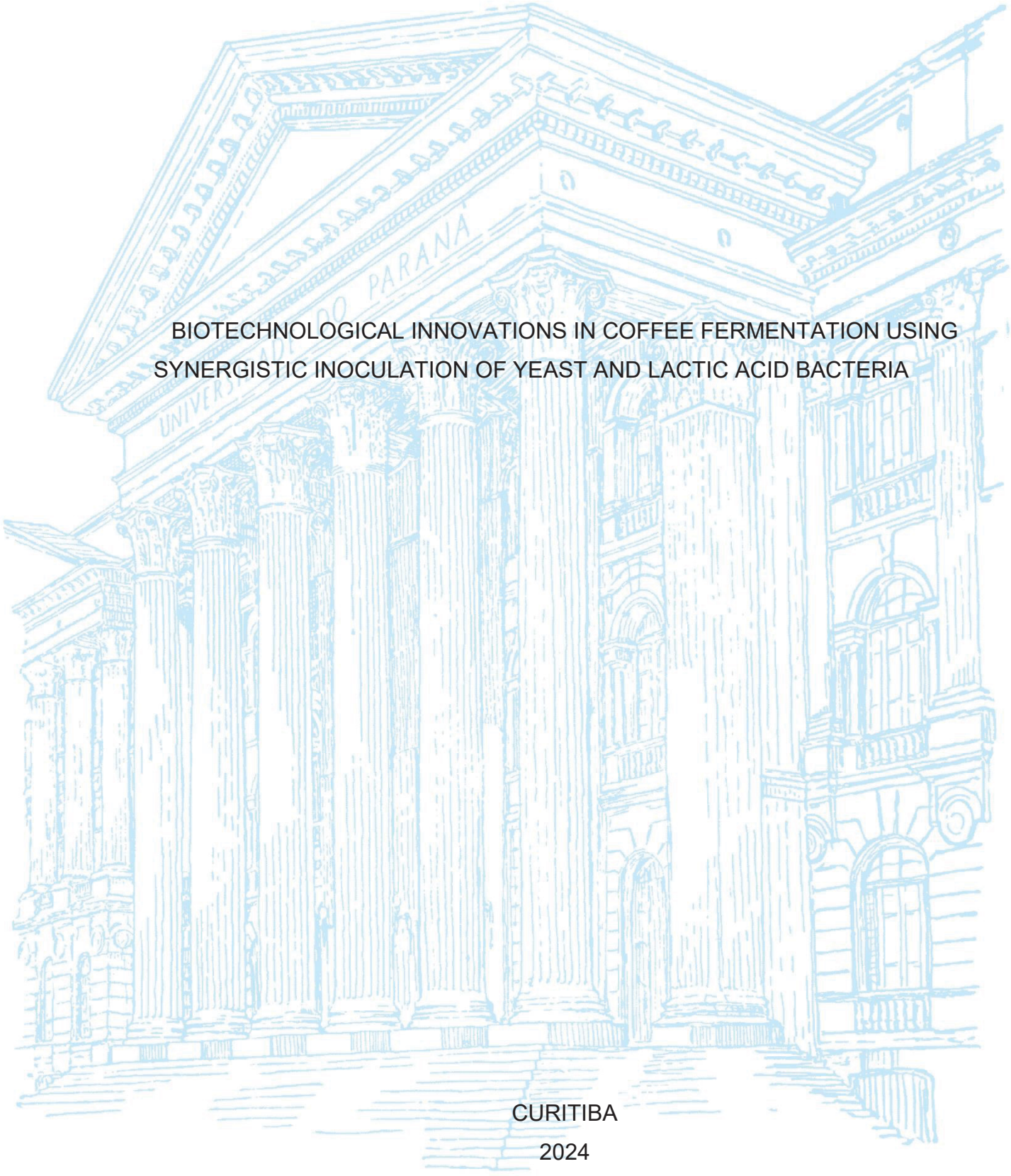

BIOTECHNOLOGICAL INNOVATIONS IN COFFEE FERMENTATION USING SYNERGISTIC INOCULATION OF YEAST AND LACTIC ACID BACTERIA

CURITIBA

2024

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Dissertação apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Gilberto Vinícius de Melo Pereira

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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 dissertação de Mestrado de **VITORIA DE MELLO SAMPAIO** intitulada: **Biotechnological innovations in coffee fermentation using synergistic inoculation of yeast and lactic acid bacteria**, sob orientação do Prof. Dr. GILBERTO VINICIUS DE MELO PEREIRA, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestra 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.

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"Um cientista no seu laboratório não é apenas um técnico: é, também, uma criança colocada à frente de fenômenos naturais que impressionam como se fossem um conto de fadas."

Marie Skłodowska-Curie

RESUMO

O mercado do café tem passado por inovações significativas, impulsionadas por novas técnicas de cultivo, métodos de processamento e tecnologias de fermentação através do uso de leveduras e bactérias ácido-láticas (BAL). Levantamentos biobibliográficos realizados no primeiro capítulo deste trabalho demonstraram o potencial sinérgico entre esses dois grupos de microrganismos, amplamente explorado em outros processos fermentativos, mas pouco conhecido na cultura do café. Assim, o objetivo deste trabalho foi investigar os efeitos da inoculação de BAL e leveduras na fermentação e qualidade do café. Cinco diferentes protocolos de inoculações foram realizados: i) fermentação espontânea (controle), ii) fermentação inoculada com *P. fermentans* YC5.2, iii) fermentação inoculada com *P. pentosaceus* LPBF07, iv) co-inoculação de *P. fermentans* YC5.2 e *P. pentosaceus* LPBF07 (fermentação simultânea) e v) fermentação sequencial (um período inicial de 36 horas fermentando exclusivamente com *P. pentosaceus* LPBF07, seguido da inoculação de *Pichia fermentans* YC5.2 após esse período). Uma alta diversidade de bactérias (p. ex., *Pseudomonas, Enterobacter e Pantoea*) e leveduras (p. ex., *Rhodotorula, Naganishia* e *Kurtzmaniella*) foi observada no tratamento espontâneo. No entanto, a inoculação de *P. fermentans* YC5.2 mostrou-se altamente eficaz na inibição do crescimento de leveduras selvagens, enquanto a introdução de *P. pentosaceus* LPBF07 foi eficaz na inibição de bactérias. Os protocolos de inoculação simultânea e sequencial demonstraram uma interação sinérgica eficaz entre *P. pentosaceus* LPBF07 e *P. fermentans* YC5.2, inibindo o crescimento de leveduras e bactérias selvagens simultaneamente. Esse efeito sinérgico melhorou a eficiência do consumo de açúcares da polpa do café e a produção de metabólitos primários durante o processo de fermentação, tais como ácido lático e etanol. A análise por HS-SPME/GC identificou um total de 93 compostos voláteis na fração líquida fermentada, categorizados em dez grupos químicos: ésteres (23), aldeídos (18), hidrocarbonetos (18), álcoois (12), ácidos (12), furanos (3), piranos (2), cetonas (2), terpenos (2) e fenóis (2). A fermentação espontânea resultou no menor conteúdo de compostos voláteis, enquanto a fermentação sequencial produziu a maior quantidade, aproximadamente três vezes maior. Para determinar o efeito dos diferentes tratamentos na qualidade e no perfil sensorial da bebida final, as amostras foram preparadas de acordo com as recomendações da *Specialty Coffee Association* (SCA). Os tratamentos de co-inoculação resultaram em bebidas com um perfil volátil mais ricos e avaliações sensoriais aprimoradas em comparação aos demais tratamentos. Os compostos com maior impacto foram o etil linoleato, 1-hexanol, 2-heptanol, benzeneacetaldeído, álcool benzílico e álcool feniletílico. Este estudo ressalta o potencial significativo da técnica de inoculação sequencial, não apenas na geração de compostos aromáticos desejáveis, mas também na produção de cafés especiais de alta qualidade, evidenciando uma nova abordagem para o aprimoramento dos perfis sensoriais no setor cafeeiro.

Palavras-chave: Cafés especiais; Fermentação de café; Levedura; BAL; Atributos sensoriais.

ABSTRACT

The coffee market has undergone significant innovations, driven by new cultivation techniques, processing methods, and fermentation technologies through the use of yeasts and lactic acid bacteria (LAB). Biobibliographic surveys carried out in the first chapter of this work demonstrated the synergistic potential between these two groups of microorganisms, widely explored in other fermentation processes, but little known in coffee cultivation. Thus, the objective of this work was to investigate the effects of LAB and yeast inoculation on coffee fermentation and quality. Five different inoculation protocols were carried out: i) spontaneous fermentation (control), ii) fermentation inoculated with *P. fermentans* YC5.2, iii) fermentation inoculated *with P. pentosaceus* LPBF07, iv) co-inoculation of *P. fermentans* YC5.2 and *P. pentosaceus* LPBF07 (simultaneous fermentation), and v) sequential fermentation (an initial period of 36 hours fermenting exclusively with *P. pentosaceus* LPBF07, followed by the inoculation of *P. fermentans* YC5.2 after this period). A high diversity of bacteria (*Pseudomonas, Enterobacter*, and *Pantoea*) and yeasts (*Rhodotorula, Naganishia*, and *Kurtzmaniella*) was observed in the spontaneous treatment. However, the inoculation of *P. fermentans* YC5.2 proved highly effective in inhibiting the growth of wild yeasts, while the introduction of *P. pentosaceus* LPBF07 was effective in inhibiting bacteria. The simultaneous and sequential inoculation protocols demonstrated an effective synergistic interaction between *P. pentosaceus* LPBF07 and *P. fermentans* YC5.2, simultaneously inhibiting the growth of wild yeasts and bacteria. This synergistic effect improved the efficiency of sugar consumption from the coffee pulp and the production of primary metabolites during the fermentation process, such as lactic acid and ethanol. HS-SPME/GC analysis identified a total of 93 volatile compounds in the fermented liquid fraction, categorized into ten chemical groups: esters (23), aldehydes (18), hydrocarbons (18), alcohols (12), acids (12), furans (3), pyrans (2), ketones (2), terpenes (2), and phenols (2). Spontaneous fermentation resulted in the lowest volatile compound content, while sequential fermentation produced the highest amount, approximately three times higher. To determine the effect of different treatments on the quality and sensory profile of the final beverage, the samples were prepared according to the recommendations of the Specialty Coffee Association (SCA). Co-inoculation treatments resulted in beverages with a richer volatile profile and improved sensory evaluation compared to the other treatments. The compounds with the most significant impact were ethyl linoleate, 1-hexanol, 2-heptanol, benzeneacetaldehyde, benzyl alcohol, and phenylethyl alcohol, which are considered important contributors to the aroma. This study highlights the significant potential of the sequential inoculation technique, not only in generating desirable aromatic compounds, but also in producing high-quality specialty coffees, evidencing a new approach to enhancing sensory profiles in the coffee sector.

Keywords: Specialty coffee; Coffee fermentation; Yeast; LAB; Sensory attributes.

SUMÁRIO

1 CHAPTER 1: HOW YEAST HAS TRANSFORMED THE COFFEE MARKET BY CREATING NEW FLAVORS AND AROMAS THROUGH MODERN POST-HARVEST FERMENTATION SYSTEMS

This chapter was published as a scientific article in 2024 in the journal Trends in Food Science & Technology: https://doi.org/10.1016/j.tifs.2024.104641

1.1 ABSTRACT

Background: Building on the success of traditional fermented foods, the coffee industry has experienced a surge in research on the use of yeast in coffee bean fermentation. As the coffee industry continually seeks innovative approaches, it becomes crucial to understand the nuances of yeast's impact on coffee quality.

Scope and approach: This review explores the transformative impact of yeast on coffee fermentation and product quality. By investigating traditional (wet and dry processing) and emerging techniques (carbonic maceration and induced fermentation), it aims to understand how yeast selection and fermentation methods affect coffee's flavor, aroma, and quality. Additionally, this review provides critical discussions on underexplored topics, such as optimal storage conditions and health properties of fermented coffee, as well as regulatory, safety, and environmental concerns, offering a complete view of yeast's role in enhancing coffee's market appeal and quality.

Key Findings and Conclusions: Based on criteria such as pectinolytic activity, stress tolerance, and aroma production, *Saccharomyces cerevisiae* and *Pichia fermentans* are the most studied yeasts affecting coffee quality. Their impact on coffee quality is evident through the accumulation of aroma-enhancing compounds, such as alcohols, aldehydes, organic acids, esters, ketones, and terpenoids. Recent protocols involving co-inoculation between distinct yeast groups and lactic acid bacteria have emerged, exhibiting metabolic synergism and producing coffees with heightened body perception, improved aroma, and enhanced acidity. However, ongoing research is needed to explore novel fermentation systems, storage methods, and sustainable yeast biomass disposal practices, ensuring a more efficient and sustainable future for the coffee market.

1.2 INTRODUCTION

Coffee is one of the most popular and consumed beverages in the world and ranks among the most traded commodities on a global scale (“Food Outlook – Biannual Report on Global Food Markets,” 2023). The journey of coffee, from bean to cup, involves intricate processing methods that play a crucial role in shaping its flavor, aroma, and overall quality (Pereira et al., 2019). The fermentation stage in post-harvest processing is particularly important as it significantly influences the final chemical composition of the beans. This process involves the breakdown of sugars and mucilage surrounding the coffee beans, leading to the development of unique aromatic compounds (Elhalis et al., 2023a). Recent research has focused on selecting new yeast strains to improve coffee fermentation (Elhalis et al., 2021; Jimenez et al., 2023; Ferreira et al., 2023). In traditional wet processing, yeasts are added to enhance the breakdown of mucilage and facilitate the drying process of the beans. This method involves placing coffee beans in water tanks, where yeast-driven biochemical reactions produce desirable sensory characteristics. Selected yeasts have also been applied by aspersion in dry coffee processing, significantly reducing the usual 30-day sun drying period for green beans and help standardize the sensory profiles of the resulting beverages (Ferreira et al., 2023). Dry coffee processing, also known as natural method, is one of the primary techniques employed in the post-harvest processing of coffee beans. This distinctive method involves drying the whole cherries, including the fruit pulp, before extracting the coffee beans.

Emerging approaches, such as carbonic maceration and induced fermentation, have expanded yeast use in coffee processing (Cassimiro et al., 2023). Carbonic maceration involves fermenting coffee cherries in a carbon dioxide-rich environment, promoting unique biochemical interactions between the yeast and coffee beans (Brioschi et al., 2021). Induced fermentation, on the other hand, uses yeast metabolism to create anaerobic conditions in closed bioreactors. This is achieved by placing an airlock at the top of the fermentation tank, allowing the exit of gases while preventing the entry (Pereira et al., 2022). As the understanding of yeast's impact on coffee fermentation continues to evolve, it opens avenues for further exploration and refinement of techniques, ultimately molding the sensory profile and quality of coffee.

This review explores the transformative role of yeast in coffee fermentation, focusing on how various yeast strains and fermentation methods impact coffee's flavor,

aroma, and overall quality. It examines traditional and emerging fermentation techniques, the metabolic activities of specific yeasts, and the sensory and health benefits of yeast-fermented coffee. Additionally, the study addresses emerging topics such as optimal storage methods and sustainable yeast biomass disposal practices to enhance the quality and market appeal of fermented coffee.

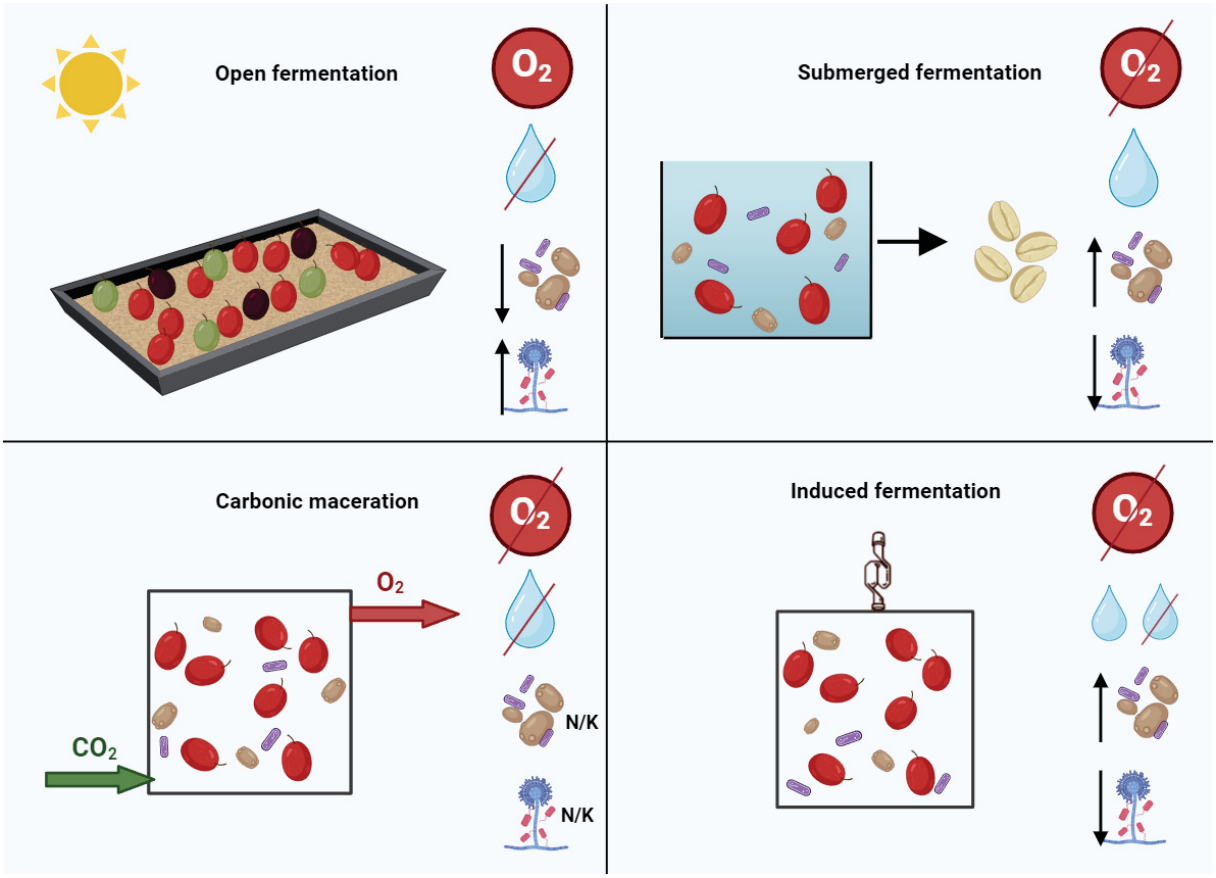
1.3 FERMENTATION PROCESS

The incorporation of the term "Fermentation" into coffee processing is relatively recent. Prior to the pivotal work of Agate and Bhat in 1966, the practice of adding depulped fruits to water tanks was exclusively aimed at washing the coffee—an expression that persists to describe this particular processing technique known as "washed coffees." However, as time has progressed, scientific investigations uncovered the intricate role played by diverse microorganisms in the removal of coffee pulp through fermentative metabolism (Vale et al., 2021).

In modern coffee processing, diverse fermentation methods have been implemented, with a primary emphasis on expeditiously removing coffee pulp and decreasing coffee fruit moisture from 65% to 11%. This focus aims to ensure optimal conditions for the storage and transportation of coffee. This intricate process involves the action of hundreds of species of microorganisms, encompassing yeasts, bacteria, and filamentous fungi, during coffee fermentation (Neto et al., 2018). The fermentation process has garnered global attention due to the consistently high quality achieved in "washed coffee." The quest for excellence in coffee production has elevated fermentation as a subject of worldwide interest, reflecting the intricate interplay of science and tradition in the pursuit of superior coffee processing methods.

There are four primary fermentation methods employed in coffee processing, as shown in Figure 1: open fermentation, submerged fermentation, carbonic maceration, and induced fermentation (Ferreira et al., 2023). Among these, open fermentation (commonly known as natural or dry processing) and submerged fermentation (often referred to as washed or wet processing) emerge as the most widely adopted techniques globally (Junqueira et al., 2019). In contrast, carbonic maceration and induced fermentation represent relatively recent applications in coffee processing.

FIGURE 1 - Illustration of the main fermentation methods employed in coffee processing.



SOURCE: Author (2024)

LEGEND: ↓: low incidence. ↑: high incidence. N/K: not known.

Open fermentation involves the straightforward method of exposing the entire coffee fruit to the sun until the complete removal of the husk, pulp, and coffee mucilage (Ferreira et al., 2023). Brazil, being the largest producer of natural coffee, benefits from a dry season during harvest, facilitating the proper drying of coffee fruits. However, a notable point of discussion arises concerning the use of the term "fermentation" for this processing, given the high incidence of aerobic microorganisms. The main microorganisms reported in this method includes a range of *Bacillus* and *Candida* species, as well as Gram-negative species of the genera *Serratia, Enterobacter,* and *Acinetobacter* (Silva et al., 2013). An alternative variation of this method involves the removal of coffee peels before drying, streamlining the drying process, and earning the designation of semi-dry processing. The semi-dry method, as observed by Vilela et al., (2010), exhibits the presence of different yeasts, including species of *Pichia, Torulaspora*, and *Rhodotorula*, likely due to sugar release after husk removal.

In submerged fermentation, coffee fruits are immersed in tanks filled with large volumes of water, creating anaerobic conditions conducive to a high prevalence of fermentative microorganisms. These microorganisms include various yeasts (e.g., *Saccharomyces cerevisiae, S. bayanus, Kluyveromyces marxianus, Pichia anomala, P. kluyveri,* and *Hanseniaspora uvarum*) and LAB (e.g., *Lactobacillus, Lactococcus,* and *Pediococcus*) (Junqueira et al., 2019). These microorganisms facilitate the removal of coffee pulp and mucilage, aiding in the drying process. Microbial activity produces ethanol, lactic acid, and compounds like esters, higher alcohols, aldehydes, and ketones, which permeate the beans and influence the final coffee composition (Pereira et al., 2015; Pereira et al., 2016; Lee et al., 2017).

Carbonic maceration involves injecting carbon dioxide to create anaerobic conditions. While widely used in wine fermentation, scientific research on coffee fermentation remains limited. In a recent study by Brioschi et al. (2021), carbonic maceration was explored across various temperatures and fermentation time. The findings indicated that a fermentation time of 96 hours at 38ºC resulted in the highest sensory quality of the coffee beverage. However, it is noteworthy that certain molecules, such as trigonelline, lipids, and formic acid, which increased during the process, are not directly linked to microbial metabolism but rather intrinsic to the coffee itself. This raises a crucial point: the positive impact of carbonic maceration on coffee quality remains uncertain. Further research is imperative to elucidate the specific mechanisms at play and determine whether this process genuinely enhances the quality of coffee. The current knowledge gap underscores the need for additional studies and comprehensive investigations to provide a clearer understanding of the relationship between carbonic maceration and coffee quality.

Induced fermentation is a process in which microorganisms themselves create anaerobic conditions. This is achieved by placing an airlock at the top of the fermentation tank, allowing the exit of gases while preventing entry (Pereira et al., 2022). Various fermentation configurations have been tested, involving the use of whole fruit (i.e., without pulping) or pulped fruit in static and closed fermentation tanks, either without (Martinez et al., 2022; Pereira et al., 2022) or with (Cassimiro et al., 2022) the addition of water, and employing spontaneous fermentation or utilizing starter cultures. Overall, this process appears to enhance the chocolatey and caramel perception of coffee beverages (Ferreira et al., 2023). One notable study by Mota et al. (2020) found the most favorable results when employing *Toluspora delbrueckii* for

a fermentation period of 70 hours. Various metabolites that have been reported to increase during induced fermentation are directly linked to microbial metabolism, including lactic acid, acetic acid, esters, and aldehydes. However, these investigations highlight the crucial role of inoculating starter cultures for the successful implementation of this method. These findings underscore the significance of precise control over the fermentation process through the introduction of specific microbial strains. Examples of such starter cultures include *T. delbrueckii, S. cerevisiae,* and *Candida parapsilosis*, emphasizing the importance of tailored microbial interventions to optimize the outcomes of induced fermentation in coffee processing. Further research in this area is essential to refine our understanding of the intricate interplay between microbial activities and the sensory attributes of coffee beverages.

1.4 YEASTS DIVERSITY

Coffee pulp, rich in polysaccharides (4.3% dry weight), reducing sugars (10.9% dry weight), crude protein (7.2-8.1% dry weight), and pectin (3.83% dry weight), creates a highly favorable environment for microbial growth (Pereira et al., 2020). Following the mechanical removal of the coffee peel, the exposure of the pulp and mucilage facilitates the integration of both epiphytic and endophytic microorganisms. The primary microbial groups encompass yeast, lactic acid bacteria, filamentous fungi, and acetic acid bacteria (Vale et al., 2021).

Despite the variability in coffee post-harvest processing methods, microbial dynamics follow a consistent pattern. Initially, there is a prevalence of lactic acid bacteria (LAB) (~10^5 – 10^7 CFU/mL), inducing coffee pulp-mass acidification through consistent lactic acid production. Subsequently, yeasts (~10^7 – 10^8 CFU/mL) prevail, breaking down mucilage and enhancing the aroma complexity of the beans (Elhalis et al., 2023; Zhang et al., 2019). The temporal occupation of the coffee pulp-bean mass is dictated by inter-species fitness to the environment and their ecological interactions. Recent studies using high-throughput amplicon sequencing have revealed over 300 bacteria (LAB, AAB, aerobic bacteria, and enterobacteria) and 150 fungal (yeast and filamentous fungi) associated with coffee fermentation (Cruz-O'Byrne et al., 2023; Neto et al., 2018; Pereira et al., 2022). The complex microbial population of coffee fermentation allow interactions within and between the different groups (i.e., especially yeast-yeast, yeast-bacteria, and bacteria-bacteria), promoting the stabilization of

dominant species and, utterly, the contribution of organoleptic characteristics (Neto et al., 2018).

Synergistics or competition + commensalism effects can be observed between LAB and yeast. The production of hydrophilic organic acids by both homo- and heterofermentative LAB results in pH reduction. This reduction, in turn, stimulates yeast growth through intracellular adaptive responses facilitated by plasma membrane and vacuolar ATPase pumps. These pumps play a crucial role in maintaining internal pH homeostasis by either expelling protons from the cytosol or sequestering them into the vacuole lumen (Peetermans et al., 2021). From the yeast's perspective, the secretion of amino acids (such as glutamine, glutamate, proline, and phenylalanine) during the exponential growth phase serves as a source of essential nutrients for auxotrophic LAB. Remarkably, this nutrient provision occurs without the need for direct physical interaction between the two organisms, facilitating nitrogen overflow (Ponomarova et al., 2017). Finally, indigenous *Saccharomyces* and non-*Saccharomyces* displays a multiplex array of positive and negative interactions, including release of beneficial molecules, substrate competition, production of lethal substances, and cell contact-dependent effects (Zilelidou & Nisiotou, 2021).

The presence and, oftentimes, dominance of yeast in different coffee fermentation methods is governed by biotic and abiotic factors (e.g., pH, temperature, osmotic pressure, nutritional restriction, and competition) that directly influences the ecological interactions and modulates the microbial dynamics during the spontaneous process. Unlikely other food matrices such as grape, apple, and cocoa (Liu et al., 2021; Viesser et al., 2021), non-*Saccharomyces* species dominate the coffee fermentation process from end-to-end, where Pichia sp. and *Hanseniaspora* sp. are prevalently reported in studies conducted in Australia, Brazil, China, Colombia, and Honduras (Vale et al., 2021; Junqueira et al., 2019; Elhalis et al., 2020; Evangelista et al., 2015). This distinctiveness can be explained by the absence of *Saccharomyces*-selective stressors, such as high osmotic pressure, presence of SO_2, and increasing alcohol concentrations (Jolly et al., 2014), enabling the prevalence of the well-adapted and high numbered non-*Saccharomyces* cells.

The diversity of yeast strains is often influenced by the geographical origin of the coffee. Different regions have distinct microclimates, soil compositions, and altitude levels, all of which contribute to the selection and prevalence of specific yeast species. This geographical influence is a key factor in the terroir of coffee, where the local

environment imparts unique characteristics to the beans. In Brazil and Ecuador, the yeast diversity in coffee is notably characterized by a prevalence of species from the *Candida* and *Pichia* genera. Meanwhile, in Mexico, a higher abundance of *Cryptococcus* and *Pichia* species is observed. Australia and Colombia showcase a richer array of *Candida* species, while in India, *Saccharomyces* emerges as the predominant yeast genus in coffee fermentation (Schwan et al., 2023; Cruz-O'Byrne et al., 2023; Elhalis et al., 2020; Pereira et al., 2022).

The methods employed during coffee processing, such as natural (dry) or washed (wet) fermentation, also influence yeast diversity. The conditions created during these processes, including exposure to air, moisture levels, and fermentation duration, shape the microbial communities, leading to variations in yeast composition and abundance.

1.5 YEAST METABOLISM

Non-*Saccharomyces* yeasts have become crucial in coffee fermentation due to their complex metabolism and production of aromatic compounds. These volatile aroma compounds play key roles in yeast metabolism, including signaling for animal vectors, regulating fungal growth, and facilitating communication among yeast cells or colonies (Dzialo et al., 2017). The aroma-producing capabilities of yeasts is attributed to i) the release of primary aroma (e.g., methoxypyrazines and terpenes) via enzymatic hydrolysis of glycoside precursors; ii) or the production of secondary aroma (e.g., volatile fatty acids, higher alcohols, esters, and aldehydes) via metabolic pathways (Padilla et al., 2016). Although not thoroughly elucidated in coffee fermentation, monoterpenes can be produced either by hydrolyzation of glycoside precursors via β-glucosidase or by de novo synthesis as described in *S. cerevisiae* (Han et al., 2023).

Volatile fatty acids are organic acids with six or fewer carbons, such as acetic, butyric, isobutyric, isovaleric, and valeric acid. They are produced during carbohydrate catabolism through various metabolic routes, including glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway (Bhatia & Yang, 2017). Acetate esters, such as ethyl acetate, isoamyl acetate, and isobutyl acetate, which represent the principal volatile compounds in yeast metabolism, are synthesized through the condensation of acetyl-CoA or acetate with higher alcohols, facilitated by the action of acyltransferases. Due to its lipophilic nature and small size, acetate esters can

passively trespass the membrane and readily diffuse into the extracellular medium (Dzialo et al., 2017). Higher alcohol production in non-*Saccharomyces* species, such as *Torulaspora delbrueckii, Pichia fermentans*, and *Hanseniaspora uvarum* is strain-specific, relying on the Elrich pathway and specific amino acid uptake (Hazelwood et al., 2008). Gobert (2017) found that *Starmerella bacillaris, Metschnikowia pulcherrima,* and *P. membranifaciens* prefer different nitrogen sources, with slower consumption and lower biomass formation compared to *S. cerevisiae.*

1.6 CONDUCTING TESTS TO SELECT COFFEE YEASTS

Despite the pioneering study by Agate and Bhat (1966), which used different yeast species to accelerate coffee mucilage degradation, it was only in the last decade that significant efforts in yeast selection were truly undertaken. The studies gain complexity by adopting different selection approach, including (i) assessing pectinolytic activity to accelerate mucilage breakdown, (ii) evaluating stress tolerance faced by yeast cells during the fermentation process, and (iii) promoting volatile aroma production for coffee flavor development. Furthermore, certain studies have incorporated specific selection criteria, such as antimicrobial properties to prevent the growth of mycotoxin-producing fungi and the absence of undesirable metabolite production (e.g. butyric acid, propionic acid and sulfides). These comprehensive methodologies have led to the selection of species from the genera *Saccharomyces, Pichia, Candida, Torulaspora,* and *Hanseniaspora.*

The primary step undertaken by most studies entails isolating wild yeasts from spontaneous coffee fermentations or, occasionally, from environmental samples linked to coffee farms (e.g., soil, fruit, and leaf). The species level identification is reached by a combination of biochemical (growth in different carbon or nitrogen sources) and genotypic (sequencing of the small subunit - SSU - rRNA) methods using the internal transcribed ITS region of yeasts (Shankar et al., 2022; Zhang et al., 2023). The yeast species are linked to coffee processing in countries of the southern hemisphere, with Brazil (54 strains) having the highest number of selected yeasts, followed by Ecuador (21 strains), Colombia (13 strains) and Australia (13 strains). In some cases, yeast associated with other fermentation processes are submitted to screening procedures, as exemplified by *S. cerevisiae* from wine production and bakery (Tinoco et al, 2019).

1.6.1 Production of pectinolytic enzymes

Polygalacturonases and pectin lyases are crucial in coffee fermentation, breaking down pectin in the mucilage surrounding coffee beans. Yeast strains producing these enzymes accelerate mucilage breakdown, aiding bean extraction and influencing coffee flavor by releasing aromatic compounds (Pereira et al., 2019; Elhalis et al., 2023; Salem et al., 2023). Finally, mucilage degradation is important for preventing the development of spoilage bacteria and fungi caused by residual mucilage in underfermented coffee (Haile & Kang, 2019). Thus, polygalacturonases and pectin lyases activity is the most used criterion for coffee yeast selection.

The enzyme activity can be estimated by measuring the increase in reducing sugars released from pectin dispersion using 3,5-Dinitrosalicyclic acid (DNS) (Siridevi et al., 2019; Pereira et al., 2014), by the formation of a clear halo (clear zone in plates) around the colonies (Elhalis et al., 2021; Shankar et al., 2022). Other absorbance methodologies can be used like measurement in liquid media. Shankar et al., 2022 and Elhalis, Cox & Zhao (2020) selected *Pichia kudriavzevii* based on the greatest polygalacturonase activity (120,3 U/g), followed by *Hanseniaspora uvarum* (90,7 U/g) and based on pectin degradation index percentage (131,11 ± 1,01). In the same way, Pereira et al. (2014) observed that *Saccharomyces* sp. (2,637.00 U/mL to 2,557.51 U/mL) and *P. fermentans* (2,344.86 U/mL) displayed higher pectin lyase activity in coffee pulp media. Silva et al. (2013) also reported a notable capacity for pectin degradation for *S. cerevisiae* strains, ranging from 2,557.51 U/mL to 2,637.00 U/mL. Additionally, *Candida parapsilosis* exhibited substantial pectin lyase activity at 2,636.76 U/mL, followed by *P. guilliermondii* at 2,344.86 U/mL.

1.6.2 Relisience to stressful conditions

pH, temperature, sugar content, and fermentation products create selection pressure on yeast growth during coffee fermentation. Analyzing yeast strains that survive these stresses provides insights into their ability to grow and complete fermentation. Microbial cells must adapt their physiology or behavior to changing environmental stresses. (Pereira et al., 2012). The specific changes that occur during coffee fermentation include temperature from 25 to 43ºC, ethanol concentration of from

2 to 12%, acetic acid from 1 to 3%, pH from 2 to 8 and osmotic pressure with fructose and glucose concentration up to 50% (Pereira et al., 2014; Shankar et al., 2022).

This approach can be performed adjusting basal media or controlling incubation parameters at specific conditions. Pereira et al. (2014) used capacity growth of 144 wild yeast under coffee-associated stress conditions. The authors found nine stress-tolerant strains, possessing the following characteristics, which were pre-selected for further investigation: (i) growth capacity in a typical pH range of coffee fermentation (pH 2.0 to pH 8.0); (ii) osmotic pressure tolerance (growth detected in the presence of up to 50% glucose and fructose); (iii) heat tolerance (ability to grow at temperatures of 37 to 43 °C); and (iv) metabolite accumulation tolerance (growth capacity up to 12 to 15% ethanol, 2% lactic acid and 2% acetic acid). The nine stress tolerant species were identified as belonging to *Saccharomyces* genus*, P. fermentans, P. kluyveri, Pichia guilliermondii, H. opuntiae* and *C. glabrata*. To investigate the physiological adaptation of 6 yeasts, Elhalis et al. (2021) followed the previous stress conditions with few modifications such as pH (2-6), ethanol concentration (2-10%) and temperature (25-40ºC). *H. uvarum, P. kudriavzevii* and *P. fermentans* showed higher resistance to pH, ethanol and osmotic pressure induced by high sugar concentration, growing at pH 2 and high ethanol, fructose, and glucose concentrations. *H. uvarum* and *P. kudriavzevii* exhibited notable resilience to combined stress factors, withstanding concentrations of acetic acid at 3% and growth at 40ºC. The resistance exhibited by these yeast strains is intricately linked to the regulation of gene expression.

In *Saccharomyces* sp, ADE genes are activated under acetic acid stress producing the stress protectant γ-aminobutyric acid (GABA) (Zhang et al., 2019). High osmolarity glycerol (HOG) genes also play an important role under toxic levels of acetic acid, in addition to regulating both acute responses and long-term adaptation of yeast cells to osmotic stress (Gonzalez et al., 2016; Mollapour & Piper, 2007). Genes related to amino acid biosynthesis also improves stress tolerance; tryptophan pathway is related to ethanol resistance while proline is believed to enhance the stability of proteins and membranes under stress conditions (Hirasawa et al., 2007; Shima & Takagi, 2009). In the same way, *Pichia* sp. presents different genetic mechanisms to protect itself. Genes associated with the pentose phosphate pathway, citrate cycle, and peroxisome, as well as those involved in biological processes like proton efflux, electron transfer chain, and oxidative phosphorylation, are regulated under acid stress

conditions (Fletcher et al., 2015; Li et al., 2019). Additionally, the expression of antioxidant enzyme genes plays a role in preventing oxidative damage to biomolecules (Li et al., 2019).

Thus, understanding the relationship between resistance and gene expression is pivotal for unraveling the molecular mechanisms that confer resilience to yeast cell. It involves deciphering how specific genes are activated or suppressed in response to external factors, such as environmental stressors or the presence of inhibitory substances (Causton et al., 2001). This dynamic interplay at the genetic level contributes significantly to the yeast's ability to withstand adverse conditions and fulfill its role in various applications, such as fermentation processes.

1.6.3 Metabolite production

Yeast metabolism during coffee fermentation produces volatile compounds shaping the final product's aromatic profile. Different yeast strains generate unique aroma compounds, guiding yeast selection. Key compounds for selection include acetaldehyde, ethyl acetate, isoamyl acetate, ketones (3-pentanone, acetoin, 2,3-butanedione), and terpenes (linalool, β-citronellol), contributing to the development of floral, fruity, buttery, creamy, sweet, citrus, and citronella aromas, among others (Pereira et al., 2019).

Yeast metabolism can be studied in synthetic media, and volatile compounds can be quantified through gas chromatography. Pereira et al., (2014) used a coffee pulp simulation media to select yeast with active and distinctive odors from coffee fermentation. The medium consisted of 50% (v/v) fresh coffee pulp extract, 2.0 g/l citric pectin, 15 g/l fructose, 15 g/l glucose, 5.0 g/l yeast extract, and 5.0 g/l soya peptone, maintaining a pH of 5.5. The inclusion of citric pectin, naturally present in coffee pulp, served as an energy source for yeast, complemented by glucose and fructose. Moreover, the incorporation of fresh coffee pulp guaranteed the availability of nitrogen, trace elements, and growth factors - essential elements inherent during the wet fermentation of coffee beans (Pereira et al., 2014). The authors specifically selected *P. fermentans* YC5.2 due to its notable production of ethyl acetate and isoamyl acetate.

The genus *Pichia* emerges as a prominent producer of key aroma compounds within the diverse spectrum of wild coffee yeast (Table 1). The central hypothesis proposes that *Pichia* generates secondary aromatic compounds as a protective

mechanism against other yeast species (Masoud & Jespersen, 2006; Ramos et al., 2010). Notably, volatile compounds produced by this genus, including ethyl propionate, isobutyl acetate, isoamyl alcohol, isobutyl alcohol, and phenyl ethyl alcohol, have demonstrated inhibitory effects on the growth of filamentous fungi and the formation of ochratoxin A (Masoud et al., 2005).

TABLE 1: Yeast strains and their impact on coffee sensory profiles.

Yeast	Strain	Variety*	Treatment	Sensory Profile	Coffee Grade**	Reference
Saccharomyces cerevisiae	CCMA 0543	Mundo Novo[a]	Dry	Sweet; caramelized sugars; citrus fruits; malic; acidity	84.75	Bressani et al., (2021a)
	CCMA 0543	Mundo Novo[a]	Semi-dry	Sweet; caramelized sugars; citrus fruits	84.92	Bressani et al., (2021a)
	CCMA 0543	Catuaí Vermelho[a]	Dry	Chocolate; caramel; fruity; spices	85.7	Bressani et al., (2021b)
	CCMA 0543	Catuaí Vermelho[a]	Self-induced anaerobic fermentation	Chocolate; caramel; nuts; honey; fruits	83.9	Bressani et al., (2021c)
	CCMA 0543	Catuaí Amarelo[a]	Semi-dry	Not evaluated	81.3	Martinez et al., (2017)
	CCMA 0543	Catuaí Amarelo[a]	Wet	Not evaluated	81.4	Martinez et al., (2017)
	CCMA 0543	Topázio Amarelo[a]	Self-induced anaerobic fermentation/ dry	Caramel; nuts; fruity	ND	Martins et al., (2023)
	CCMA 0543	Topázio Amarelo[a]	Self-induced anaerobic fermentation/ semi-dry	Caramel; honey	ND	Martins et al., (2023)
	CCMA 0200	Mundo Novo[a]	Wet	Caramel; herbaceous; brown sugar; honey	79	Martins et al., (2019)
	CCMA 0200	Catuaí[a]	Wet	Caramel; yellow fruits; brown sugar; herbaceous; floral	81	Martins et al., (2019)
	MERIT.ferm	ND	Semi-dry	Roasted; nuts; sweet; smoky	ND	Wang et al., (2020a)
	CCMA 0543	Catuaí Amarelo[a]	Dry	Fruity; floral	81.58	Bressani et al., (2018)
	CCMA 0543	Catuaí Amarelo[a]	Semi-dry	Banana; cashew; citrus	84	Bressani et al., (2018)
	CCMA 0543	Bourbon Amarelo[a]	Dry	Sweet; caramel; citric fruits	ND	Bressani et al., (2020)
	CCMA 0543	Bourbon Amarelo[a]	Semi-dry	Dairy; caramel; red fruits; fruity; citric	ND	Bressani et al., (2020)
	CCMA 0543	Canário Amarelo[a]	Dry	Caramel; citric fruits	ND	Bressani et al., (2020)
	CCMA 0543	Canário Amarelo[a]	Semi-dry	Red fruits; dairy; caramel	ND	Bressani et al., (2020)

Yeast	Strain	Variety*	Treatment	Sensory Profile	Coffee Grade**	Reference
	UFLA YCN727	Acaiá[a]	Semi-dry	Caramel	81.08	Evangelista et al., (2014)
	UFLA YCN724	Acaiá[a]	Semi-dry	Caramel; herbaceous	79.33	Evangelista et al., (2014)
	White wine	Catuaí Amarelo[a]	Wet	Not evaluated	81.83	Tinoco et al., (2019)
	Sparkling wine	Catuaí Amarelo[a]	Wet	Not evaluated	81.73	Tinoco et al., (2019)
	CCMA 0543	Arara[a]	Self-induced anaerobic fermentation/ dry	Sweet; fruity; chocolate; nuts; citrus; green; sour	84	Ferreira et al., (2024)
	CCMA 0543	Arara[a]	Self-induced anaerobic fermentation/ wet	Sweet; nuts; cocoa; fruity; citric	84.85	Ferreira et al., (2024)
	Lalvin EC1118	Catuaí Vermelho[a]	Dry	Nuts; spices; cocoa; floral	ND	Rabelo et al., (2024)
	MTCC 173	Sln.2R[b]	Dry	Chocolate; sweet; fruity; buttery; caramel; floral; nuts; herbal	ND	Prakash et al., (2022)
	CCMA 0200	Mundo Novo[a]	Semi-dry	Not evaluated	80.13	Ribeiro et al., (2017a)
	CCMA 0200	Ouro Amarelo[a]	Semi-dry	Not evaluated	83.25	Ribeiro et al., (2017a)
	CCMA 0543	Mundo Novo[a]	Semi-dry	Not evaluated	82.63	Ribeiro et al., (2017a)
	CCMA 0543	Ouro Amarelo[a]	Semi-dry	Not evaluated	82.88	Ribeiro et al., (2017a)
	CCMA 0200	Mundo Novo[a]	Semi-dry	Green	73.75	Ribeiro et al., (2017b)
	CCMA 0200	Ouro Amarelo[a]	Semi-dry	Astringent	79.92	Ribeiro et al., (2017b)
	CCMA 0543	Mundo Novo[a]	Semi-dry	Almond; milk; chocolate	75.88	Ribeiro et al., (2017b)
	CCMA 0543	Ouro amarelo [a]	Semi-dry	Lemon; caramel; Sweet	82.17	Ribeiro et al., (2017b)
Pichia fermentans	YC5.2	Catuí[a]	Wet	Citric; lactic; phosphoric; velvety; toffee	89	Pereira et al., (2015)
	YC5.2	Catuí[a]	Supplemented wet	Floral; Sicilia; lemon; vanilla	87.5	Pereira et al., (2015)
	YC5.2	Catuí[a]	Wet	Not evaluated	91.0	De Carvalho Neto et al., (2020)
	YC5.2	Mundo Novo[a]	Wet in bioreactor	Vanilla; yellow fruit; citric; liqueur	87.2	Sampaio et al., (2024)

Yeast	Strain	Variety*	Treatment	Sensory Profile	Coffee Grade**	Reference
Pichia guilliermondii	CCMA 1740	Vitoria[b]	Wet	Cereal; almond; caramel; chocolate	78.77	Bravim et al., (2023)
	UFLA YCN731	Acaiá[a]	Semi-dry	Caramel; acidic	74.17	Evangelista et al., (2014)
Pichia kudriavzevii	CBS5147	Sln 795[a]	Dry	Sweet; honey; berry-like; malt chocolate	ND	Shankar et al., (2022)
Pichia kluyveri	CCMA 1743	Robusta Tropical[b]	Dry	Fruity; almond	80.95	Silva et al., (2021)
	FROOTZEN	ND	Semi-dry	Fruity; acidic; roasted; caramel	ND	Wang et al., (2020a)
Candida parapsilosis	CCMA 0544	Novo Mundo[a]	Dry	Cocoa; citric	83.92	Bressani et al., (2021a)
	CCMA 0544	Novo Mundo[a]	Semi-dry	Citric; caramelized sugars	84.42	Bressani et al., (2021a)
	CCMA 0544	Catuaí Vermelho[a]	Dry	Chocolate; fruity; caramel; winey; spices	86.1	Bressani et al., (2021b)
	CCMA 0544	Catuaí Vermelho[a]	Self-induced anaerobic fermentation	Chocolate; caramel; citric; fruity; spices	84.5	Bressani et al., (2021c)
	CCMA 0544	Topázio Amarelo[a]	Self-induced anaerobic fermentation	Caramel; honey; chocolate; citrus	85.9	Jimenez et al., (2023)
	CCMA 0544	Catuaí Amarelo[a]	Semi-dry	Not evalueted	81	Martinez et al., (2017)
	CCMA 0544	Catuaí Amarelo[a]	Wet	Not evalueted	81.3	Martinez et al., (2017)
	CCMA 0544	Catuaí Amarelo[a]	Dry	Almond; caramel; fruity	81.33	Bressani et al., (2018)
	CCMA 0544	Catuaí Amarelo[a]	Semi-dry	Almond; caramel; fruity	80.67	Bressani et al., (2018)
	CCMA 0544	Bourbon Amarelo[a]	Dry	Dairy; fruity	ND	Bressani et al., (2020)
	CCMA 0544	Bourbon Amarelo[a]	Semi-dry	Caramel; dairy	ND	Bressani et al., (2020)
	CCMA 0544	Canário Amarelo[a]	Dry	Caramel; cocoa; fruits; Sweet	ND	Bressani et al., (2020)
	CCMA 0544	Canário Amarelo[a]	Semi-dry	Dairy; caramel; citric; Sweet	ND	Bressani et al., (2020)
	UFLA YCN448	Acaiá[a]	Semi-dry	Caramel	80	Evangelista et al., (2014)
Torulaspora delbrueckii	CCMA 0684	Novo Mundo[a]	Dry	Dairy; cocoa	84.42	Bressani et al., (2021a)
	CCMA 0684	Novo Mundo[a]	Semi-dry	Cocoa	84.83	Bressani et al., (2021a)
	CCMA 0684	Topázio Amarelo[a]	Self-induced anaerobic fermentation/ dry	Caramel; nuts	ND	Martins et al., (2023)

Yeast	Strain	Variety*	Treatment	Sensory Profile	Coffee Grade**	Reference
	CCMA 0684	Topázio Amarelo[a]	Self-induced anaerobic fermentation/ semi-dry	Caramel; nuts; chocolate; floral	ND	Martins et al., (2023)
	CCMA 0684	Catuaí Amarelo[a]	Semi-dry	Not evaluated	80.8	Martinez et al., (2017)
	CCMA 0684	Catuaí Amarelo[a]	Wet	Not evaluated	81	Martinez et al., (2017)
	CCMA 0684	Catuaí Amarelo[a]	Dry	Almond; caramel	81.5	Bressani et al., (2018)
	CCMA 0684	Catuaí Amarelo[a]	Semi-dry	Almond; caramel	81.5	Bressani et al., (2018)
	CCMA 0684	Bourbon Amarelo[a]	Dry	Caramel; citric fruits; sweet	ND	Bressani et al., (2020)
	CCMA 0684	Bourbon Amarelo[a]	Semi-dry	Citric fruits; caramel	ND	Bressani et al., (2020)
	CCMA 0684	Canário Amarelo[a]	Dry	Dairy; caramel; sweet	ND	Bressani et al., (2020)
	CCMA 0684	Canário Amarelo[a]	Semi-dry	Caramel; dairy; fruits; lactic	ND	Bressani et al., (2020)
	CCMA 0684	Catuaí Vermelho[a]	Dry	Chocolate; caramel; honey; yellow fruit	85.2	Bressani et al., (2021b)
	CCMA 0684	Catuaí Vermelho[a]	Self induced anaerobic fermentation	Chocolate; cucumber; caramel	83.3	Bressani et al., (2021c)
	CCMA 0684	Catucaí[a]	Dry	Sweet; fruity; nuts; fermented	84.1	Rocha et al; (2023)
	CCMA 0684	Novo Mundo	Semi-dry	Astringent	75.83	Ribeiro et al., (2017b)
	CCMA 0684	Ouro Amarelo	Semi-dry	Apple; acidic	79.96	Ribeiro et al., (2017b)
	CCMA 0684	Mundo Novo[a]	Wet	Caramel; herbaceous; brown sugar; honey	79.3	Martins et al., (2019)
	CCMA 0684	Catuaí[a]	Wet	Caramel; yellow fruits; brown sugar; herbaceous; floral	82	Martins et al., (2019)
	CCMA 0684	Topázio Amarelo[a]	Self-induced anaerobic fermentation	Citrus fruits; caramel, honey; chestnuts	86.5	Jimenez et al., (2023)
Meyerozyma caribbica	CCMA 0198	Mundo Novo[a]	Dry	Caramelized sugars; malic	84.08	Bressani et al., (2021a)
	CCMA 0198	Mundo Novo[a]	Semi-dry	Caramelized sugars	82.75	Bressani et al., (2021a)
	CCMA 0198	Bourbon Amarelo[a]	Dry	Red fruits; citric fruits; caramel	ND	Bressani et al., (2020)
	CCMA 0198	Bourbon Amarelo[a]	Semi-dry	Cocoa; caramel; dairy; sweet	ND	Bressani et al., (2020)
	CCMA 0198	Canário Amarelo[a]	Dry	Nuts; almond; citric fruits	ND	Bressani et al., (2020)
	CCMA 0198	Canário Amarelo[a]	Semi-dry	Caramel; Dairy	ND	Bressani et al., (2020)
	CCMA 1738	Robusta Tropical[b]	Dry	Chocolate; nuts; almond	82.21	Silva et al., (2021)

SOURCE: Author (2024)

LEGEND: *a *Coffea arabica* varieties and b *Coffea canephora* varieties. ** In all selected studies, the coffee grade was assessed by a panel of expert coffee tasters with Q-Grader Coffee Certificates, following the protocol established by the Specialty Coffee Association of America. ND: Not Described.

Although *Pichia* produces a diverse array of aromatic compounds, Table 1 indicates that *S. cerevisiae* strains are more commonly selected as starter cultures for coffee fermentation. *S. cerevisiae* is known for producing elevated levels of organic acids (citric, acetic), aldehydes (acetaldehyde), esters (ethyl acetate), alcohols (ethanol, glycerol), and ketones (diacetyl), contributing to the body, astringency, acidity, and aroma of coffee beverages (Bressani et al., 2021a; Evangelista et al., 2014). They also possess invertase activity, increasing the availability of reducing sugars during coffee fermentation. This benefits the growth of other aromatic microorganisms, making it a compelling candidate for co-inoculations (Evangelista et al., 2014; Pereira et al., 2014).

1.7 YEAST IMPACT ON COFFEE QUALITY

1.7.1 Volatile compound

Volatile compounds produced by yeast during coffee fermentation can remain in the beans or react during roasting, altering the coffee's chemical structure (Kim et al., 2022). Figure 2 illustrates the primary volatile compounds in coffee beverages produced from yeast fermentations. *S. cerevisiae* primarily increases the levels of butanedione, hexanal, benzyl alcohol, and acetaldehyde in coffee beverages. The metabolic routes for the formation of these compounds during yeast fermentation include the glycolytic pathway for butanedione, lipid oxidation for hexanal, the Ehrlich pathway for benzyl alcohol, and ethanol fermentation for acetaldehyde (Pereira et. al., 2019). Many other alcohols (ethanol, glycerol, hexanol, heptanol) and organic acids (citric, acetic, succinic acids) have also been observed to increase in coffee beverages through the use of *S. cerevisiae* in fermentation. These compounds are part of the primary *S. cerevisiae* metabolism, varying in concentration depending on the fermentation method or strain used (Vale et al., 2023). Interestingly, despite the high production of alcohols, coffees produced from *S. cerevisiae* fermentation are not described with alcohol-related sensory notes (Table 1), indicating loss through evaporation or reactions with other compounds during roasting. Thus, the relationship

between coffee quality and alcohol should be further studied. The production of organic acids can influence the perception of sour notes; however, off-flavor-causing acids such as isovaleric, isobutyric, and propionic should be minimized to avoid unpleasant and bitter sensations in the coffee beverage (Wu et al., 2021).

FIGURE 2: Major volatile compounds produced for selected yeast cultures in coffee beverages.

SOURCE: Author (2024)

Coffee beverages produced from *Torulaspora delbrueckii* fermentation are primarily distinguished by their composition of benzyl alcohol, phenethyl alcohol, and hexanal (Figure 2). Benzyl alcohol and phenethyl alcohol form through the reduction of their respective aldehydes, while hexanal is a product of lipid oxidation. *T. delbrueckii* also produces long-chain esters, such as octadecadienoic acid ethyl ester and hexadecenoic acid methyl ester, likely formed by reactions between fatty acids

and alcohols (Domínguez et al., 2022). Long-chain esters can enhance fruity and floral aromas and improve the mouthfeel and body perception of the coffee. However, the specific sensory effects can vary depending on the type and concentration of esters present. In significant quantities, they have the potential to induce undesirable wax-like odors (Pereira et al., 2019).

Candida parapsilosis increases the levels of phenethyl alcohol, hexanal, and heptanol (Figure 2). This yeast is distinct in producing heptanol, indicating specific fatty acid metabolism (Procopio, Qian & Becker, 2011). Some studies also *link C. parapsilosis* with producing esters (e.g., methyl salicylate, propyl acetate, isoamyl acetate, isobutyl acetate) and alcohols (e.g., ethanol, 1-hexanol, phenylethyl alcohol, benzyl alcohol) (Xue et al., 2020). *Pichia kluyveri, P. fermentans,* and *P. guilliermondii* mainly increase the proportion of esters and ketones (Figure 2). These species share the production of ethyl acetate, a key component derived from the esterification of ethanol and acetic acid.

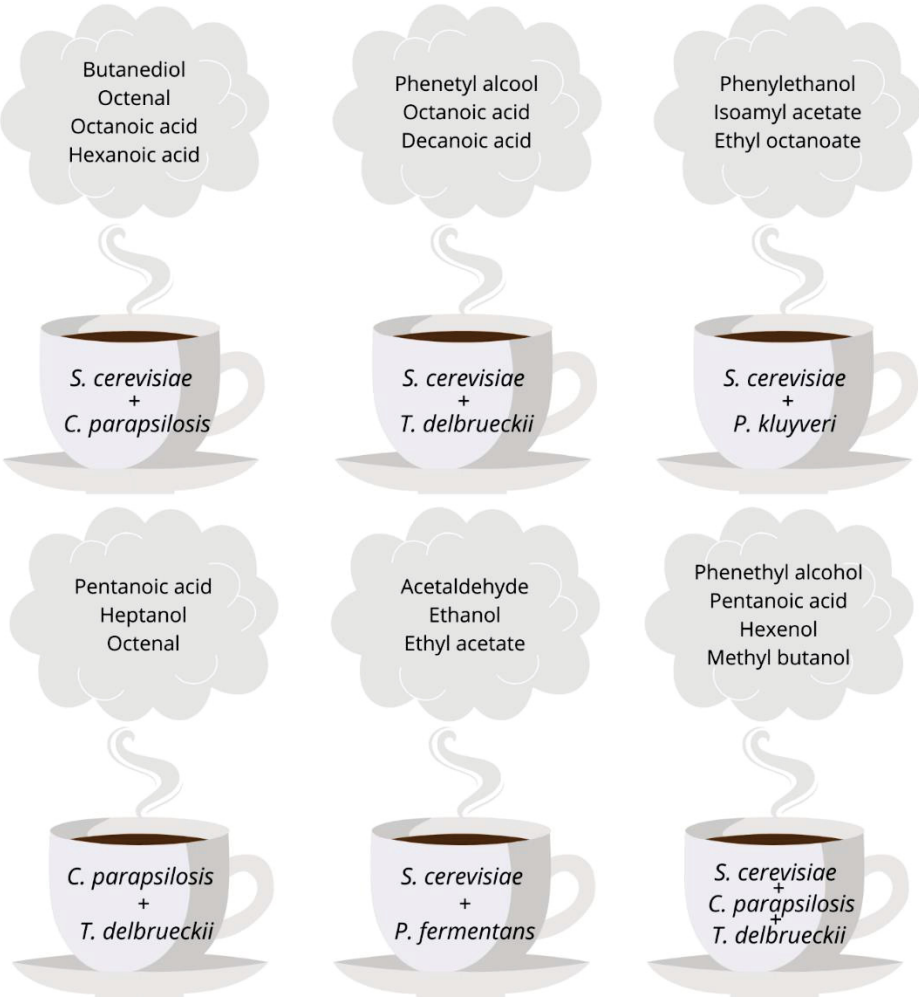
It is crucial to note that the permeation of these compounds into coffee beans varies in rates and concentrations. For example, 2-phenylethanol transfers 3-10 times more efficiently than butanal and isoamyl acetate, with rates increasing up to 45 h. Butanal transfers quickly initially but undergoes reactions, causing higher loss than isoamyl acetate, which transfers up to about 15 h (Salem et al., 2023). Further studies are needed to understand the diffusion of various compounds that yeasts may contribute to coffee quality.

1.7.2 Co-inoculation assays

Co-inoculations between yeasts and yeast + lactic acid bacteria (LAB) have been widely used in food fermentations to enhance flavor complexity, nutritional profiles, and product safety. Fig. 3 shows the primary volatile compounds in coffee from various yeast combinations. *S. cerevisiae,* the primary yeast under examination, showed notable coffee improvements when co-inoculated with *T. delbrueckii, C. parapsilosis,* and *P. kluyveri* (Bressani et al., 2021a, Bressani et al., 2021b, Bressani et al., 2021c). Each yeast combination produces a unique set of volatile compounds, potentially leading to more complex flavor profiles. The most frequently appearing compound is phenylethyl alcohol, known for its floral aroma, found in combinations: *S. cerevisiae* + *T. delbrueckii, S. cerevisiae* + *P. kluyveri,* and *S. cerevisiae* + *C.*

parapsilosis + *T. delbrueckii. S. cerevisiae* is the most likely primary phenylethyl alcohol producer due to its well-established metabolic pathways and high production yield. The absence of phenylethyl alcohol in single *S. cerevisiae* inoculation (Fig. 2) suggests that non-*Saccharomyces* yeasts modify the environment to stimulate its production. Thus, investigating the metabolic pathways of these yeasts during fermentation might offer deeper insights into optimizing and controlling the fermentation process for better consistency and quality.

FIGURE 3 - Major volatile compounds produced for co-inoculation assays in coffee beverages



SOURCE: Author (2024)

On the other hand, *S. cerevisiae* can stimulate the growth of non-*Saccharomyces* yeasts by expressing invertase, which hydrolyzes sucrose into glucose and fructose. Non-*Saccharomyces* yeasts typically do not utilize sucrose (Chin et al., 2023); however, co-culturing with *S. cerevisiae* enhances their metabolic activity through invertase expression, fostering a commensal interaction. *P. kluyveri*, when co-

inoculated with *S. cerevisiae*, significantly elevated the production of isoamyl acetate compared to yeast monocultures (Wang et al., 2020a). This ester partially survived the roasting process (Fig. 3), imparting considerable fruity and wine-like aromas to the roasted coffees. On the other hand, Pereira et al. (2014) observed a reduction in the production of esters (isoamyl acetate and ethyl acetate) when *P. fermentans* was co-inoculated with *Saccharomyces* sp. compared to single *P. fermentans* culture. This indicates that the growth and volatile compound production of *P. fermentans* were suppressed by the presence of *Saccharomyces* sp. in mixed-culture fermentations. Therefore, the choice of yeast strains and their combinations is crucial for optimizing the aromatic profile of the final coffee product.

Traditionally associated with dairy fermentations, LAB have gained attention in coffee fermentation for enhancing flavor, aroma, and quality (Cassimiro et al., 2023). Observations of the high prevalence of this group in spontaneous coffee fermentation have led to experimental studies showing that LAB are responsible for creating an acidic environment that promotes mucilage swelling and activates endogenous pectinase (Avallone et al., 2002; Cassimiro et al., 2023; Elhalis et al., 2023a; Pereira et al., 2016). The integration of yeast and LAB in coffee fermentation has emerged as a significant advancement in the quest for enhancing the sensory and quality attributes of coffee. Yeast and LAB play distinct but complementary roles in the fermentation process, contributing to the development of complex flavor profiles and improving the overall quality of the final coffee product. Studies on co-inoculations such as *Pichia fermentans* with *Pediococcus acidilactici* or *Pediococcus pentosaceus* have shown enhanced metabolite production and volatile composition (Sampaio et al., 2024; Vale et al., 2019). Other effective combinations include *Lactobacillus plantarum* with *S. cerevisiae* and *Leuconostoc mesenteroides* with *S. cerevisiae* (Cassimiro et al., 2022; Cassimiro et al., 2023; Borém et al., 2024). These studies commonly report an increase in coffee acidity and overall quality, sometimes adding up to 5 points in coffee grading. Thus, co-inoculation of LAB and yeasts is a potential avenue to explore in coffee processing, leading to new perspectives on coffee quality.

1.7.3 Sensory atributes

Table 1 provides detailed insights into the impact of different yeasts on the sensory quality of coffee beverages. *S. cerevisiae* is the most extensively tested, followed by *T. delbrueckii, C. parapsilosis,* and *P. fermentan*s. The preferred use of *S.*

cerevisiae may be related to its well-documented metabolism, high fermentation efficiency, and established safety in food applications (Elhalis et al., 2020).

A great sensory diversity in coffee is evident when examining different yeast strains and fermentation methods. *S. cerevisiae* commonly produces coffee with pronounced flavors of sweet, caramel, chocolate, and citrus fruits. On the other hand, *P. fermentans* tend to produce floral and citric flavors (Table 1). This suggests that coffee producers can use specific yeast strains to create distinct coffees tailored for specific market niches. However, it is crucial to consider the method used and the variety of coffee, as yeast metabolism is influenced by environmental conditions essential for their growth.

The highest coffee grade (91.0) was achieved using *P. fermentans* YC5.2 in submerged fermentation (Neto et al., 2020). The fermentation process in this study was performed in a stirred-tank bioreactor with controlled temperature, agitation, and oxygen levels. Replicating these conditions on coffee farms is challenging due to the need for advanced infrastructure and high-quality equipment. However, it shows that temperature and aeration control are crucial for high-quality results. The next highest scores were with *S. cerevisiae* (85.7), *T. delbrueckii* (87.2), and *C. parapsilosis* (86.1), all using natural fermentation (dry processing). These findings highlight that the choice of processing method is fundamental for manipulating the sensory quality of coffee through yeast fermentation.

Arabica (*Coffea arabica*) is the most widely studied coffee specie, while Conilon (*Coffea canephora*) is less frequently mentioned. Studies often focus on Brazilian varieties, such as Catuaí Vermelho, Topázio Amarelo, and Novo Mundo. Arabica coffee is the most consumed worldwide and preferred in specialty coffee production for its superior quality and complexity. Conilon, known for its higher caffeine content and bitter characteristics, has inferior sensory attributes. However, recent studies have shown that yeast fermentation can enhance Conilon's acidity and body, thereby improving its overall sensory experience (Silva et al., 2021; Cassimiro et al., 2023). Further studies with different yeasts and fermentation methods are essential to elevate Conilon coffee in the market. Optimizing these factors can enhance Conilon's sensory qualities, making it more competitive with Arabica and opening new opportunities in specialty coffee production.

1.7.4 Health properties

Coffee contains over 1,000 phytochemicals, including phenolic compounds (chlorogenic acids), diterpenes (cafestol and kahweol), and alkaloids (caffeine and trigonelline). These compounds can reduce due to these bioactive compounds, regular coffee consumption is generally associated with reduced risk of type 2 diabetes, prevention of neurological diseases, protection against depression, anti-cancer and liver-protective effects, as well as cardiovascular and gastrointestinal health benefits (Pereira et al., 2020).

Chlorogenic acids (caffeic, coumaric, and ferulic acids) are generated during the esterification of quinic acid and migrate from the inner to the outer layers of coffee cherries (Martinez et al., 2017; Fagan et al., 2011). Although chlorogenic acids are not linked to yeast metabolism, their concentration was observed to increase during the fermentation process (Bressani et al., 2021b). Yeast enzymatic activity during fermentation may release phenolic compounds from the coffee fiber, requiring further studies to confirm this hypothesis.

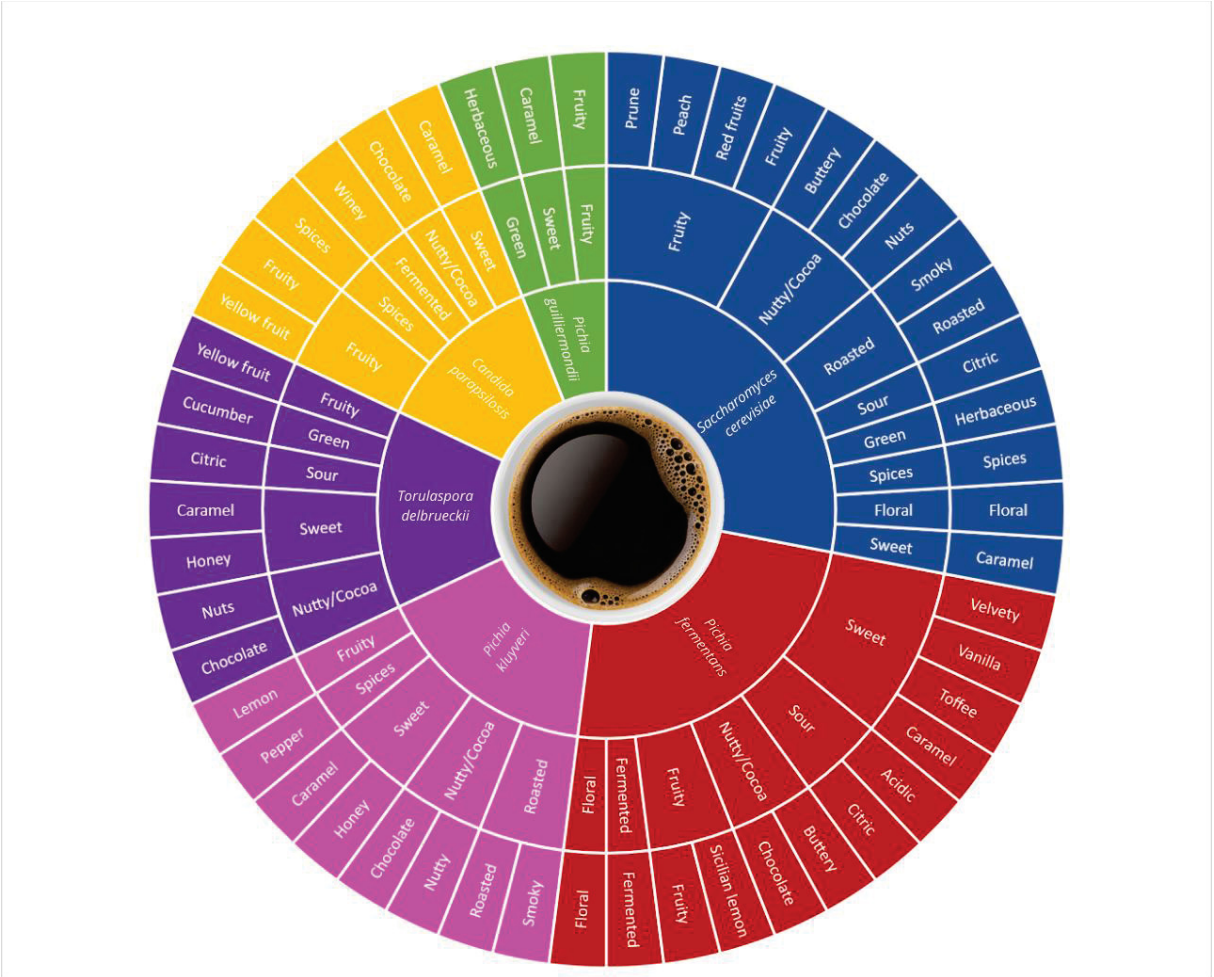
Some alkaloids, such as caffeine and trigonelline, degrade during roasting to form flavor-active metabolites through the Maillard reaction (Cassimiro et al., 2023; Gonzalez-Rios et al., 2007; Lee et al., 2016; Bruyn et al., 2017). These alkaloids possess antioxidant properties and, similar to chlorogenic acids, their levels have been observed to increase with co-inoculation and carbonic maceration processes (Bressani et al., 2018; Brioschi et al., 2021; Martinez et al., 2017). The mechanism behind this increase is unknown and requires further investigation.

Yeast fermentation reduces harmful compounds and enhances consumer safety by demonstrating antagonistic activity against toxigenic fungi (Masoud et al., 2005). For example, *P. fermentans* reduced ochratoxin A levels in coffee cherries by up to 88% (Pereira et al., 2015). Additionally, yeast fermentation reduces compounds like βN-alkanoyl-5-hydroxytryptamides (C-5HTs) and diterpenes (cafestol and kahweol), which are linked to gastric irritation and increased blood cholesterol (Tinoco et al., 2019). Thus, yeast-fermented coffee enhances safety and health benefits, paving the way for healthier and more functional coffees. However, while most studies on fermented coffee focus on sensory quality, its health benefits remain largely unexplored. More research is needed to fully understand and maximize these benefits.

1.8 CHARACTERIZATION OF ACTIVE-AROMA WHEEL OF YEAST-FERMENTED COFFEE BEVERAGES

The aroma wheel, a structured tool for describing and categorizing the complex aromas present in coffee, plays a crucial role in the coffee market. It serves multiple purposes, from enhancing quality control to aiding marketing strategies and improving consumer experience (Kwok et al., 2020). To help understand and harness the distinct aromas produced by different yeasts, the first aroma wheel was constructed linking specific yeast strains to their potential aromas in coffee beverages (Figure 4). The consistent caramel, chocolate, roasted, citric, and toffee aromas in all beverages result from Maillard-driven pyrolysis reactions (Wang et al., 2020a). However, their perceptions can also be influenced by yeast fermentation through compounds such as 2,3-butadione, benzyl alcohol, and ethyl salicylate (Mota et al., 2020; Neto et al., 2020).

FIGURE 4 - Characterization of the active-aroma wheel of yeast-fermented coffee beverages.



SOURCE: Author (2024)

LEGEND: The sensory descriptors were constructed based on studies that adopted a panel of expert coffee tasters with Q-Grader Coffee Certificates, following the protocol established by the Specialty Coffee Association of America

Notably, both *S. cerevisiae* and *P. fermentans* are frequently associated with fruity-like perceptions, indicating a trend where these yeast strains are preferred for creating fruit-forward coffee profiles. This trend is due to the extensive research and studies conducted with these yeast species, leading to a better understanding of how to optimize their use in coffee fermentations. *S. cerevisiae* and *P. fermentans* also seem to promote floral sensations in coffee, which are highly appreciated in the market. Additionally, some specific sensory perceptions identified include red fruits and spices (*S. cerevisiae*), cucumber, green, and yellow fruit (*T. delbrueckii*), and herbaceous notes (*C. parapsilosis*). Further studies should be conducted to determine whether these sensations originate from the coffee fruit itself or are yeast-driven reactions. This will allow coffee producers to use different yeasts to target various market segments, enhancing both the sensory profile and market appeal of their coffee products.

1.9 SUPPLEMENTATION NEEDS

Although the potential of yeast supplementation in coffee fermentation is promising, research in this area is still developing. While some studies have examined the impact of temperature and external carbon supplementation on yeast activity and fermentation outcomes (Pereira et al., 2015; Ladino-Garzon et al. 2023), factors such as micronutrients, pH adjustments, and oxygen requirements remain underexplored.

Coffee pulp contains sufficient carbohydrates, proteins, and fats for yeast proliferation (Pereira et al., 2020). Studies with external sugar supplementation (e.g., sucrose, and sugarcane juice) showed no significant effect on yeast growth but resulted in different sensory profiles compared to non-supplemented batches (Pereira et al., 2015; Ladino-Garzon et al., 2023). For instance, coffee from sucrose-supplemented fermentation had 'vanilla' taste and 'sicilian lemon' and 'floral' aromas, while non-supplemented fermentation produced 'caramel' taste and 'lactic' and 'citric' acid perceptions (Pereira et al., 2015). This is likely due to changes in yeast metabolism and the formation of different end-metabolites, altering the coffee's chemical composition.

To the best of our knowledge, no studies have evaluated the effect of additional nitrogen supplementation in coffee fermentation. However, additional supplementation with ammonium salts (e.g., ammonium sulfate) or free amino acids can be tested to support more robust fermentation. This is particularly important during high-demand phases of yeast activity. Wine and beer studies show amino acid supplementation can improve specific metabolite production. In wine, amino acids like alanine, phenylalanine, and isoleucine increased fruity esters (Zhu et al., 2021). In beer, valine, leucine, and isoleucine boosted higher alcohols and esters (Ferreira & Guido, 2018). These findings suggest targeted amino acid supplementation can enhance coffee sensory qualities by influencing flavor and aroma pathways.

While yeast can ferment anaerobically, it requires oxygen for the biosynthesis of essential membrane components like fatty acids and sterols. Thus, a brief aeration period at fermentation start is necessary under strictly anaerobic conditions (Walker & Stewart, 2016). Coffee fermentation methods such as carbonic maceration and self-induced anaerobic fermentation provide low oxygen due to minimal air exposure, limiting yeast growth and fermentation efficiency. Studies have shown slow yeast growth in these methods (Braga et al., 2023), suggesting coffee pulp may lack sufficient fatty acids (e.g., oleic acid) and sterols (e.g., ergosterol). Research should focus on improving yeast growth by introducing initial oxygen or supplementing ergosterol and oleic acid at fermentation start.

Yeast grows optimally at pH 4.5-6.5, supported by the natural acidity of coffee pulp, while the optimal temperature range (28-30°C) may require adjustments. Most coffee studies use ambient temperatures (15-35°C), affecting yeast metabolism and fermentation consistency. Thus, stable temperature control is imperative for the successful yeast fermentation of coffee. The fermentation temperature can also be controlled to optimize the metabolism of *Saccharomyces* and non-*Saccharomyces* yeasts. In general, lower temperatures favor non-*Saccharomyces* yeasts, while *Saccharomyces* thrives near 30°C. For instance, 15°C promoted *P. fermentans* during controlled coffee fermentation, producing isoamyl acetate and ethyl acetate, while 28-37°C favored *Saccharomyces* sp., producing acetaldehyde and ethanol (Pereira et al., 2014). These findings highlight the potential for future studies to balance the metabolisms of different yeast species in coffee fermentation, thereby directing flavor formation towards desirable levels.

Specific minerals (magnesium, calcium, and iron) and vitamins (biotin, thiamine, riboflavin, and pantothenic acid) are critical for various biochemical processes within yeast cells (Walker and Stewart 2016). It is known that coffee pulp contains all essential minerals, including potassium (329 mg/L), calcium (4600 mg/L), magnesium (1100 mg/L), and iron (136 mg/kg) (Rojas-Orduña et al., 2023). However, coffee pulp may lack sufficient levels of these vitamins. Therefore, tests of mineral and vitamin supplementation and their proportions can be conducted to optimize yeast growth and fermentation performance. These tests can assist in determining the optimal levels and combinations of these nutrients to meet the specific metabolic needs of yeast, ultimately enhancing the efficiency of coffee fermentation and the quality of the final product.

1.10 SAFETY, MARKET GROWTH, AND ENVIRONMENTAL IMPACT OF MICROBIAL STARTER CULTURES IN COFFEE PROCESSING

The European Food Safety Agency (EFSA) and the United States Food and Drug Administration (FDA) have established specific criteria for evaluating the safety of microbial commercialization in the food industry. These criteria include programs like the Qualified Presumption of Safety (QPS) and Generally Recognized as Safe (GRAS), which assess factors such as taxonomy, pathogenicity, and antibiotic resistance. While *S. cerevisiae* is a widely recognized and safe yeast for coffee fermentation, other yeasts like *Candida* species require thorough pathogenicity testing due to their potential as opportunistic human pathogens (Pereira et al., 2022, Pereira et al., 2022). In general, these yeasts have been selected based on their impact on the quality of coffee bean, but safety criteria are often overlooked.

The market for microbial starter cultures in coffee processing is growing significantly, although the selection of strains is less extensive than in other fermented beverage industries (Pereira et al., 2019). Dominated by Lallemand's LalCaféTM brands, this market includes five S. cerevisiae strains (LalCafe BSC™, LalCafe ORO™, LalCafe CIMA™, LalCafe INTENSO™, LalCafe BRIOSA™). These cultures are applied to whole fruit or pulped coffee using submerged or "dry" protocols, and they can be used synergistically in mixed fermentations.

Selvatech™ markets coffee starter cultures as microbial consortia produced through backslopping, rather than defined/pure strains. The main disadvantage of

these undefined cultures is their sensitivity to temperature and substrate changes, leading to inconsistent microbial diversity and fermentation reproducibility (Smid et al., 2014). Unlike Lallemand, Selvatech recommends immediate application after washing the coffee fruit, using a natural post-harvest processing method. Coffee fruits should be grouped in heaps for 48–72 h, then spread in a single layer and dried until reaching 11% humidity.

In addition to commercial starter cultures, patent BR1020160117569 proposes using lactic acid bacteria (LAB) to efficiently remove coffee mucilage and enhance the sensory profiles of the final coffee beverage (Soccol et al., 2022). Additionally, PaliniAlves™ has developed a rotating cylinder for the controlled fermentation of coffee cherries (BR202021015732Y1) (Palini & Alves, 2022). This new bioreactor reduces the likelihood of contamination and allows coffee producers to rotate the coffee beans, resulting in a homogeneous product. However, this rotating cylinder lacks control over temperature, pH, and agitation. Thus, further modifications are needed to integrate these control parameters to ensure optimal fermentation conditions and consistent quality of the final product.

As there is no specific legislation on the disposal of waste generated during the coffee fermentation process, the use of yeast in coffee fermentation can have significant environmental impacts. These impacts include increased biochemical oxygen demand (BOD) and chemical oxygen demand (COD) in water bodies, which harm aquatic life and cause eutrophication. Excessive organic matter from spent yeast can alter soil nutrient balance and microbial diversity, reducing soil health and fertility. The decomposition of this organic matter releases greenhouse gases like methane and carbon dioxide, contributing to global warming. Additionally, certain yeast strains may spread pathogens if not properly treated before disposal. To mitigate these environmental impacts, it is essential to develop and implement appropriate waste management practices for yeast biomass. This can include composting, anaerobic digestion, wastewater treatment, land application guidelines, and continuous research and monitoring (Szlachta, Prask, Fugol, & Luberański, 2018).

1.11 OPTIMAL STORAGE CONDITIONS FOR YEAST-FERMENTED COFFEE BEANS

The ideal storage parameters for fermented coffee beans involve maintaining stable humidity (50–60%) and temperature levels (10–15 °C) to prevent mold growth, flavor loss, and degradation of volatile compounds (Borém et al., 2021). Monitoring specific volatile compounds is essential for controlling the storage of fermented coffee beans. The increase of key compounds, such as hexanal, linalool, 1-hydroxy-2-propanone, 2-methyl tetrahydro-3-furanone, furfural, pyridine, trans-2-nonenal, 2-methylbutanal, 3-methylbutanal, and 2,3-pentanedione, can be used as markers of inadequate storage and depreciation of sensory quality in fermented coffee beans (Bonnländer et al., 2006; Borém et al., 2021). Additionally, 2-phenylethanol, butanal and isoamyl acetate should be tracked to ensure the beans retain their fruity and floral aromas formed during fermentation (Salem et al., 2023).

Yeast-fermented coffees are expected to have more volatile compounds than non-fermented coffees. Hermetic packaging with CO_2 injection is recommended to reduce oxygen exposure and prolong freshness. For instance, high-barrier packaging, such as PICS bags, effectively limits moisture and oxygen, maintaining quality and volatile compounds for up to 12 months (Ribeiro et al., 2011). Additionally, refrigerated high-barrier packaging helps conserve aroma, flavor, and body descriptors, preserving fruity, sweet, and chocolate notes, citric acidity, and a creamy body after nine months (Salvio et al., 2023). These conditions retain the unique flavors and aromas of fermented coffee beans during storage.

Interestingly, Salvio et al. (2023) recently demonstrated that coffee beans undergoing yeast fermentation significantly enhanced storage quality compared to naturally fermented coffees. The study found that yeast-fermented coffees retained sensory qualities such as acidity, body, and aftertaste even after six months of storage. In contrast, naturally fermented coffees showed a decline in these attributes over the same period. Therefore, incorporating specific yeast strains in the fermentation process is recommended for producing high-quality coffee with enhanced storage stability. Further studies are necessary to better understand the mechanisms behind the range of compounds accumulated in coffee beans through yeast fermentation that enhance storage quality.

1.12 CONCLUSION AND OUTLOOK

Recent research on yeast selection has identified *Saccharomyces, Pichia, Candida, Torulaspora,* and *Hanseniaspora* as key players in the intricate process of coffee fermentation. The yeasts' metabolic activities and production of aromatic compounds significantly contribute to the rich and diverse sensory experiences that coffee enthusiasts seek in their cups. However, unlike the well-established use of *S. cerevisiae* in wine and other alcoholic beverages, determining the best yeast for coffee fermentation remains a complex endeavor. Research indicates that various yeasts, both *Saccharomyces* and non-*Saccharomyces*, can serve specific roles in coffee fermentation. For example, *S. cerevisiae* is noted for its ability to enhance sweetness and caramel notes in coffee beverages, while non-*Saccharomyces*, especially *Pichia* species, enhance fruity perceptions. However, non-*Saccharomyces* yeasts pose challenges in handling and consistency, necessitating further research and validation for their safety and scalability in commercial applications compared to *S. cerevisiae*.

The choice of yeast should align with the producer's desired outcomes, fermentation methods, and specific goals, ensuring the production of high-quality and diverse coffee products. For instance, understanding the optimal use of yeasts in coffee fermentation involves examining different methods and their impact on yeast performance. Methods like carbonic maceration, with high CO_2 concentrations, may favor *S. cerevisiae* due to its resilience. This yeast adapts well to high CO_2 levels, adjusting membrane fluidity and regulating proteins and enzymes to maintain cellular stability and protect intracellular components. On the other hand, non-*Saccharomyces* yeasts may be more suited for traditional wet fermentation, as many studies have demonstrated their adaptability to this process worldwide. Continued research and experimentation will further enhance our understanding and application of these yeasts in coffee fermentation, paving the way for innovations to refine and elevate the quality of the final coffee beverage.

Finally, coffee fermentation benefits from the co-inoculation of *Saccharomyces* with non-*Saccharomyces* yeasts and LAB, offering a multifaceted impact on the sensory and microbiological aspects of the final coffee product. These interactions result in a broader range of flavor compounds that contribute to the complexity and uniqueness of the coffee aroma and taste profile. However, ongoing research in this field will likely uncover novel yeast strains and fermentation approaches, continuously raising the standards of coffee production. This study serves as a foundation for future

investigations, highlighting the significance of microbial interventions in shaping the sensory experience of coffee consumers.

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2 CHAPTER 2: MODULATION OF AROMA AND CHEMICAL COMPOSITION OF COFFEE BEANS THROUGH SIMULTANEOUS AND SEQUENTIAL INOCULATION OF *Pichia fermentans* AND *Pediococcus pentosaceus* DURING WET FERMENTATION

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2.1 ABSTRACT

The combined inoculation of yeast and lactic acid bacteria (LAB) is a promising approach to enhance microbial metabolism and the quality of fermented food products. In this study, we investigated the effects of simultaneous and sequential inoculation of LAB and yeast on coffee fermentation. Fermentations were conducted using single and combined protocols with *P. fermentans* YC5.2 and *P. pentosaceus* LPBF07. A temporal analysis utilizing Illumina high-throughput rRNA Gene Sequencing revealed a synergistic interaction between the two microbial groups. This positive synergy led to increased consumption of coffee pulp sugar and the production of metabolites, surpassing the results observed in single cultures and the spontaneous process. Furthermore, the combined inoculation processes demonstrated a more significant role in suppressing wild microbiota compared to single cultures. Notably, the sequential process emerged as particularly effective in promoting a more intricate aroma profile and elevated sensorial score, attributed to the formation of distinctive compounds such as benzeneacetaldehyde, 1-hexanol, 2-heptanol, benzyl alcohol and phenylethyl alcohol. These results suggest that implementing a sequential inoculation process could enable coffee farmers to standardize on-farm processing and produce high-value-added products.

2.2 INTRODUCTION

Coffee is one of the most popular and consumed beverages in the world and is considered one of the most traded commodities on a global scale (“Food Outlook – Biannual Report on Global Food Markets,” 2023). The journey of coffee, from bean to cup, involves intricate processing methods that play a crucial role in shaping its flavor,

aroma, and overall quality (Pereira et al., 2019). The fermentation stage in post-harvest coffee processing holds paramount importance as it significantly influences the final quality and flavor profile of the coffee beans. This crucial step involves the breakdown of sugars and mucilage surrounding the coffee beans, leading to the development of unique aromatic compounds and flavors (Pereira et al., 2019)

In modern coffee processing, diverse fermentation methods have been implemented, with a primary emphasis on removing coffee pulp and decreasing coffee fruit moisture from 65% to 10%. This focus aims to ensure optimal conditions for the storage and transportation of coffee. In the wet process, coffee fruits are immersed in tanks filled with large volumes of water, creating anaerobic conditions conducive to a high prevalence of fermentative microorganisms. These microorganisms include mainly yeasts (e.g., *Saccharomyces cerevisiae, S. bayanus, S. cerevisiae, Kluyveromyces marxianus, Pichia anomala,* and *P. kluyveri, Hanseniaspora uvarum*) and LAB (e.g, *Lactobacillus, Lactococcus,* and *Pediococcus*) (de Oliveira Junqueira et al., 2019a). In the initial stages of coffee fermentation, there is a prevalence of lactic acid bacteria (LAB) (~ 10^8 – 10^9 CFU/mL), inducing coffee pulp-mass acidification through consistent lactic acid production. Subsequently, during the later stages of coffee fermentation (~ 10^7 – 10^8 CFU/mL), yeasts dominate, contributing to mucilage breakage and enhancing the aroma complexity of the beans (Elhalis et al., 2023)

Given that the activities of both yeast and LAB significantly contribute to the aroma profiles of coffee beans, incorporating both cultures into the manufacturing process is crucial. While massive studies have employed selected yeasts and, more recently, LAB (Pereira et al., 2014, 2015; Pereira et al., 2016) the use of co-inoculation protocols is scarce. The interaction between different yeast strains and LAB can lead to the production of a broader range of compounds, contributing to a more nuanced and rich sensory experience in the final coffee beverage. Furthermore, the synergistic action of compatible microbial strains may result in a faster and more efficient conversion of sugars into flavor-active metabolites, reducing the overall fermentation time.

This study aims to examine the effects of simultaneous and sequential inoculation of specific strains, *Pichia fermentans* YC5.2, and *Pediococcus acidilactici* LPBF07, on the coffee bean fermentation process. By exploring the interactions between these microorganisms, we aim to understand their potential in modulating the aroma and chemical composition of coffee beans.

2.3 MATERIAL AND METHODS

2.3.1 Production of starter cultures

The yeast *Pichia fermentans* YC5.25 and the lactic acid bacterium *Pediococcus pentosaceus* LPBF07 were isolated from different coffee fermentation processes and selected by Pereira et al., (2014) and Junqueira et al., (2016), respectively. The microbial strains belong to the Microbiological Culture Collection of the Department of Bioprocess Engineering and Biotechnology, UFPR, PR, Brazil. *P. fermentans* YC5.2 was reactivated in 10 mL of YEPG liquid medium (20 g/L glucose, 10 g/L yeast extract, 10 g/L soy peptone and pH 5.0, at 30 °C, 150 rpm for 24 h), while *P. pentosaceus* LPBF07 was reactivated in MRS broth (De Man, Rogosa, and Sharpe - Merck Millipore, Burlington, MA) at 30 °C and 150 rpm. The strains were transferred to an Erlenmeyer flask containing 1 L of YEPG and MRS, respectively. Each culture from this pre-inoculum was transferred to a fermenter (New Brunswick™ BioFlo® 110 - Eppendorf, Hamburg, Germany) with 9 L of culture medium, and fermentation was carried out for 24 h at 350 rpm and aeration at 10 L/min.

After this period, the culture was centrifuged and the cells recovered and resuspended in 1 L of whey cryoprotectant solution (10%), obtaining a solution with approximately 9.61 Log CFU/mL and 9.06 Log CFU/mL of *P. fermentans* YC5.2 and *P. pentosaceus* LPBF07, respectively. The cells were transferred to sterile stainless-steel trays and frozen at -80ºC for 24 hours. After this period, the cultures were transferred to a lyophilizer (Thermofisher). Freeze-drying was carried out for 48 h at -50 ºC and a pressure of 960 mbar. The dried biomass was then recovered, ground, vacuum-packed, and stored at 4 ºC until it was applied in the field.

2.3.2 On-farm inoculation protocols

The field experiments took place at California farm (23°08′58.42″ S 50°01′39.33″ W; 650 m above sea level) situated in the Norte Pioneiro region of Paraná (Jacarezinho, Paraná, Brazil). The coffee fruits (*Coffea arabica* var. Mundo Novo) were harvested in May 2023 and mechanically pulped. Approximately 8 kg of pulped coffee beans were transferred to cylindrical fermentation tanks (50 x 37 x 54 cm) and then 3 L of water was added. Before inoculation, the freeze-dried strains were rehydrated in mineral water. Appropriate concentrations of inoculum were used to achieve an initial

cell concentration of around 6 log CFU/mL. Five inoculation protocols were carried out: i) spontaneous fermentation (control), ii) fermentation inoculated with *P. fermentans* YC5.2, iii) fermentation inoculated with *P. pentosaceus* LPBF07, iv) co-inoculation of *P. fermentans* YC5.2 and *P. pentosaceus* LPBF07 (simultaneous fermentation) and v) sequential fermentation (an initial 36-hour period of fermenting solely with *P. pentosaceus* LPBF07, followed by the inoculation of *Pichia fermentans* YC5.2 after this duration). All fermentation protocols were carried out in triplicate for 72 hours. Approximately 50 mL of the liquid fraction from the fermentations were collected every 12 h (0, 12, 24, 36, 48, 60, and 72 h)) for physicochemical and microbiological analysis. After completing the fermentations, approximately 100 g of coffee beans were collected, frozen, and stored for physicochemical analysis. Subsequently, the beans were spread out on a raised bed to sun-dry until they achieved a moisture content of 11%.

2.3.3 Assessment of fermentation microbial diversity by high-throughput sequencing

High-throughput sequencing was used to access the microbial diversity of samples collected at 0 and 72 h from the fermentation liquid fraction. DNA extraction was performed using the Power Soil Kit (Qiagen, Carlsbad, CA, USA) and its concentration was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The V3-V4 variable regions of the 16S rRNA gene for bacteria were amplified from the total DNA extracted using primers 341F and 805R, and for fungi, the ITS region was amplified using primers 3F and 805R (da Silva Vale et al., 2023). The amplicons were quantified using the Qubit DNA HS kit (Thermo) and sequenced using the MiSeq Reagent 500 v2 kit (Illumina), in paired 2 x250 b. The resulting fasta files were filtered and demultiplexed using bcl2fastq (Illumina). Taxonomic identification was conducted using the QIIME2 software package, version 1.9.0 (da Silva Vale et al., 2021a).

2.3.4 Analysis of the liquid fraction of coffee fermentation

The consumption of glucose and fructose, production of lactic and acetic acids, and ethanol from both spontaneous and inoculated fermentations were determined by high-performance liquid chromatography (HPLC). Aliquots of 2 mL, collected from the

samples at 0, 12, 24, 36, 48, 60, and 72 h, were centrifuged at 10,000 rpm for 10 min and filtered through a 0.22 μm pore size filter (Millipore Corp., Billerica, MA, USA). The samples were, then, evaluated using an HPLC system (Agilent Technologies, Waldbronn, Germany). A Hiplex-H column (300 × 7.7 mm) (Bio-Rad, Richmond, CA, USA) was employed to separate the compounds through an isocratic mobile phase composed of 4.0 mM H2SO4, with a flow rate of 0.5 mL min-1 during 30 minutes. The column and the RID detector were maintained at temperatures of 70 and 50 °C, respectively, throughout the entire run. Organic acids were quantified using DAD at 210 nm, while sugars were estimated on the RID (Pregolini et al., 2021).

The volatile organic compounds (VOCs) generated during fermentation were identified by Gas Chromatography Coupled with Mass Spectrophotometry (GC/MS). Aliquots (3 mL) were added to hermetically sealed vials (20 mL), both collected at 0 and 72 h. Solid Phase Microextraction (SPME) was utilized for sample analysis, employing a DVB/CAR/PDMS Fiber (Supelco Co., Bellefonte, PA, USA). The SPME fiber was exposed for 30 min at 60 °C. Thermal desorption of the compounds occurred at 260 °C, and they were 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 settings within the GC were: column oven at 60 °C, injection at 260 °C, and detector at 250 °C. Helium served as the carrier gas with a flow rate of 1 mL/min, column pressure of 57.4 kPa, and a split ratio of 1:20. Mass spectrometry covered a range of 30–250 (m/z) with an ion source temperature of 250 °C. The identification of volatiles was accomplished by comparing each mass spectrum either with spectra from authentic compounds or with spectra in reference libraries (de Oliveira Junqueira et al., 2019a).

2.3.5 Analysis of the volatile profile of green coffee beans

The coffee beans collected immediately after fermentation and the dried beans at 11% moisture were subjected to GC/MS analysis to determine their volatile profile. Briefly, the coffee beans were ground to 3g and transferred to a hermetically sealed 20 mL vial. The injection parameters applied to the green beans followed the procedures reported in the preceding section.

2.3.6 Sensory analysis

To determine the effect of the starter cultures on the quality and sensory profile of the final drink, the samples were prepared according to the recommendations of the Specialty Coffee Association (SCA). Each sample, containing 8.25 g of coffee and 150 mL of water, was prepared, resulting in a total of five samples. Certified Q-Graders were subsequently tasked with evaluating and scoring attributes such as flavor, acidity, aftertaste, aroma, body, sweetness, balance, uniformity, clean cup, and overall quality on a scale ranging from 6 to 10, with intervals of 0.25.

2.3.7 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)(da Silva Vale et al., 2023).

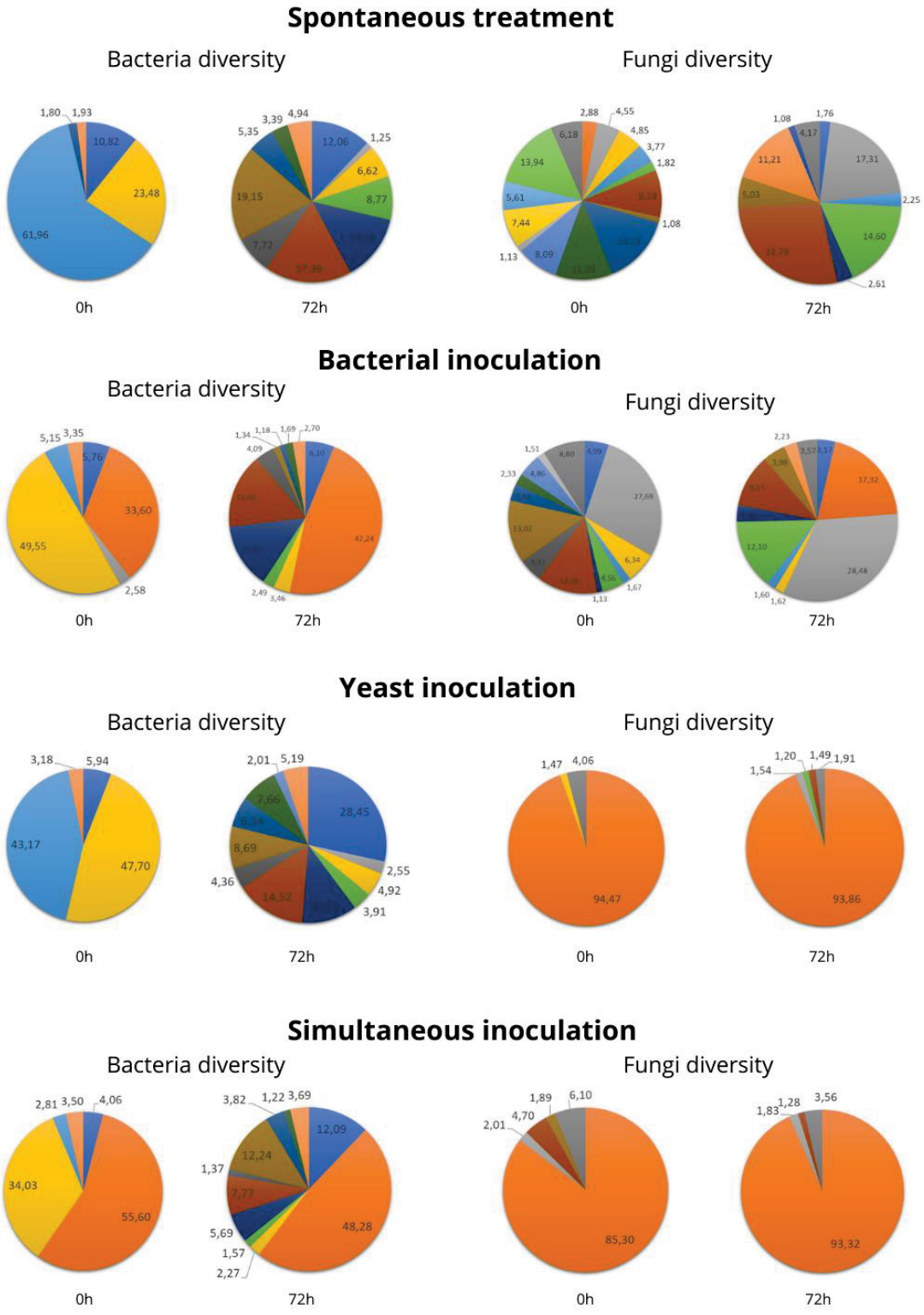
2.4 RESULTS AND DISCUSSION

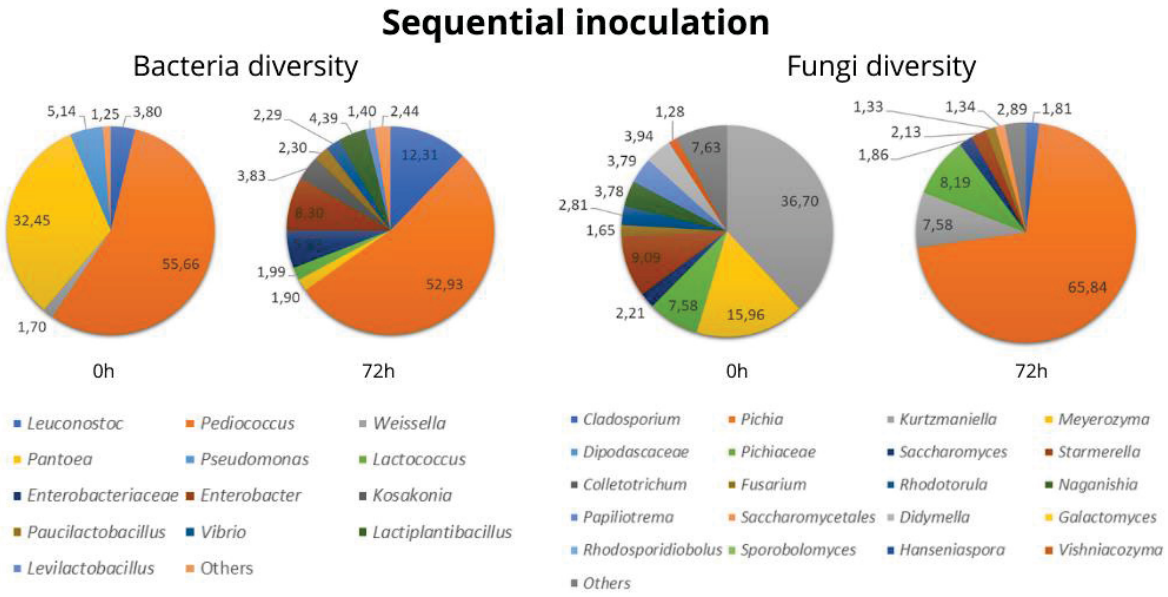
2.4.1 Microbiota dynamics during inoculated treatments

A total of 794.812 sequences from the hypervariable region V3-V4 of the 16S rRNA gene and 935.555 from the Internal Transcribed Spacer (ITS) rDNA gene were obtained from all samples. The sequences corresponded to 224 different microbial genera after searching in the SILVA database. This extensive diversity is consistent with findings from prior studies utilizing NGS in coffee fermentations conducted in Colombia (173 genera), Honduras (409 genera), Brazil (62) and Ecuador (20 genera) (Vale et al., 2021b; De Bruyn et al., 2017; de Carvalho Neto et al., 2018; Junqueira et al., 2019b). Illumina's technology has revolutionized the approach to studying microbial diversity in traditional fermentations. By generating data on millions of rDNA amplicons in a single run, it enables precise phylogenetic analysis of microbial taxa, revealing a broader bacterial diversity landscape, including uncultivable microorganisms, sub-dominant populations, and late-growing species (Pereira et al., 2022). The comprehensive list of bacteria and fungi at the genus level is provided in the Appendix

A (Table S1 and S2), along with the rarefaction curves (Figure S1 and S2). Figure 5 illustrates the relative abundance of bacteria and fungi with >1% of read sequences.

FIGURE 5 - Composition of bacteria and fungi at beginning (0 h) and end (72 h) of spontaneous, lactic acid bacteria inoculation, yeast inoculation, and simultaneous and sequential processes.





SOURCE: Author (2024)

LEGEND: Only bacteria and yeast with prevalence greater than 1% are shown. The complete list of minor microbial group is reported in the Supplementary Information (Table S1 and Table S2).

2.4.2 Spontaneous process and single cultures

At the beginning of the spontaneous fermentation process, a notable yeast diversity was observed, marked by the co-dominance (over 10% of reads) of *Rhodotorula, Naganishia,* and *Sporobolomyces* (Figure 5). After 72 hours of spontaneous process, there was a significant increase in reads belonging to *Kurtzmaniella* (17.31%), co-dominating with *Starmorelle*. Interestingly, *Kurtzmaniella* remained dominant throughout the process with *P. pentosaceus* LPBF07 single inoculation, reaching sequence reads above 28%. *Kurtzmaniella* is frequently encountered in soil, flowers, and fruits, showing a frequent phylogenetic relationship with *Candida* species (Lachance & Starmer, 2008; Lopes et al., 2019). This yeast grows well on hexose sugars (glucose, fructose, mannose, and galactose) and is able to assimilate pentose sugars such as ribose, xylose, arabinose, and lyxose. Furthermore, it can grow in the presence of complex plant-derived polysaccharides (Arrey et al., 2021). These characteristics support its growth and dominance during the spontaneous process. Furthermore, the synergy with LAB can be associated with the acid production mechanisms (by LAB) and the supply of vitamins (by *Kurtzmaniella*). Subsequent investigations could further explore the application of this yeast in coffee fermentation processes. However, *P. fermentans* YC5.2 inoculation proved highly

effective in inhibiting the growth of wild yeasts with dominance over 94% throughout the fermentation process (Figure 5).

Concerning the bacterial profile, spontaneous processes and single-yeast inoculation revealed a high prevalence of *Pseudomonas, Enterobacter,* and *Pantoea*. These bacterial groups are associated with native soil, the water source used in the process, the air surrounding the fermentation tank, and human-related factors (Junqueira et al., 2019a). Notably, these bacteria have the potential to impact coffee with off-flavors, producing compounds such as butyric and propionic acids (Pereira et al., 2016). The introduction of *P. pentosaceus* LPBF07 in the single inoculation process exhibited the inhibition of the growth of these bacteria, consistent with findings from previous studies (Vale et al., 2019). After 72 h, the OTU readings attributed to *Pediococcus* increased to 47%, suppressing the growth of *Pseudomonas*, *Enterobacter, Pantoea*, and other wild species. This suppression is beneficial, given that some indigenous species detected in the spontaneous process, such as *Enterobacter* and *Bacillus*, can produce off-flavor metabolites that decrease coffee flavor quality (e.g. butyric acid, propionic acid and sulfides).

2.4.3 Simultaneous and sequential inoculations

The microbiome analysis unveiled the successful performance of co-inoculation protocols (simultaneous and sequential), showing a synergistic interaction between *P. pentosaceus* LPBF07 and *P. fermentans* YC5.2, as demonstrated by Illumina reads proportion. In the case of yeast, the proportion of *Pichia* reads in the simultaneous protocol was similar to the process using single-yeast inoculation (over 84% throughout the fermentation process). The same was observed for bacteria, with *Pediococcus* representing over 48% of the total sequence reads, similar to the process using single *P. pentosaceus* LPBF07 inoculation. Sequential fermentation behaved as described in the inoculation protocol, where *Pediococcus* represented over 55% of the population at the beginning of the process and remained high until the end. On the other hand, the yeast population underwent a drastic change after *P. fermentans* YC5.2 inoculation, reaching levels of 70% at the end of the process.

The synergistic interaction between LAB and yeast plays a pivotal role in controlled fermentation processes, offering significant benefits to both microbial metabolism during fermentation process. It is well-established that LAB-produced

organic acids reduce pH levels, thereby stimulating yeast growth through intracellular adaptive responses facilitated by vacuolar ATPase pumps and the plasma membrane (Peetermans et al., 2021). Conversely, yeast secretes amino acids like proline, glutamate, glutamine, and phenylalanine during the exponential growth phase, which serve as essential nutrients for LAB growth. Previous research has already established a beneficial symbiotic relationship between *Pichia* and *Pediococcus* in coffee fermentation (Pregolini et al., 2021). However, while such symbiosis has been observed to enhance sensory attributes in wine, sourdough, and yogurt, limited information exists regarding these mechanisms in the context of coffee fermentation (Junqueira et al., 2019).

Regardless of the protocol (single yeast, single bacteria, simultaneous, and sequential process), inoculation proved to be very effective in inhibiting the growth of wild yeasts and bacteria. However, it became evident that yeast and LAB inoculation are important for controlling the growth of wild yeasts and bacteria, respectively. The prevalence of *Pichia* and *Pediococcus* over the wild microbiota can be attributed to the notable stress tolerance and metabolite adaptation capabilities exhibited by these microbial groups (Pereira et al., 2014; de Carvalho Neto et al., 2018). These attributes are essential in facilitating the coffee bean fermentation process, underscoring the significant role played by *Pichia* and *Pediococcus* in this context (Pereira et al., 2014; Muynarsk et al., 2019).

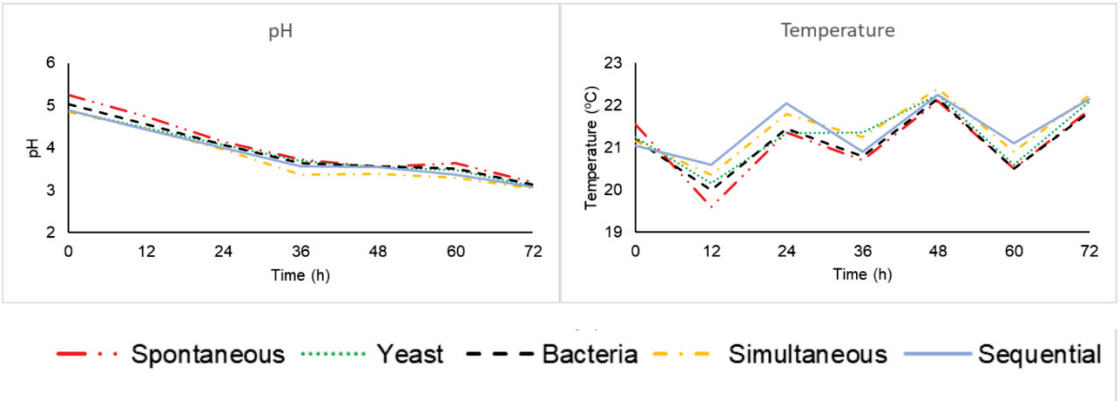
The combined protocols stand out in the formation of coffee aromas due to the increased predictability of metabolites produced during fermentation. These include key flavor compounds such as acetaldehyde, isoamyl acetate, and ethyl acetate from yeast metabolism, as well as lactic acid, 1-butanol, benzaldehyde, 2-nonenal, and 2-pentanone from LAB activity. In contrast, traditional methods may yield a broader spectrum of compounds from indigenous microflora, some of which may not contribute positively to coffee quality.

2.4.4 Physical-chemical profile, sugar consumption, and metabolite production

The pH and temperature of the starter culture-added fermentations and spontaneous process are shown in Figure 6. All fermentations were characterized by an initial pH value of around 5. The final pH values demonstrated a strong correlation with bacterial growth and acid production. Across all treatments, final pH values

converged around 3.1. The reduction in pH levels below 4.5 is a widely used method by coffee producers to determine the end of fermentation of the coffee beans during the wet processing. Throughout the treatments, temperature fluctuations followed a consistent pattern, decreasing at night and rising during the day (Figure 6).

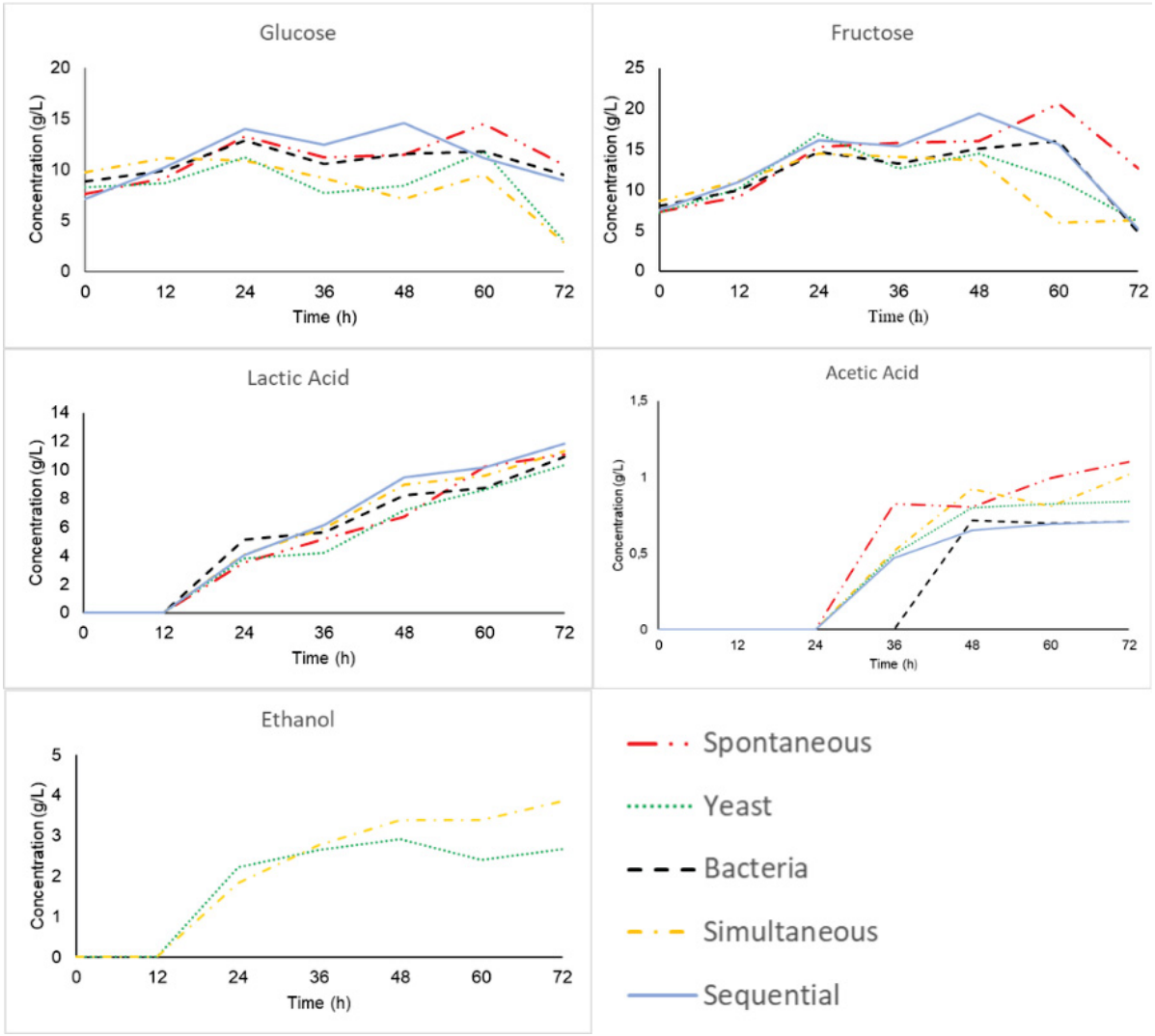
FIGURE 6 - pH and temperature during coffee fermentations conducted spontaneously, single lactic acid bacteria inoculation, single yeast inoculation, and simultaneous and sequential processes



SOURCE: Author (2024)

The initial content of glucose (7.0-9.7 g/L) and fructose (7.1-8.6 g/L) (Figure 7) increased up to 24 hours of fermentation, attributable to the enzymatic action of microbial invertases on complex sugars (Manoochehri et al., 2020). Subsequently, these simple sugars were progressively consumed over the course of 72 hours of fermentation. In terms of residual glucose, processes involving single-yeast and simultaneous inoculation demonstrated higher efficiency, resulting in values below 3.5 g/L. Regarding fructose, all except for the spontaneous treatment showed a low residual content, ranging from 4.7 to 6.2 g/L (Figure 7). The elevated residual sugar content in the spontaneous process could potentially serve as an available carbon source for the growth of filamentous fungi and other undesirable microorganisms during the subsequent coffee drying process. The spontaneous process demands more extended periods for the adaptation of wild microflora to the fermentation environment, leading to delays in the overall process. Additional factors, such as a limited microbial presence at the process's initiation and competition among wild microflora, contribute to the inefficiencies observed in the spontaneous process.

FIGURE 7 - Sugar consumption and organic acids production (g/L) during coffee fermentations conducted spontaneously, single lactic acid bacteria inoculation, single yeast inoculation, and simultaneous and sequential processes



SOURCE: Author (2024)

The single and co-inoculation processes showed to be relevant for the conversion of sugars into essential metabolites. This has been highlighted in various fermentation processes, such as the modernization of wine and bread production chains. Several authors have also demonstrated the adaptability of yeast LAB in coffee processing (Pereira et al., 2015; Pereira et al., 2016; Wang et al., 2020). Lactic acid was the primary end-metabolite formed during all fermentation processes. It is primarily generated through the Embden–Meyerhof–Parnas (EMP) pathway of LAB (Salvetti et al., 2013). However, yeasts can also produce lactic acid as a primary compound through the reduction of acetaldehyde by the enzyme acetaldehyde dehydrogenase (Ilmén et al., 2013; Song et al., 2015). The protocols involving LAB (sequential, single-

bacteria and simultaneous) accumulated lactic acid more rapidly up to 48 hours of fermentation (8,2-9,4 g/L) (Figure 7). However, the extended fermentation time of 72 hours revealed that both the wild microbiota in the spontaneous process and single yeast fermentation efficiently accumulated lactic acid. The final values for all processes were approximately 11 g/L. Lactic acid is crucial as a contributor to the breakdown of mucilage present in the coffee pulp and facilitates the drying process (Pereira et al., 2015; Elhalis et al., 2023). Moreover, recent studies have highlighted lactic acid as a substantial contributor to both the acidity and body of coffee beverages. This acid permeates the beans during the fermentation process and remains present in the final beverage (De Carvalho Neto et al., 2020). Coffee beverages with high lactic acid concentration tend to exhibit sensory perceptions of sour and acid (Pereira et al., 2020), enhancing the beverage's added value.

Acetic acid accumulated during the fermentations, though in concentrations considerably lower than that of lactic acid. After 72 hours, all processes displayed their peak values, with the highest reaching 1.1 g/L in the case of spontaneous fermentation (Figure 8). Acetic acid can be produced by yeast through the alcoholic fermentation or through the pentose phosphate pathway in heterofermentative LAB. In low concentrations, acetic acid has the potential to enhance fruity and wine-like perceptions in coffee beverages (Bressani et al., 2021). Nevertheless, when exceeding concentrations of 1 mg/mL, it may adversely impact the final quality of the beverage by imparting an undesirable vinegar-like flavor.

Ethanol was accumulated in the single-yeast and simultaneous fermentations, reaching the maximum value of 2.67 and 3.85 g/L, respectively (Figure 7). Ethanol is associated with the growth of *P. fermentans* YC5.2 in fermentative processes, produced through the EMP pathway. The non-detection of ethanol in spontaneous, single-bacteria, and sequential processes may be associated with the delayed growth of *P. fermentans* YC5.2 in the sequential process, as well as the high prevalence of non-alcoholic yeasts in spontaneous and single-bacteria processes. The impact of ethanol on the sensory profile of coffee is not yet well elucidated, but studies indicate its influence on the liquor perception and enhances the alcohol flavor.

2.4.5 Volatile compounds and sensory profile

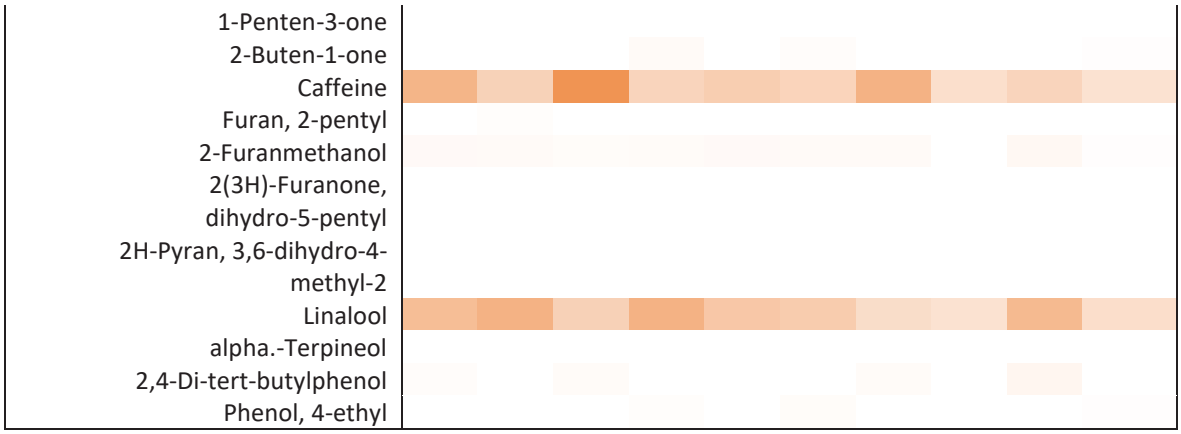
2.4.5.1 Volatile composition of the fermentation liquid fraction

The HS-SPME/GC analysis identified a total of 93 volatile compounds in the fermented liquid fraction, categorized into ten chemical groups: esters (23), aldehydes (18), hydrocarbons (18), alcohols (12), acids (12), furans (3), pyrans (2), ketones (2), terpenes (2) and phenols (2) (Table 2). Spontaneous fermentation yielded the lowest content of volatile compounds, whereas sequential fermentation produced the highest, approximately three times greater. This distinction is directly attributed to the metabolic activities of yeast and LAB. The dominant class of volatile compounds produced was esters, initially present in minimal amounts across all assays. However, there was a noticeable increase in ester production in inoculated fermentations. One notable compound was ethyl linoleate, an ester formed by ethyl alcohol and linoleic acid, commonly employed in industries as a flavoring agent due to its intensely sweet and fruity aroma. This compound exhibited an approximately 35% increase in sequential fermentation compared to spontaneous fermentation. Methyl salicylate, characterized by a sweet and fruity odor, was present in all fermentations but had higher levels in assays inoculated with *P. fermentans* YC5.2 (single-yeast, simultaneous, and sequential). The accumulation of esters at the end of coffee fermentation is desirable, as even at low concentrations these compounds can diffuse into coffee beans and modulate the sensory profile of the final beverage (Hadj Salem et al., 2020).

TABLE 2 - Heatmap of the volatile compounds identified in the liquid fraction of spontaneous (S), bacteria inoculation (B), yeast inoculation (Y), simultaneous (Si) and Sequential (Se) fermentation treatments in 0 h and 72 h


Treatments
Compounds
0
72
0
72
0
72
i0
i72
e0
e72
Phenylethyl Alcohol
1-Butanol, 3-methyl
1-Hexanol
1-Octanol
1-Octen-3-ol
2-Heptanol
2-Hexanol, 5-methyl
2-Nonen-1-ol
2-Propyl-1-pentanol
3-Ethyl-4-methylpentan-1-ol
Benzyl alcohol
Oleyl alcohol, trifluoroacetate
9,12-Octadecadienoic acid
1,2-Benzenedicarboxylic acid
Dodecanoic acid
Lactic acid
n-Decanoic acid
n-Hexadecanoic acid
Nonanoic acid
Octanoic acid
Oxalic acid
Pentadecanoic acid
Tetradecanoic acid
Tridecanoic acid
Benzaldehyde, 2,4-dimethyl
2,4-Decadienal
2,4-Dodecadienal
2-Butenal
2-Heptenal
2-Octenal
Benzaldehyde
Benzaldehyde, 3-methyl
Benzeneacetaldehyde
Butanal, 3-hydroxy
Butanal, 3-methyl
Decanal
Hexanal
Nonanal
Octanal
Pentadecanal
2-Dodecenal
Tridecanal
Benzenepropanoic acid, ethyl ester
Butyric acid, 4-pentadecyl ester
Phthalic acid, isobutyl nonyl ester


1-Butanol, 3-methyl-, acetate
8,11,14-Docosatrienoic acid, methyl ester
9,12,15-Octadecatrienoic acid, ethyl ester
9,12,15-Octadecatrienoic acid, methyl ester
9,12-Octadecadienoic acid, methyl ester
Acetic acid, 2-phenylethyl ester
Acetic acid, phenylmethyl ester
Dichloroacetic acid, tridec-2-ynyl ester
Decanoic acid, ethyl ester
Dodecanoic acid, ethyl ester
Ethyl 9-hexadecenoate
Ethyl heptadec-9-enoate
Formic acid, 2-phenylethyl ester
Hexadecanoic acid, ethyl ester
Hexadecanoic acid, methyl ester
Linoleic acid ethyl ester
Methyl salicylate
Octanoic acid, ethyl ester
Tetradecanoic acid, ethyl ester
1,3-Hexadiene, 3-ethyl-2-methyl
2-Methyltetracosane
10-Methylnonadecane
Annulene
Benzene, 1,2,3-trimethyl
Benzene, 1,2,4-trimethyl
Benzene, 1-ethyl-2-methyl
Benzene, 1-ethyl-3-methyl
Benzene, ethenyl
Benzene, propyl
Dodecane, 2,6,11-trimethyl
Ethylbenzene
Heneicosane
Heptadecane
Nonane, 3-methyl-5-propyl
Pentadecane
Tetradecane



SOURCE: Author (2024)

Aldehydes, compounds whose production is linked to the metabolism of amino acids found in coffee pulp by yeast and LAB, play a significant role (De Carvalho Neto et al., 2020). Among the noteworthy aldehydes produced, benzeneacetaldehyde (imparting floral and honey notes) and benzaldehyde (conveying nutty and chocolate aromas) are prominent, widely reported, and associated with the metabolism of LAB and *Pichia*, respectively. Additionally, aromatic aldehydes such as hexanal (offering fruity notes), nonanal (providing fruity and floral characteristics), and decanal (imparting citrus notes) stem from the oxidation of unsaturated fatty acids (Gänzle & Schwab, 2009). Their consistent presence across all assays at similar levels suggests that they are not fermentation by-products but rather the result of the oxidation of fatty acids present in coffee beans.

While the precise connection between alcohols and the ultimate quality of a coffee beverage remains unclear, it is established that a higher concentration of these compounds enhances sensory perception. Phenylethyl alcohol, in particular, displayed a noteworthy alteration, presenting an almost fivefold increase in concentration in single-yeast and simultaneous inoculations compared to other treatments. This alcohol, known for its floral scent, is produced by yeast through (i) the use of the amino acid L-phenylalanine as a substrate via the Ehrlich pathway (Vale et al., 2023) or (ii) the use of glucose as a substrate through the shikimate pathway (Mitri et al., 2022). The delayed growth of *P. fermentans* YC5.2 in sequential fermentation may be the reason why phenylethyl alcohol did not present a pronounced increase in concentration in this assay.

Benzyl alcohol, known for its fruity and floral notes, exhibited an increase in all inoculated assays, suggesting its production as a byproduct of microorganism

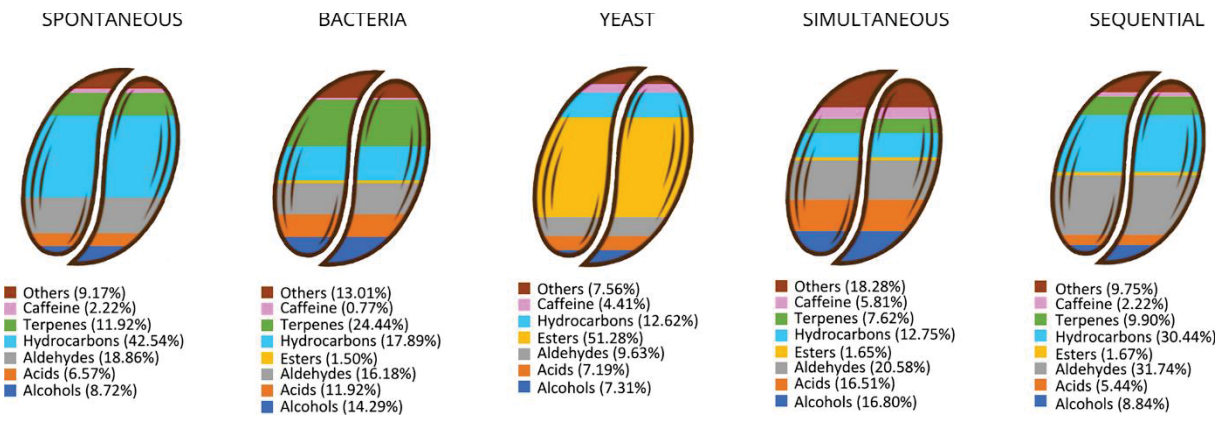
metabolism. Conversely, 2-Hexanol, 5-methyl (characterized by green and fruity aromas), was consistently present across all assays with no significant increase, indicating its origin from the coffee beans themselves.

Within the class of terpenes, linalool, known for its floral and fruity aroma, shows high amounts in all assays. While all initial treatments showed a comparable content of linalool, the inoculated assays exhibited a notable increase, likely associated with the production of this compound through the mevalonate (MVA) pathway in yeast metabolism (Vale et al., 2023). Ultimately, the distinctions noted in the generation of volatile compounds among all treatments underscore that the inoculation process not only substantially enriches the complexity of compounds but also that the method of inoculation (such as simultaneous and sequential approaches) plays a crucial role in shaping the formation and concentrations of these compounds. Consequently, these factors collectively contribute to influencing the ultimate outcome of the fermentation process.

2.4.5.2 Volatile composition of the green beans and sensory profile

A total of 90 volatile compounds were identified in green coffee beans, analyzed both before and after the drying process. These volatiles were classified into eight chemical groups: alcohols, acids, aldehydes, esters, hydrocarbons, terpenes, caffeine, and others, as illustrated in Fig. 8. For a detailed list of all compounds identified in green coffee beans, please consult Appendix A, Table S3.

FIGURE 8 - Profile of the volatile compounds identified in green beans of spontaneous, bacteria inoculation, yeast inoculation, simultaneous (Si) and sequential (Se) inoculation after drying.



SOURCE: Author (2024)

Coffee beverages resulting from both spontaneous and inoculated fermentation processes underwent assessment following the SCA methodology by certified Q-Graders. The scoring outcomes were as follows: 84.5 for the spontaneous process, 85.0 for single bacteria inoculation, 86.0 for simultaneous processing, 87.2 for single yeast inoculation, and 88.5 for the sequential process. While all coffees scored above 84 and can be categorized as specialty coffees, the spontaneous process yielded the lowest score among the evaluations. This could be attributed to its higher concentration of hydrocarbons and lower levels of volatiles, with no detection of esters (Fig. 8).

The single yeast inoculation exhibited the most pronounced difference, with the concentration of esters approximately fifty times higher compared to the other treatments. These esters and other low molecular weight flavor compounds (e.g., aldehydes, alcohols) are produced by yeast metabolism during the mucilage removal process (Pereira et al., 2014). Such compounds are associated with the floral and fruity notes in coffee beverages and may contribute to the higher score observed in the single-yeast fermentation coffee.

Both the simultaneous and sequential processes exhibited a greater balance among compounds, including alcohols, esters, aldehydes, acids, hydrocarbons, and terpenes (Fig. 8). However, the sequential process showed twice the concentration of alcohols and a higher concentration of aldehydes compared to the simultaneous process. This was reflected in the sensory analysis, as the simultaneous process achieved the highest score among the treatments. Additionally, coffee beans fermented from the simultaneous treatment resulted in a beverage with greater sensory complexity, presenting intense floral, fruity, and caramel perceptions. A complex sensory description was also related to coffee beverages from single yeast inoculation (vanilla, yellow fruit, citric, and liqueur-like perception) and the simultaneous process (green apple, green grape, white wine, and herbal-like perception), compared to single bacteria (citric, syrup, and creamy-like perception) and spontaneous treatment (caramel, citric, and creamy-like perception). It is evident that yeast inoculation is indispensable for achieving complex, fruity-like notes.

2.5 CONCLUSION

P. fermentans YC5.2 and *P. pentosaceus* LPBF07 demonstrated a beneficial synergy in coffee fermentation, resulting in increased coffee pulp sugar consumption and metabolite production. This surpassed the outcomes observed in single cultures and the spontaneous process. The co-inoculation treatments yielded coffee beverages with a richer volatile profile and enhanced sensory evaluation compared to single inoculations and spontaneous processes. The compounds with major impact were ethyl linoleate, 1-hexanol, 2-heptanol, benzeneacetaldehyde, benzyl alcohol and phenylethyl alcohol, that are reportedly important contributors to aroma. This research underscores the significant potential of sequential inoculation in generating desirable aroma compounds and producing specialty coffees. Further studies should prioritize investigating the scalability and economic advantages of this protocol in enhancing efficiency, consistency, and profitability within the coffee industry. Embracing and optimizing the utilization of starter cultures can empower coffee producers to meet expanding market demands while ensuring the production of high-quality, value-added coffee products.

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3 CONCLUSION AND OUTLOOK

Co-inoculation of yeasts with lactic acid bacteria (LAB) has proven to be a promising approach for enhancing the complexity and quality of coffee, resulting in richer and more distinct sensory profiles. The study demonstrated that the proper selection of yeasts, such as *Saccharomyces*, *Pichia*, and *Torulaspora*, is crucial to achieve the desired sensory characteristics. The interaction between yeast and LAB can intensify acidity and the production of aromatic compounds, enriching the coffee's sensory profile. However, the choice of strains and their combinations is critical to achieving the desired balance of sensory characteristics and avoiding the production of undesirable metabolites.

This pioneering study explored five different inoculation protocols using *Pichia fermentans* YC5.2 and *Pediococcus pentosaceus* LPBF07. The sequential inoculation method, an innovative approach previously underexplored, proved highly effective in suppressing wild microorganisms. This resulted in coffee with more pronounced fruity and floral notes, achieving higher sensory scores than those obtained from individual cultures or spontaneous fermentation processes. The increased production of volatile compounds, such as ethyl acetate, also contributed to the higher sensory scores of the coffee fermented using this method. These findings align with the literature, which associates *P. fermentans* with high ester production and coffee profiles within this sensory profile.

To advance the application of these techniques, additional studies are essential to investigate the metabolic differences between simultaneous and sequential co-inoculated fermentations. Such research could optimize fermentation conditions, maximizing sugar consumption and production of desirable compounds. Evaluating the economic viability of sequential inoculation on a large scale and its impact on production efficiency is crucial to determine the practical applicability of these techniques in the coffee market. Controlled environment surveys may also reveal the maximum potential of fermentation, although they require high-quality infrastructure and equipment.

In summary, this study provided a solid foundation for future research and innovations in coffee fermentation, highlighting the potential of combined inoculation techniques to improve product quality and consistency. Adapting and optimizing these practices could significantly transform the coffee industry, meeting the growing demand for high-quality and uniquely characterized products. It also emphasizes the importance of choosing the right strain to align with the producer's goals and desired outcomes. The choice of yeast, whether *Saccharomyces* or non-*Saccharomyces*, should be tailored to the fermentation methods and specific objectives, ensuring the production of high-quality and diverse coffee products. Continued exploration and refinement of these fermentation methodologies hold the promise of advancing coffee production, paving the way for exceptional and innovative coffee experiences.

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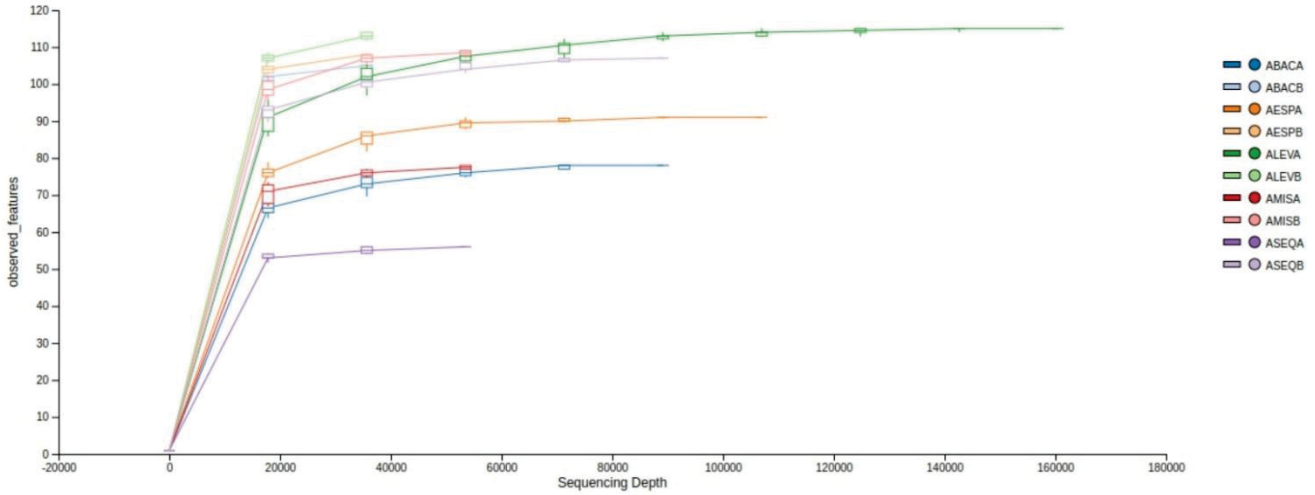
APPENDIX A

Supplementary materials to Chapter 2.

(A)



(B)

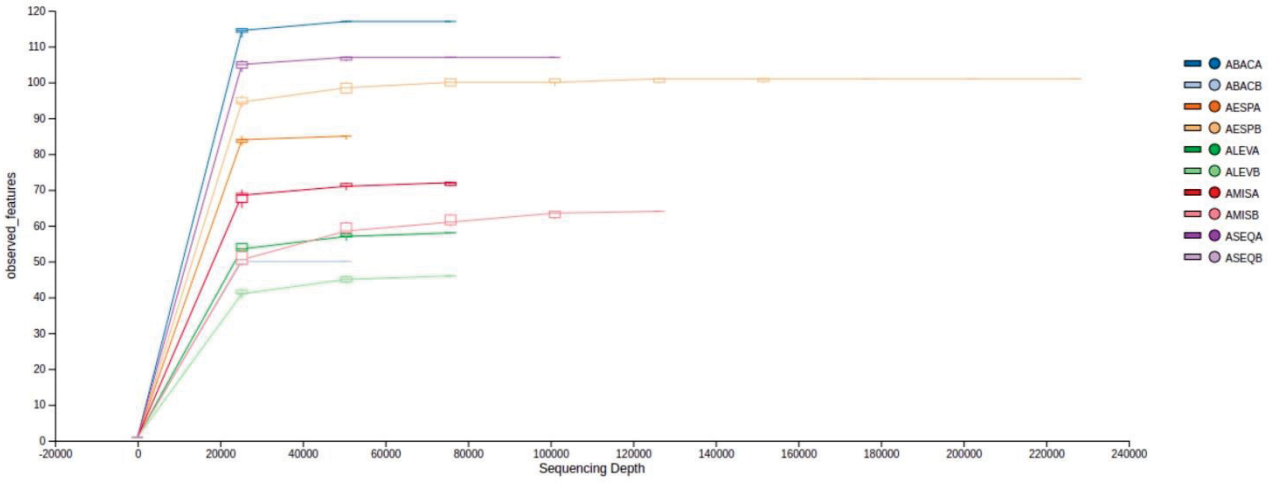


Figure S1: Alpha rarefaction curves of the OTUs (Operational taxonomic units) observed from the fermentation liquid fraction samples. (A) Bacterial analysis, (B) Fungal analysis.

Table S1: Relative abundance (%) of bacteria identified in the liquid fraction of spontaneous (S), bacteria inoculation (B), yeast inoculation (Y), simultaneous (Si), and Sequential (Se) fermentation treatments in 0h and 72h.

Microorganisms	Treatments									
	S (0h)	S (72h)	B (0h)	B (72h)	Y (0h)	Y (72h)	Si (0h)	Si (72h)	Se (0h)	Se (72h)
Achromobacter	ND	0,015242	ND	ND	ND	ND	ND	ND	ND	ND
Acinetobacter	ND	ND	ND	ND	0,00187	0,00402	ND	ND	ND	ND
Actinomycetospora	ND	ND	ND	ND	0,011218	ND	ND	0,013212	ND	ND
Akkermansia	ND	ND	ND	ND	ND	ND	ND	0,004404	ND	ND
Anaerostipes	ND	ND	ND	ND	ND	ND	ND	0,005872	ND	ND
Azospirillum	0,001985	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bacteroides	0,002978	0,008709	ND	ND	ND	ND	ND	ND	ND	ND
Bifidobacterium	ND	ND	ND	ND	0,005609	ND	ND	ND	ND	ND
Blautia	ND	ND	ND	0,014797	0,00187	0,016082	ND	0,022019	ND	ND
Bradyrhizobium	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,004146
Burkholderia	0,00397	0,017419	ND	0,029595	ND	0,036184	ND	0,027891	ND	0,006219
Cellulomonas	ND	ND	ND	0,024662	0,005609	ND	ND	ND	ND	ND
Cellulosimicrobium	ND	ND	0,003687	ND	ND	ND	ND	ND	ND	ND
Chryseobacterium	ND	ND	ND	0,007399	ND	ND	ND	ND	ND	ND
Clostridium	ND	ND	ND	ND	ND	0,010051	ND	ND	ND	ND
Cnuibacter	0,00397	ND	ND	ND	ND	ND	ND	ND	ND	ND
Collinsella	ND	ND	ND	ND	ND	ND	ND	0,004404	ND	ND
Comamonas	ND	ND	ND	ND	ND	0,006031	ND	ND	0,003714	ND
Cronobacter	0,093304	0,130642	0,127208	0,512972	0,082262	0,673421	0,092264	0,361117	0,061285	0,298511
Curtobacterium	ND	ND	ND	ND	ND	ND	0,018453	0,13652	ND	ND
Devosia	ND	0,008709	ND	ND	ND	ND	ND	ND	ND	ND
Dorea	ND	ND	ND	ND	ND	ND	ND	0,010276	ND	ND
Dyadobacter	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,003109
Enterobacter	0,112163	15,90785	0,070978	17,39173	0,094103	14,51775	0,10362	7,768415	0,076142	8,295069

Enterococcus	ND	0,023951	0,011983	0,160304	0,024305	0,068347	ND	ND	ND	0,015547
Epilithonimonas	ND	ND	ND	0,009865	0,00187	ND	ND	ND	ND	ND
Erwinia	0,005956	0,037015	0,032263	ND	0,034899	ND	0,056778	ND	ND	ND
Faecalibacterium	ND	0,004355	ND	ND	ND	ND	ND	ND	ND	ND
Flavipsychrobacter	ND	ND	ND	ND	ND	0,006031	ND	ND	ND	ND
Frateuria	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,002073
Fundicoccus	ND	ND	ND	ND	0,001246	ND	ND	ND	ND	ND
Fusobacterium	ND	ND	ND	ND	ND	ND	ND	0,004404	ND	ND
Gluconobacter	0,685883	0,139352	0,808414	0,1825	0,260496	0,733727	0,907026	0,184962	0,490278	0,078774
Gordonia	ND	ND	ND	ND	ND	ND	ND	0,005872	ND	ND
Halotalea	ND	ND	ND	ND	ND	0,006031	ND	ND	ND	ND
Humisphaera	ND	ND	ND	ND	ND	ND	ND	0,002936	ND	ND
Hymenobacter	ND	0,006532	ND	ND	ND	ND	0,017033	0,063122	ND	ND
Hyphomicrobium	ND	ND	ND	ND	ND	ND	ND	0,002936	ND	ND
Intestinimonas	ND	ND	ND	ND	ND	ND	ND	0,004404	ND	ND
Janthinobacterium	ND	ND	ND	0,012331	ND	ND	ND	0,004404	ND	ND
Klebsiella	ND	0,524746	ND	0,900168	ND	0,75584	ND	0,378732	ND	0,462277
Klenkia	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,002073
Kluyvera	ND	ND	ND	0,012331	ND	ND	ND	ND	ND	ND
Kosakonia	0,09926	4,093453	ND	7,719246	0,073537	4,36417	0,07665	1,374005	ND	3,826739
Lacipirellula	ND	0,006532	ND	ND	ND	ND	ND	ND	ND	ND
Lacrimispora	ND	ND	ND	ND	ND	ND	ND	0,004404	ND	ND
Lacticaseibacillus	0,00397	0,010887	0,01014	0,288547	ND	0,082419	ND	0,016148	ND	0,061153
Lactiplantibacillus	ND	1,680928	ND	3,388577	0,041754	7,658907	0,058197	1,218402	ND	4,393702
Lactobacillus	ND	ND	0,006453	ND	0,00187	ND	ND	ND	ND	ND
Lactococcus	0,033748	2,490909	0,014749	8,767387	0,012464	3,905842	0,053939	1,569243	0,029714	1,991107
Leuconostoc	6,345661	6,103164	10,81911	12,06471	5,940933	28,44852	4,062456	12,08861	3,79594	12,30527
Levilactobacillus	ND	0,37233	ND	0,355135	ND	2,006191	ND	0,776548	ND	1,404451
Limnospira	ND	ND	ND	ND	ND	0,010051	ND	ND	ND	ND

Liquorilactobacillus	ND	0,063144	ND	0,525303	ND	0,85233	ND	0,252488	ND	0,171022
Luteibacter	ND	ND	0,011062	ND	ND	ND	ND	ND	ND	ND
Massilia	ND	ND	ND	ND	0,001246	ND	ND	ND	ND	ND
Mediterraneibacter	ND	ND	ND	ND	ND	0,008041	ND	ND	ND	ND
Methylobacterium	0,00397	0,015242	0,003687	0,012331	0,049233	0,070357	0,048261	0,01468	ND	0,013474
Methylophilus	0,004963	0,071853	0,016592	0,036993	0,018073	0,042214	0,011356	0,026423	0,005571	ND
Methylotenera	0,010919	0,043547	0,014749	0,044392	0,018696	0,060306	ND	0,029359	ND	ND
Microbacterium	ND	ND	ND	ND	ND	ND	ND	ND	0,013	ND
Mycobacterium	ND	ND	ND	ND	0,00187	ND	ND	ND	ND	ND
UNIDENTIFIED	0,203482	13,82629	0,197667	13,3866	0,562747	11,50243	0,201561	5,711811	0,007428	5,93808
Niallia	ND	ND	ND	ND	ND	0,006031	ND	ND	ND	ND
Niastella	ND	0,017419	ND	ND	ND	ND	ND	ND	ND	ND
Nocardioides	ND	0,058789	0,004609	ND	0,001246	ND	ND	ND	ND	0,004146
Novosphingobium	ND	ND	ND	ND	0,002493	ND	ND	ND	ND	ND
Ochrobactrum	ND	ND	0,001844	ND	ND	ND	ND	ND	ND	ND
Paenarthrobacter	ND	0,004355	ND	ND	ND	ND	ND	ND	ND	ND
Pantoea	50,33252	3,462016	23,48273	6,624248	47,70196	4,918988	34,03123	2,265054	32,44935	1,904041
Paucilactobacillus	ND	1,343436	ND	19,14521	ND	8,686126	ND	12,2354	ND	2,304128
Pediococcus	33,60332	47,23583	0,016592	0,256486	0,022435	0,231174	55,59688	48,27809	55,65955	52,92654
Phocaeicola	ND	ND	0,001844	ND	0,00187	ND	0,004258	ND	ND	ND
Phreatobacter	ND	ND	ND	0,004932	ND	ND	ND	ND	ND	ND
Pseudocitrobacter	ND	ND	ND	ND	ND	0,020102	ND	0,016148	ND	ND
Pseudomonas	5,15554	0,017419	61,9649	0,009865	43,17444	ND	2,811923	ND	5,144205	0,003109
Quadrisphaera	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,002073
Rahnella	ND	ND	ND	ND	0,092856	ND	ND	ND	ND	ND
Rhizocola	ND	0,015242	ND	ND	ND	ND	ND	ND	ND	ND
Rhizorhabdus	ND	0,017419	ND	ND	ND	ND	ND	ND	ND	ND
Rosenbergiella	0,221349	0,163303	0,192655	0,540101	0,113422	0,544767	0,370476	0,289187	0,135569	0,203153
Roseomonas	0,001985	ND	ND	ND	ND	ND	ND	ND	ND	ND

Serratia	ND	0,178544	ND	0,108513	ND	0,174888	ND	0,073398	ND	0,161693
Solibacillus	ND	ND	ND	ND	ND	ND	ND	0,002936	ND	ND
Solirubrobacter	ND	ND	ND	ND	ND	ND	ND	0,00734	ND	ND
Sphingomonas	ND	ND	ND	ND	0,005609	ND	0,009936	0,013212	ND	ND
Spirosoma	ND	ND	ND	ND	ND	ND	ND	0,004404	ND	ND
Streptomyces	ND	ND	ND	ND	ND	ND	ND	0,01468	ND	ND
Symbiopectobacterium	ND	ND	ND	0,027128	ND	ND	ND	ND	ND	ND
Tatumella	0,069482	0,328783	0,094023	0,831114	0,04674	0,685482	0,12917	0,364053	ND	0,224919
Vibrio	0,42086	1,180134	1,803031	5,351682	0,798315	6,340208	0,775018	3,81815	0,43085	2,289617
Weissella	2,578762	0,374507	0,295896	1,252836	0,783981	2,546938	0,56352	0,529932	1,697402	0,70378

ND: Non-detected

Table S2: Relative abundance (%) of eukaryotes identified in the liquid fraction of spontaneous (S), bacteria inoculation (B), yeast inoculation (Y), simultaneous (Si), and Sequential (Se) fermentation treatments in 0h and 72h.

Microorganisms	**Treatments**									
	S (0h)	S (72h)	B (0h)	B (72h)	Y (0h)	Y (72h)	Si (0h)	Si (72h)	Se (0h)	Se (72h)
Aaosphaeria	0,006500767	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acremonium	ND	ND	ND	ND	ND	ND	0,002050861	0,004807164	ND	ND
Agaricus	ND	ND	ND	ND	ND	ND	ND	0,005493902	ND	ND
Alternaria	0,40694802	0,101464269	ND	0,036136383	0,012528616	ND	0,007178015	ND	0,03408622	ND
Apiospora	ND	ND	ND	ND	ND	ND	ND	0,002746951	ND	ND
Apiotrichum	0,127415035	ND	0,077045237	0,060050166	0,04214171	ND	0,034864643	ND	0,275380779	ND
Arxiella	0,04940583	ND	ND	ND	ND	ND	ND	0,001373475	ND	ND
Atractiella	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,012266176
Aureobasidium	0,624073641	ND	0,143084011	0,048358983	ND	ND	0,046144381	ND	0,159667031	ND
Bisifusarium	ND	ND	ND	0,117974662	ND	ND	ND	ND	ND	ND
Brunneofusispora	ND	ND	ND	ND	0,014806547	ND	ND	ND	ND	ND

Bullera	ND	0,007957982	0,077045237	ND	ND	ND	ND	ND	ND	ND
Candida	0,257430377	ND	0,194971619	0,097249384	0,055809292	0,004086637	0,024610336	0,010301066	0,391094526	0,03066544
Capnodiales	ND	ND	ND	ND	0,003416895	ND	ND	ND	ND	ND
Cercospora	0,139116416	ND	ND	ND	ND	0,006129955	0,016406891	ND	0,079833516	ND
Chaetocapnodium	ND	ND	ND	0,06323867	ND	ND	0,009228876	0,007554115	ND	ND
Chloridium	0,011701381	ND	ND	ND	ND	ND	ND	ND	ND	ND
Chordomyces	ND	ND	ND	ND	ND	ND	ND	ND	0,057408371	ND
Cladosporium	5,113503393	3,678577113	1,044041573	2,123543916	0,143509607	0,333060891	0,825471698	0,289116581	0,754381873	1,950321987
Clonostachys	0,007800921	ND	ND	ND	ND	ND	ND	ND	ND	ND
Colletotrichum	5,094001092	1,078306541	0,036164091	0,484121248	0,037585849	0,046996322	0,719852338	0,035710361	0,657505247	0,337319841
Coniochaeta	ND	ND	ND	ND	ND	ND	ND	ND	0,201826304	ND
Coprinellus	ND	ND	0,017295869	ND	ND	ND	ND	ND	ND	ND
Cryptococcus	ND	ND	ND	ND	ND	ND	ND	ND	0,052923342	ND
Curvibasidium	ND	ND	0,1336499	ND	ND	ND	ND	ND	ND	ND
Curvularia	0,026003068	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cutaneotrichosporon	ND	ND	ND	0,311942012	ND	ND	0,041017227	ND	ND	ND
Cyphellophora	ND	ND	ND	0,052078905	0,009111721	ND	ND	ND	ND	ND
Cystobasidiomycetes	ND	ND	ND	ND	ND	ND	ND	ND	0,006279041	0,012266176
Cystobasidium	0,083209819	ND	ND	ND	ND	ND	ND	ND	0,335480167	ND
Debaryomyces	0,005200614	ND	ND	0,033479296	ND	ND	ND	ND	ND	ND
Derxomyces	0,027303222	ND	0,12421579	ND	ND	ND	ND	ND	0,11302273	ND
Diaporthe	0,014301688	ND	ND	ND	ND	ND	ND	0,001373475	ND	0,012266176
Didymella	1,552383181	ND	1,193414991	0,035073548	0,397498833	0,008173273	0,236874487	ND	4,133402702	ND
Dioszegia	ND	ND	ND	ND	ND	ND	0,006152584	ND	ND	ND
Dothiora	0,027303222	ND	ND	ND	ND	ND	ND	ND	ND	ND
Erythrobasidium	0,30033544	ND	0,158807528	ND	0,037585849	ND	0,007178015	0,004120426	0,154284997	ND
Exophiala	ND	ND	ND	ND	ND	ND	0,009228876	ND	0,034983226	ND
Exutisphaerella	0,089710586	0,262613401	ND	0,034542131	ND	ND	ND	ND	ND	ND
Filobasidium	0,516160907	ND	0,779886476	ND	0,00227793	ND	ND	ND	0,38391848	ND

Fusarium	13,34737499	4,625576954	1,138382679	6,075695094	0,104784793	0,25950143	1,899097621	0,587160752	1,736603218	1,435142594
Fusicolla	0,085810126	ND	ND	0,055267409	ND	ND	ND	ND	ND	ND
Fusidium	ND	ND	ND	ND	ND	ND	ND	0,002060213	ND	ND
Galactomyces	0,696882232	0,483447398	7,858614129	0,954957061	ND	ND	0,170221493	ND	ND	ND
Ganoderma	ND	0,06565335	ND	ND	ND	ND	ND	ND	ND	ND
Gibellulopsis	ND	ND	ND	ND	ND	ND	ND	ND	0,019734127	ND
Hannaella	0,083209819	ND	0,281450966	0,046764731	ND	ND	ND	0,001373475	ND	0,067463968
Hanseniaspora	0,370543724	0,968884291	0,028302332	1,306755378	0,014806547	0,048017981	0,033839212	0,042577739	0,667372311	0,674639681
Humicola	ND	ND	ND	0,003188504	ND	ND	ND	ND	ND	ND
Hyphopichia	ND	ND	ND	0,031353626	0,012528616	0,007151614	ND	ND	0,0618934	ND
Kazachstania	0,024702915	ND	ND	0,049953235	ND	0,02451982	0,094339623	0,098203494	0,048438313	0,926096289
Kockovaella	ND	ND	ND	ND	0,010250686	ND	ND	ND	ND	ND
Kodamaea	0,128715188	ND	ND	0,081306862	ND	0,007151614	0,032813782	ND	0,082524533	0,036798528
Kurtzmaniella	28,3966508	33,08530957	4,805106999	20,90861746	0,808665247	1,544748672	2,023174733	1,848011208	38,51832583	8,157007053
Kwoniella	0,217125621	ND	0,047170553	ND	ND	ND	0,019483183	ND	ND	ND
Langdonia	0,007800921	ND	ND	ND	ND	ND	ND	ND	0,013455087	ND
Leptobacillium	ND	ND	ND	ND	ND	ND	ND	0,006180639	ND	ND
Loculosulcatispora	ND	ND	ND	ND	0,053531361	ND	ND	ND	ND	ND
Magnaporthales	0,002600307	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malassezia	ND	ND	ND	0,021788113	ND	ND	ND	ND	ND	ND
Marquandomyces	ND	ND	ND	ND	ND	ND	ND	0,001373475	ND	ND
Meira	0,04550537	ND	0,004717055	0,036136383	ND	ND	ND	ND	0,087906568	ND
Metschnikowia	0,413448787	ND	0,029874684	ND	0,207291655	0,01225991	0,147662018	0,014421492	0,13006584	ND
Meyerozyma	6,502067244	1,878083718	5,121149704	0,959739818	1,470403991	0,068451165	0,426579163	0,095456543	16,74620118	0,282122048
Microdochium	ND	ND	ND	ND	0,003416895	ND	ND	ND	ND	ND
Microstromatales	0,024702915	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mortierella	0,023402762	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mucor	0,067607978	0,069632341	0,072328181	0,133385767	ND	0,004086637	ND	ND	ND	ND
Myrmecridium	ND	ND	ND	ND	ND	ND	ND	0,002060213	ND	ND

Naganishia	2,388381829	ND	11,82094058	ND	ND	0,002043318	0,035890074	ND	3,971941659	ND
Nakaseomyces	0,239228229	20,11578864	3,042500668	0,279525551	94,67989385	94,30833674	85,75369155	94,09268212	0,132756858	70,81876725
UNIDENTIFIED	3,147671425	4,440553876	4,21861979	16,34852479	0,113896514	0,199223539	0,990566038	0,22593671	0,745411815	1,882858019
Neocucurbitaria	0,032503835	ND	ND	ND	ND	ND	ND	ND	ND	ND
Neopyrenochaeta	ND	ND	ND	0,238074994	0,12642513	ND	0,010254307	ND	ND	ND
Neosetophoma	ND	ND	ND	0,001062835	ND	ND	ND	ND	ND	ND
Papiliotrema	4,984788205	0,053716378	8,542587148	0,119037497	0,375858495	0,017368206	0,439909762	0,031589935	3,978220699	0,036798528
Paramonodictys	0,023402762	ND	ND	ND	ND	ND	ND	ND	ND	ND
Penicillium	0,785292665	ND	0,336483278	0,134448601	0,036446884	ND	0,124077112	ND	0,025116162	ND
Phaeoacremonium	ND	ND	ND	ND	ND	ND	ND	ND	0,031395203	ND
Phaeosphaeria	ND	ND	ND	ND	ND	0,004086637	ND	ND	ND	ND
Phaeosphaeriaceae	0,04940583	ND	ND	ND	ND	ND	ND	0,002746951	ND	ND
Phallus	ND	ND	ND	0,021256696	ND	ND	ND	ND	0,017940116	ND
Pichia	4,676651845	14,05180646	1,921413859	17,63136638	0,159455119	1,205557826	0,64704676	0,887951873	7,950162358	8,807114382
Plectosphaerella	ND	ND	ND	ND	ND	ND	ND	0,002746951	ND	ND
Pseudallescheria	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,018399264
Pseudocoleophoma	0,00390046	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pseudopithomyces	0,00390046	ND	ND	0,002657087	ND	ND	ND	ND	ND	ND
Pseudothielavia	ND	ND	ND	ND	0,012528616	0,021454843	0,010254307	ND	ND	ND
Pyrenochaetopsis	ND	0,115390737	ND	0,004782757	ND	ND	ND	0,012361279	ND	ND
Rhinocladiella	0,026003068	ND	ND	ND	ND	ND	ND	ND	ND	ND
Rhizopycnis	ND	ND	ND	ND	0,010250686	ND	ND	ND	ND	ND
Rhodosporidiobolus	0,382245105	0,007957982	5,918332049	0,044107644	ND	ND	0,01538146	0,004807164	0,099567643	ND
Rhodotorula	3,617026809	0,107432755	14,28953285	0,047296148	0,249433365	0,011238251	0,309680066	ND	2,951149064	ND
Rhytidhysteron	ND	ND	ND	ND	0,004555861	ND	ND	ND	ND	ND
Saccharomyces	1,162337156	3,362247334	0,915108728	3,153962248	ND	0,354515734	ND	0,311778925	2,319656985	1,999386691
Saccharomycopsis	ND	ND	ND	0,011691183	ND	ND	ND	ND	ND	ND
Saitozyma	ND	ND	ND	0,001594252	ND	ND	ND	ND	ND	ND
Sarocladium	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,085863232

Septobasidium	0,179421172	ND	0,044025849	0,043576227	ND	ND	ND	ND	ND	ND
Sesquicillium	ND	ND	ND	ND	ND	ND	ND	0,026782771	0,005382035	ND
Setophoma	ND	ND	ND	ND	ND	ND	0,017432322	ND	ND	ND
Sordariomycetes	ND	ND	ND	ND	0,009111721	ND	ND	ND	ND	ND
Sporobolomyces	0,185921939	ND	14,72192959	0,020193861	0,018223442	ND	0,020508614	ND	0,156976014	ND
Starmerella	12,69079751	10,62589527	9,706127455	27,52476405	0,735771478	1,498774009	4,7292863	1,293813867	9,543244649	2,287641828
Symmetrospora	ND	ND	ND	ND	ND	ND	ND	ND	0,059202382	ND
Torula	ND	ND	ND	ND	ND	ND	ND	0,004807164	ND	ND
Tricellula	ND	ND	0,316042705	ND	ND	ND	ND	ND	ND	ND
Trichoderma	ND	ND	ND	ND	ND	ND	ND	ND	0,028704185	ND
Vishniacozyma	0,26263099	0,610775107	0,470133178	0,170584984	0,010250686	0,003064978	0,039991797	0,029529722	1,341920669	0,067463968
Wallemia	ND	ND	0,022012925	0,004782757	ND	ND	0,008203445	ND	0,062790406	ND
Wickerhamiella	0,146917336	ND	0,146228714	ND	ND	ND	0,006152584	ND	0,23680953	0,024532352
Wickerhamomyces	0,093611046	0,202928537	0,176103398	0,034010713	0,012528616	ND	0,008203445	0,007554115	0,313055022	0,036798528
Xenoramularia	ND	ND	ND	ND	0,003416895	ND	ND	0,001373475	ND	ND
Xeromyces	ND	ND	0,025157628	ND	ND	ND	ND	ND	ND	ND
Zalaria	ND	ND	ND	ND	ND	ND	ND	ND	0,090597585	ND
Zygosporium	ND	ND	ND	ND	ND	ND	ND	0,002060213	ND	ND

ND: Non-detected

Table S3: Compounds identified (Area*10^{-5}) in the coffee bean of spontaneous (S), bacteria inoculation (B), yeast inoculation (Y), simultaneous (Si), and Sequential (Se) fermentation treatments before (1) and after (2) drying.

Compounds	**Treatments**									
	S (1)	S (2)	B (1)	B (2)	Y (1)	Y (2)	Si (1)	Si (2)	Se (1)	Se (2)
Acid										
2-Butenoic acid, 3-methyl	ND	ND	ND	ND	ND	ND	ND	0,315	ND	ND
Butanoic acid, 2-methyl	ND	ND	ND	0,225	ND	0,196	ND	0,638	ND	ND

Butanoic acid, 3-methyl	0,476	0,350	0,491	0,723	0,161	0,567	0,370	1,864	0,761	0,902
Dodecanoic acid	ND	ND	ND	0,220	ND	ND	ND	ND	0,208	ND
Hexanoic acid	ND	ND	ND	ND	ND	ND	ND	0,170	ND	0,255
n-Decanoic acid	ND	0,144	ND	ND	ND	ND	ND	ND	ND	ND
Alcohol										
1,2-Benzenedicarboxylic acid	0,120	0,336	ND	0,589	0,311	0,455	0,331	0,251	0,348	0,212
1-Butanol, 2-methyl	ND	ND	ND	ND	ND	0,080	0,108	ND	ND	ND
1-Butanol, 3-methyl	ND	0,136	ND	0,431	0,316	0,144	0,352	0,266	0,085	0,317
1-Dodecanol	ND	ND	ND	ND	0,174	ND	ND	0,180	ND	0,328
1-Hexanol	ND	0,695	ND	0,617	ND	0,612	ND	0,749	ND	0,528
1-Hexanol, 2-ethyl	ND	0,126	ND	ND	ND	ND	ND	ND	ND	ND
1-Nonanol	0,230	ND	0,207	ND	ND	ND	ND	ND	ND	ND
1-Nonanol, 4,8-dimethyl	ND	ND	ND	ND	0,202	ND	0,125	ND	ND	ND
1-Octen-3-ol	0,371	ND	0,602	ND	0,273	ND	0,438	ND	0,453	ND
1-Tetradecanol	0,128	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Heptanol	ND	ND	0,734	ND	ND	ND	0,565	ND	ND	ND
2-Hexanol, 5-methyl	0,501	ND	ND	ND	0,416	ND	ND	ND	0,588	ND
3-Ethyl-4-methylpentan-1-ol	ND	ND	0,308	ND	ND	ND	ND	ND	0,234	ND
Benzyl alcohol	ND	ND	0,213	ND	ND	ND	ND	ND	0,135	ND
n-Pentadecanol	ND	ND	ND	0,099	ND	ND	ND	ND	ND	ND
Phenylethyl Alcohol	0,353	0,145	0,727	0,959	0,672	0,404	0,975	2,102	0,431	1,051
Aldehyde										
13-Methyltetradecanal	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,121
2-Butenal, 2-methyl	ND	ND	ND	ND	ND	ND	ND	ND	0,148	ND
2-Butenal, 3-methyl	0,236	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Hexenal	ND	ND	0,136	ND	ND	ND	ND	ND	ND	ND
2-Nonenal	ND	ND	0,171	ND	ND	ND	ND	ND	ND	ND
2-Octenal	1,027	ND	ND	ND	0,844	ND	0,620	ND	0,661	ND
Benzaldehyde	10,436	ND	16,248	ND	5,618	ND	16,556	0,291	15,542	0,234
Benzeneacetaldehyde	21,334	0,405	18,369	0,460	16,505	0,332	26,587	1,602	22,936	1,730
Butanal, 2-methyl	2,819	ND	ND	ND	1,541	ND	3,387	0,081	3,651	0,125
Butanal, 3-methyl	8,512	ND	14,356	ND	4,359	ND	13,490	0,210	12,200	0,321

Decanal	0,285	0,196	0,263	0,224	0,286	0,166	0,218	0,266	0,211	0,794
Dodecanal	ND	ND	ND	0,080	ND	ND	ND	ND	ND	ND
Heptanal	ND	0,163	ND	ND	ND	ND	ND	ND	ND	0,268
Hexanal	1,172	0,608	1,860	0,527	0,873	0,446	0,880	0,766	0,955	1,357
Methional	ND	ND	ND	ND	ND	ND	0,517	ND	0,195	ND
Nonanal	0,686	0,874	0,718	0,917	0,668	0,600	0,389	0,675	0,482	2,069
Octanal	0,182	ND	ND	0,175	ND	ND	ND	ND	ND	0,374
Pentadecanal	1,351	0,138	1,308	ND	1,143	0,087	1,113	0,146	1,590	0,386
Pentanal	0,197	ND	0,179	ND	ND	ND	0,162	ND	ND	0,203
Tetradecanal	0,199	ND	0,165	ND	0,142	ND	0,166	ND	0,150	ND
Caffeine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Caffeine	2,311	0,280	21,541	0,114	2,055	0,748	5,163	1,140	3,619	0,558
Ester										
Benzeneacetic acid, ethyl ester	1,026	ND	0,424	ND	4,662	ND	5,636	ND	0,919	ND
Benzoic acid, ethyl ester	ND	ND	ND	ND	0,173	ND	0,224	ND	ND	ND
Butanoic acid, 3-methyl-, ethyl ester	1,002	ND	0,496	ND	1,415	ND	1,673	ND	0,559	ND
Butyric acid, 3-pentadecyl ester	ND	ND	0,255	ND	ND	ND	ND	ND	ND	ND
Hexadecanoic acid, ethyl ester	ND	ND	ND	ND	ND	ND	0,267	ND	ND	ND
Hexadecanoic acid, methyl ester	ND	ND	ND	0,221	ND	7,425	ND	0,324	0,217	0,421
Hexanoic acid, ethyl ester	ND	ND	ND	ND	0,405	ND	0,293	ND	ND	ND
Methyl salicylate	0,497	ND	ND	ND	ND	ND	1,012	ND	0,493	ND
Methyl salicylate Benzoic acid, 2-hydroxy-, methyl ester	ND	ND	ND	ND	0,167	ND	ND	ND	ND	ND
Pentadecanoic acid, ethyl ester	ND	ND	ND	ND	0,211	ND	ND	ND	ND	ND
Furan										
Furan, 2-pentyl	0,836	0,450	ND	0,432	0,553	0,310	0,643	0,613	0,598	0,875
Hydrocarbon										
10-Methylnonadecane	ND	ND	ND	ND	ND	ND	ND	0,465	ND	ND
1-Nonadecene	ND	ND	ND	ND	ND	0,154	ND	ND	ND	ND
1-Pentadecene	ND	0,228	ND	ND	ND	ND	ND	ND	ND	0,400
2,6,10-Trimethyltridecane	ND	ND	ND	ND	ND	ND	0,335	ND	0,429	0,194

2,6,10-Trimethyltridecane										
Heneicosane	ND	ND	ND	ND	0,514	ND	ND	ND	ND	ND
9-methylheptadecane	ND	0,312	ND	0,233	ND	0,183	ND	0,241	0,432	ND
Benzene, 1,2,3-trimethyl	ND	ND	ND	ND	0,201	ND	ND	ND	ND	ND
Benzene, 1,2,4-trimethyl	0,301	ND	0,255	ND	ND	ND	ND	ND	ND	ND
Decane	ND	0,159	ND	ND	ND	ND	ND	ND	ND	ND
Decane, 3,3,8-trimethyl	ND	0,358	ND	ND	ND	ND	ND	ND	ND	ND
Dodecane	ND	1,748	ND	ND	ND	0,577	0,148	0,994	0,137	3,045
Dodecane, 2,6,10-trimethyl	0,196	ND	ND	ND	0,197	ND	ND	ND	ND	ND
Eicosane	0,190	ND	ND	ND	ND	ND	ND	ND	0,198	ND
Ethylbenzene	ND	ND	ND	ND	0,413	ND	ND	ND	ND	ND
Heneicosane	0,664	0,592	0,779	0,373	0,300	0,115	0,465	0,236	0,179	0,349
Heptadecane	0,154	ND	ND	ND	0,147	ND	0,346	ND	1,544	0,245
Heptadecane, 7-methyl	ND	0,385	ND	ND	ND	ND	ND	ND	ND	ND
Hexacosane	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,210
Hexadecane	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,181
Nonadecane	ND	0,219	ND	ND	ND	ND	ND	ND	ND	ND
Nonadecane, 9-methyl	ND	ND	ND	ND	ND	0,185	ND	ND	ND	0,665
Nonane, 5-butyl	ND	0,142	ND	ND	ND	0,081	ND	ND	ND	ND
Pentadecane	0,273	ND	ND	ND	0,438	ND	ND	ND	0,320	1,539
Styrene	ND	ND	ND	ND	7,375	ND	ND	ND	ND	ND
Tetracosane	ND	ND	ND	ND	ND	0,125	ND	ND	ND	ND
Tetradecane	1,747	0,738	1,533	1,340	1,680	0,604	1,575	0,564	ND	ND
Tridecane, 1-iodo	ND	ND	ND	ND	0,321	ND	ND	ND	ND	ND
Undecane	ND	0,202	ND	0,384	ND	0,115	ND	ND	ND	0,830
Ketone										
2(3H)-Furanone	ND	ND	ND	ND	ND	ND	ND	0,212	ND	ND
2-Pentadecanone	0,180	0,292	0,191	0,306	ND	ND	ND	0,300	0,229	0,478
2-Pentadecanone, 6,10,14-trimethyl	ND	ND	ND	ND	0,157	0,244	ND	ND	ND	ND
Butyrolactone	ND	ND	ND	ND	ND	ND	ND	0,273	ND	ND
Ethanone	ND	ND	ND	ND	ND	ND	ND	ND	ND	0,254

Pyrazine										
Pyrazine, 2-methoxy-3	1,135	0,709	1,395	0,654	1,137	0,728	1,211	0,907	1,171	0,845
Pyridine										
Pyridine, 2,3-dimethyl	ND	ND	ND	0,368	ND	ND	ND	0,606	ND	ND
Terpene										
D-Limonene	1,336	1,506	ND	3,601	1,185	ND	1,386	1,494	1,748	2,489
Linalool	2,741	ND	ND	ND	3,184	ND	ND	ND	3,175	ND
Linalool 1,6-Octadien-3-ol, 3,7-dimethyl	ND	ND	ND	ND	ND	ND	2,174	ND	ND	ND
Others										
2-Methoxy-4-vinylphenol	ND	ND	ND	ND	ND	ND	ND	0,506	ND	ND
Dibutyl phthalate	ND	ND	ND	0,279	ND	ND	ND	ND	ND	ND
Diethyl Phthalate	ND	ND	0,154	0,185	ND	1,266	ND	ND	ND	ND
Toluene	ND	ND	ND	ND	ND	ND	ND	0,170	ND	ND

ND: Non-detected