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Starter Culture Modulates Microbial Diversity During Wine–Coffee Fermentation: A DGGE-Based Molecular Study

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Abstract

Wine-flavored coffee is a unique post-harvest product characterized by fruity, acidic, and winey notes. It is highly favored by Indonesian consumers and has strong potential in the global market. However, maintaining consistency in flavor and quality remains a challenge due to variability in natural fermentation. This study aimed to evaluate the impact of yeast and bacterial starter culture inoculation on microbial community dynamics during wine–coffee fermentation using a molecular approach based on PCR-DGGE. The analysis results revealed that natural fermentation without inoculation involved diverse populations of bacteria, yeasts, and filamentous fungi, with eukaryotic microbes dominating from the early stages. Sequencing identified prevalent yeast genera, including *Pichia*, *Torulaspora*, *Hanseniaspora*, *Saccharomyces*, and *Candida*, as well as *Aspergillus* among filamentous fungi. Bacterial communities were dominated by lactic acid bacteria and members of *Lactobacillus*, *Klebsiella*, *Enterobacter*, *Pantoea*, and *Bacillus*. In contrast, controlled fermentation with inoculated starter cultures (*Pichia kudriavsevii* and *Klebsiella* sp.) showed a more stable microbial profile throughout the process. Shannon–Wiener diversity indices demonstrated a significant difference ($p = 0.05$) between natural and inoculated fermentations, with species dominance observed in the latter. Cluster analysis confirmed that starter culture inoculation had significant impacts on microbial succession and community structure. These findings highlight the importance of controlled fermentation using selected microbial starters to ensure consistent microbial ecology, which in turn contributes to reproducible quality in wine-flavored coffee. The molecular profiling approach provides valuable insights for improving fermentation practices and developing reliable starter culture formulations tailored to enhance flavor consistency and product quality.

Keywords: controlled fermentation, microbial diversity, PCR-DGGE, starter culture, wine-flavored coffee

Introduction

Coffee is one of the most popular and valuable non-alcoholic beverages consumed worldwide. The coffee industry sustains the livelihoods of approximately 25 million farmers and generates over USD 200 billion in annual global revenue. For many low-income producing countries, coffee exports are a vital source of foreign exchange and national income, underpinning economic stability and access to global markets (FAO, 2025). Indonesia holds a strategic position in the global coffee sector, ranking as the fourth-largest coffee

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producer after Brazil, Vietnam, and Colombia, and contributing approximately 6% of global coffee production. Indonesia's coffee exports for the 2024/25 coffee year reached 6.06 million bags, marking a 53.0% increase over the previous year (ICO, 2025). This growth was largely driven by better-than-expected harvests and favorable production conditions. In addition to volume, Indonesia is gaining international recognition for its value-added innovations, particularly in the specialty and fermented coffee segments. One type of fermented coffee that has gained popularity among farmers and consumers is grape-flavored coffee, commonly known as wine coffee, which undergoes a longer fermentation process compared to conventional coffee. This post-harvest processing enhances its flavor profile, resulting in fruity, sour, and wine-like notes that resemble the characteristics of wine (Juanda et al., 2022; Muzaifa et al., 2023).

Post-harvest processing plays a crucial role in determining the final brew quality and aroma of coffee. Fermentation, in particular, helps remove the mucilage layer adhering to the parchment skin after depulping. This natural fermentation improves aroma through microbial metabolite production, which serves as precursors for volatile compounds formed during subsequent processing stages (Pereira et al., 2017). Moreover, fermentation reduces bitterness and enhances the formation of unique flavor notes in the final brew (Cao et al., 2022; Ribeiro et al., 2017).

During the fermentation process, various microbial groups, including lactic acid bacteria (LAB), yeasts, and acetic acid bacteria, play crucial roles in the breakdown of mucilage and the development of flavor precursors (De Bruyn et al., 2017; Neu et al., 2016). Yeast species commonly involved in coffee fermentation include *Pichia kluyveri*, *Pichia anomala*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, and *Torulaspora delbrueckii* (Pereira et al., 2014; De Bruyn et al., 2017). In addition, bacterial species exhibiting pectinolytic activity, such as those belonging to the genera *Erwinia*, *Klebsiella*, *Aerobacter*, *Escherichia*, and *Bacillus*, have also been successfully isolated (Silva et al., 2008; Avallone et al., 2002; Elhalis et al., 2022). Filamentous fungi belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium* are usually present in smaller abundances and are more commonly detected during drying and storage stages (Silva et al., 2008; Vilela et al., 2010; Pereira et al., 2014). These fermentative microorganisms utilize fruit flesh (endocarp and mesocarp) as sources of carbon and nitrogen and produce ethanol, lactic acid, acetic acid, and other metabolites during fermentation (Avallone et al., 2001; Pereira et al., 2014). These microorganisms are naturally present in coffee cherries, fermentation tanks, and the surrounding environment. Consequently, spontaneous fermentation often leads to inconsistent and unpredictable outcomes in terms of microbial composition and sensory quality (Pereira et al., 2021).

To address this challenge, the use of starter cultures has been widely applied in fermented food production to achieve more controlled and consistent microbial activity during fermentation (Hansen, 2002; Manna et al., 2021; Laranjo et al., 2017). Inoculated fermentation enables producers to exert greater control over microbial succession and impact associated biochemical transformations, thereby improving the safety, consistency, and overall quality of the final product (Masoud et al., 2005).

Several studies on coffee fermentation have investigated microbial

diversity and dynamics using culture-dependent and culture-independent methods (Silva et al., 2008; Lee et al., 2023; Vale et al., 2024). However, most of these studies focus on spontaneous fermentation processes, with limited exploration of microbial community dynamics under inoculated wine-coffee fermentation conditions. Moreover, while sensory attributes and metabolite profiling have been widely examined, less attention has been paid to how starter culture inoculation impacts the structure and succession of microbial communities.

To better understand these dynamics, molecular approaches are required to capture a broader and more accurate view of microbial populations, particularly those that are difficult or impossible to culture. Denaturing Gradient Gel Electrophoresis (DGGE), a PCR-based fingerprinting technique, has been widely used in microbial ecology due to its ability to detect changes in dominant microbial species over time without the need for cultivation (Ercolini, 2004). Therefore, this study aimed to investigate the impact of starter culture inoculation on microbial community dynamics during wine-coffee fermentation using PCR-DGGE, a culture-independent method that enables profiling of dominant microbial populations throughout the fermentation process. The findings are expected to provide deeper insights into microbial succession patterns in inoculated fermentation systems and contribute to the development of more controlled and reproducible coffee fermentation practices.

Research Methods

Sampling

Coffee cherries were manually harvested at the fully ripe stage (red maturity) from local plantations in the Sumedang and Puntang regions, West Java, Indonesia. The fermentation process was carried out using the wine-flavored coffee method (Taufik et al., 2024). The harvested samples were aseptically placed in sterile plastic containers and transported to the laboratory in an icebox to maintain microbiological integrity.

Preparation of Inoculum

Bacterial (NP) and yeast (PB) isolates were used as starter cultures in controlled coffee processing. These isolates were obtained from dominant microorganisms involved in the natural processing of wine coffee, previously isolated using a culture-dependent approach by Taufik et al. (2024). The bacterial and yeast isolates, identified as *Klebsiella* sp. and *Pichia kudriavsevii*, respectively, were stored at −80°C and reactivated in Luria–Bertani (LB) medium and yeast extract peptone dextrose (YPED) medium. The cultures were incubated at room temperature for 24 hours, then transferred to fresh medium and incubated at room temperature at 150 rpm for an additional 24 hours. Subsequently, yeast and bacterial cultures were transferred to larger-volume media and re-incubated for 6 and 4 hours, respectively, until optimum cell concentrations were achieved (approximately 10^6 CFU/mL).

Inoculation and Post-harvest Processing of Wine-flavored Coffee

Three different fermentation treatments were applied: (1) natural fermentation without inoculation, (2) optimized fermentation with starter culture inoculation, and (3) optimized fermentation without starter culture inoculation (control). All fermentations were conducted simultaneously and repeated in triplicate. In treatment (1), coffee cherries were fermented for three days and sun-dried for 15 days until the moisture content reached 12%. Samples (10 g) were aseptically collected in sterile

plastic bags at the following time points: before fermentation (0 h), during fermentation (24, 48, and 72 h), during drying (192, 312, and 432 h), and after hulling.

For treatment (2), coffee cherries were inoculated with yeast and bacterial starter cultures at a ratio of 10:1 (v/v), with a total inoculum concentration of 10% (v/w). Treatments (2) and (3) were fermented for 24 hours and sun-dried for 15 days until the moisture content reached 12%. The selection of inoculum concentration and fermentation duration was based on Istiadi (2018). Samples (10 g) were aseptically collected at the following stages: before fermentation (0 h), after fermentation (24 h), and during drying (144 and 264 h). All samples were stored at −20°C until further laboratory analysis.

Moisture Content and pH Measurement

Moisture content of the coffee fruit samples was determined using the gravimetric method. Aluminum foil was shaped into a cup and weighed using an analytical balance. Three coffee fruits were placed into the aluminum foil dish and weighed. The samples were then oven-dried at 80°C and weighed at 24, 48, and 72-hour intervals until a constant weight was achieved (Madigan and Martinko, 2014). pH measurements were conducted in duplicate using a digital pH meter. One gram of coffee fruit sample (liquid fraction) was homogenized in 9 mL of deionized water. The pH of the resulting suspension was measured according to the method described by Madigan and Martinko (2014).

Microbial Community Analysis Using PCR-DGGE

1. *Total DNA Extraction*

Total DNA was extracted following the method described by Evangelista et al. (2014) with minor modifications. Ten grams of coffee fruit samples were mixed with ddH_2O for 10 minutes and centrifuged at 100 × g for 10 minutes at 4°C. The resulting pellet was used for DNA extraction using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the "DNA Purification from Tissue" protocol. DNA concentration and purity were determined using a UV/Vis microplate spectrophotometer (Thermo Scientific™ Multiskan™ Go, Finland) by measuring absorbance at 260 and 280 nm. DNA quality was further assessed by electrophoresis on a 1% agarose gel prepared in 1× TAE buffer (40 mM Tris-HCl, pH 7.4; 20 mM sodium acetate; 1.0 mM Na_2EDTA). Electrophoresis was performed at 70 V for 30 minutes, and the gel was visualized using a blue-light transilluminator. Extracted DNA was stored at −20°C until further analysis.

2. *PCR Amplification of Target DNA*

For bacterial community analysis, extracted DNA was amplified using primers 338fGC (5′-CGC CCG CCG CGC GCG GGC GGG GGG GCA CGG GGG GAC TTC TAC GGG AGG CAG CAG-3′; GC clamp underlined) and 518r (5′-ATT ACC GCT GGC TGC TG-3′), targeting the V3 region of the 16S rRNA gene (Leesing, 2005; Øvreås et al., 1997). PCR reactions were performed in a final volume of 25 µL, consisting of 12.5 µL GoTaq Green Master Mix 2× (Promega, Madison, WI, USA), 2 µL of each primer (10 µM), 2.5 µL of template DNA, and 6 µL of nuclease-free water. Amplification followed the protocol of Ramos et al. (2010) with the following conditions: initial denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 92°C for 1 minute, annealing at 52°C for 45 seconds, and extension at 72°C for 1 minute; and a final extension at 72°C for 10 minutes. For fungal community analysis, fragments of the ITS1 region were amplified using primers ITS1fGC (5′-CGC CCG CCG CGC GCG GCG GGC GGG

GCG GGT CCG TAG GTG AAC CTG CGG-3′; GC clamp underlined) and ITS2r (5′-GCT TTC TTC TTC ATC GAT GC-3′) (Lv et al., 2017). PCR reactions (25 µL) contained 12.5 µL GoTaq Green Master Mix 2×, 1 µL of each primer (10 µM), 2.5 µL of template DNA, and 9.5 µL of nuclease-free water. Thermal cycling conditions followed Lv et al. (2017). Amplified products (5 µL) were verified by electrophoresis on a 1% (w/v) agarose gel in 1× TAE buffer stained with Diamond™ Nucleic Acid Dye (Promega). Electrophoresis was performed at 100 V for 30 minutes.

3. DGGE Analysis

A total of 15 µL of amplified PCR products were analyzed by DGGE using the Bio-Rad DCode system (Bio-Rad Universal Code Mutation Detection System, Richmond, CA, USA), following the procedure described by Muyzer et al. (1993) and modified by Leesing et al. (2005). PCR products were separated on polyacrylamide gels [8% (w/v) acrylamide:bisacrylamide (37.5:1)] in 1× TAE buffer. Electrophoresis was performed using denaturing gradients ranging from 15–55% for bacterial PCR products (where 100% denaturant corresponds to 7 M urea and 40% [v/v] formamide) and 12–60% for fungal PCR products. Electrophoresis was conducted at 130 V for 5 hours and 85 V for 15 hours for bacterial and yeast samples, respectively, at 60°C. DGGE gels were stained using the silver staining method (Bassam and Peter, 2007) and subsequently photographed using a light box.

4. DGGE Band Sequencing and Identification

Visualized bands were excised from the DGGE gel using a sterile scalpel, eluted in 50 µL of nuclease-free water, and incubated at 40°C overnight for re-amplification. An aliquot of 3 µL of the eluate was used as a template for PCR re-amplification. PCR products were purified and sent for sequencing at Macrogen Inc. (Seoul, Korea). The obtained DNA sequences were edited using MEGA version 7.0 software and compared with sequences in the GenBank database using the BLASTN algorithm (Altschul et al., 2007). Sequences showing high similarity were imported into MEGA 7.0 and aligned with 16S rRNA gene fragments (for prokaryotic sequences) and ITS rRNA gene fragments (for eukaryotic sequences) using the MUSCLE algorithm. After manual trimming of the alignments, phylogenetic tree construction was performed using the Maximum Likelihood method (Tamura, 1992). Bootstrap analysis was conducted with 100 replications.

Statistical Analysis of DGGE Banding Patterns

1. Cluster Analysis

Similarity among DGGE band migration patterns was analyzed based on percent similarity. Profile similarities were visualized graphically using dendrograms. Clustering was performed using the unweighted pair group method with arithmetic mean (UPGMA). Cluster and dendrogram analyses were conducted using the Multivariate Statistical Package (MVSP) software version 3.22 (Fromin et al., 2002; Silva & Russo, 2000).

2. Shannon–Wiener Diversity Index

The Shannon–Wiener diversity index (H′) was used to evaluate the diversity of microbial taxa involved in the post-harvest processing of wine coffee. This index is commonly applied in ecology and microbiology to describe species richness and evenness (Krebs, 1985). Normality of the Shannon–Wiener diversity indices was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's

test. Since all assumptions for parametric testing were met ($P > 0.05$), differences between treatments were analyzed using an independent t-test. All statistical analyses were performed using SPSS version 23.0 (IBM Corp., Chicago, IL, USA).

3. Dominance Indices

Dominance indices were used to determine the extent to which one microbial group dominated another. High dominance values (C) indicate labile and less stable communities. As dominance indices approach one, the likelihood that a particular species dominates the community increases (Odum, 1971).

Research Results and Discussion

One of the major challenges in microbial ecology is the assessment of microbial diversity and microbial community structures present in specific environments. Changes in diversity and microbiota structure at the beginning of and throughout the fermentation process are particularly important. Recent advances in molecular biology techniques have made significant contributions to characterizing and understanding these changes. Such progress can be applied to studying the dynamics and population structure of biota in biotechnological processes, including industrial-scale fermentation (Escobar-Zepeda et al., 2016; Tamang et al., 2016; Ferrocino and Cocolin, 2017). Using the approach applied in this study, it is possible to characterize microbial dynamics during coffee processing and evaluate the impact of the main treatment on microbial community structure.

Observation of microbial diversity profiles in this study was conducted across three different fermentation variations: (1) natural fermentation, (2) controlled fermentation with inoculation of starter cultures, and (3) controlled fermentation without inoculation (control). The inoculum was added at the beginning of fermentation to evaluate the impact of starter cultures on the dynamics of prokaryotic and eukaryotic microbiota during wine coffee processing. The isolates used were PB and NP, representing yeast and bacterial isolates, respectively. NP was identified as *Klebsiella*, while PB was identified as *P. kudriavsevii* (Taufik et al., 2024).

This research focused on evaluating microbial community dynamics occurring during three variations of wine coffee processing using a molecular approach. Microbial diversity was assessed by combining PCR amplification with sequence differentiation analysis using DGGE. This method enables the separation of DNA fragments of identical length but differing sequences (Daniel et al., 2009). DGGE allows the analysis of microbiota using culture-dependent and culture-independent approaches. Thus, one major advantage of DGGE in microbial ecology studies is its ability to provide a more comprehensive visualization of the entire microbial community.

A. PCR Amplification Efficiency

In this study, the fungal primer set ITS1fGC/ITS2r and the bacterial primer set 338fGC/518r were used, each targeting hypervariable regions. Amplification efficiency was assessed by visualizing the presence of target DNA fragments through electrophoresis. Both primer sets successfully amplified target DNA sequences obtained from natural fermentation samples and controlled fermentation samples with inoculum addition (see Fig. 1 and Fig. 2), producing bands of approximately 250 bp. Yeast and bacterial inocula (PB and NP isolates) were also successfully amplified.

Figure 1

Electrophoregram of amplified ITS1 region of ITS (a) and V3 region of 16S rRNA (b) from natural fermentation samples. DNA bands were observed at approximately 250 bp. L = 1 kb ladder (Promega); BS = fresh coffee fruit; F1 = 24 h; F2 = 48 h; F3 = 72 h; P5 = 192 h; P10 = 312 h; P15 = 432 h; RH = after resting and hulling; K+ = positive control; K– = negative control.

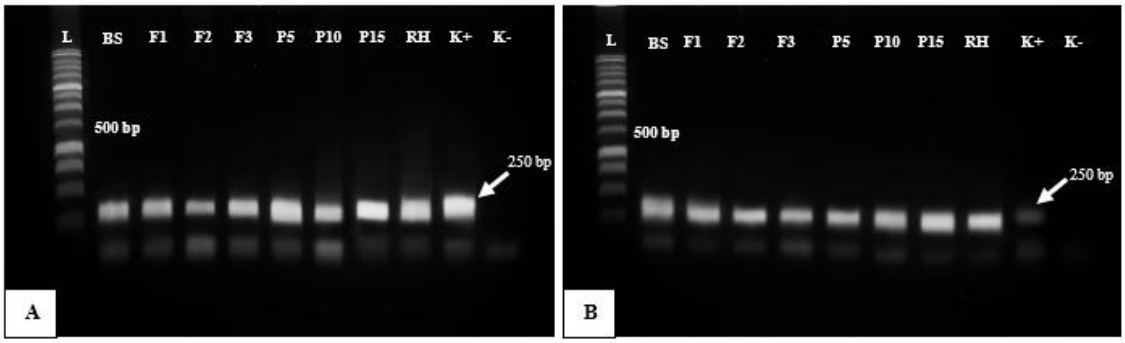
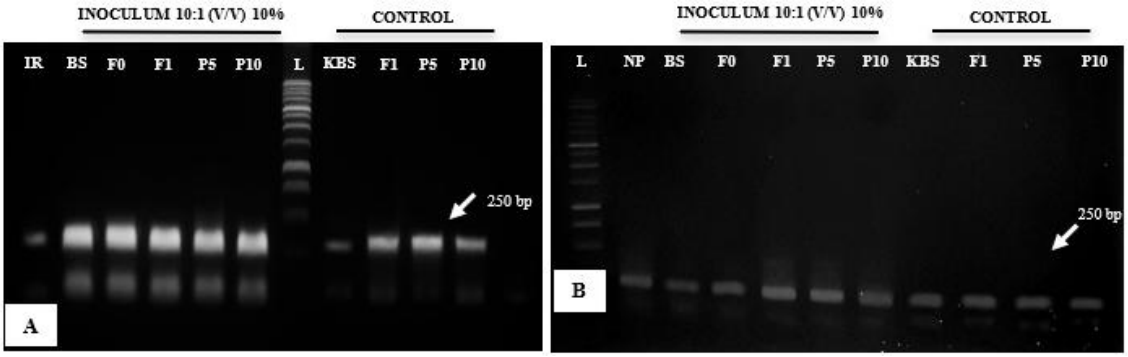


Figure 2

Electrophoregram of amplified ITS1 region of ITS (a) and V3 region of 16S rRNA (b) from controlled fermentation samples. DNA bands were observed at approximately 250 bp. L = 1 kb ladder (Promega); NP = bacterial inoculum; PB = yeast inoculum; BS = fresh coffee fruit; F1 = 24 h; P5 = 144 h; P10 = 264 h; KBS = fresh fruit control.



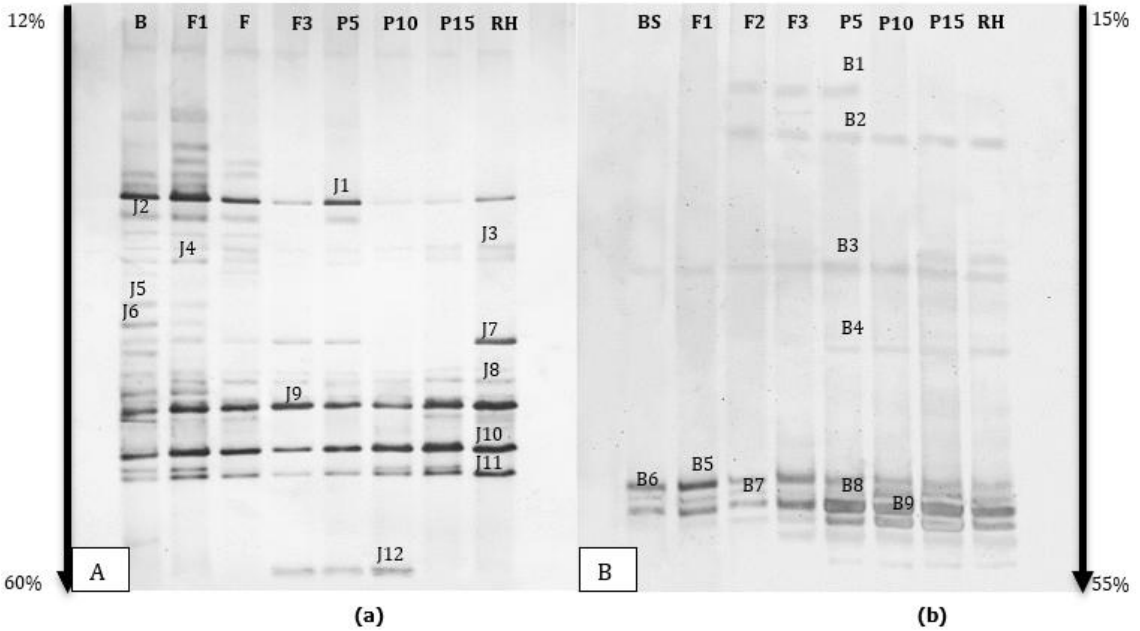
1) Variation in eukaryotic microbial population structure

The number of eukaryotic DNA bands observed in the DGGE profile of natural fermentation was 27 bands (see Fig. 3a), whereas controlled fermentation showed 18 bands at the final processing stage (see Fig. 4a). Based on band intensity, 12 major eukaryotic bands (J1–J12) were identified in the natural fermentation process. Bands J1, J3, J4, J5, J7, J8, J9, J10, and J11 were excised and sequenced and were identified as *P. kudriavsevii*, uncultured fungal isolates, *Fungal* sp., uncultured *Candida*, uncultured fungus, *Hanseniaspora* sp., *P. kluyveri*, *P. anomala*, and *T. delbrueckii*, respectively. These species persisted until the end of both natural and controlled fermentation processes. The remaining three bands (J2, J5, and J12) were detected only in natural fermentation and controlled fermentation without starter cultures and were identified as *S. cerevisiae*, *Candida railenensis*, and *Aspergillus carbonarius*, respectively. Three bands corresponding to species of the *Pichia* genus were consistently detected throughout post-

harvest coffee processing. These yeast species have previously been reported in coffee fermentation environments (Silva et al., 2008; Vilela et al., 2010; Silva, 2014), except for *P. kudriavsevii*, which was identified in this study using a molecular approach. This species is commonly reported as a dominant yeast in cocoa fermentation (Daniel et al., 2009; Pereira et al., 2012). Silva et al. (2013) further demonstrated that *Pichia* species exhibit pectinolytic activity, contributing to the degradation of pectin in the pulp layer and coffee fruit mucilage.

Figure 3

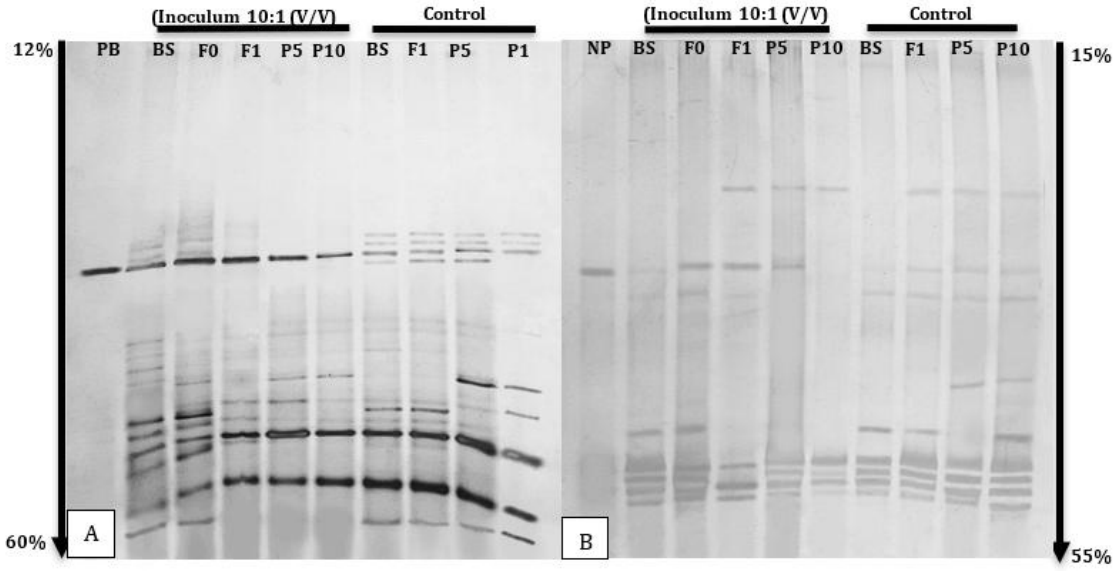
PCR-DGGE profiles of eukaryotic (a) and prokaryotic (b) communities during natural fermentation of wine-flavored coffee. BS = fresh coffee fruit; F1 = 24 h; F2 = 48 h; F3 = 72 h; P5 = 192 h; P10 = 312 h; P15 = 432 h; RH = after resting and hulling. Bands J1–J12 and B1–B9 were excised for further analysis.



Filamentous fungi were detected at low abundance during fermentation. *A. carbonarius* was observed at the final stage of fermentation and remained detectable until the tenth day of drying, but was not detected from the eleventh day of drying until the end of processing. *A. carbonarius* has been previously reported in coffee fermentation and is known for its potential to produce mycotoxins, such as ochratoxin A. In this study, controlled fermentation with starter cultures was associated with reduced detection of filamentous fungi, including *A. carbonarius*, based on DGGE profiles. However, no mycotoxin analyses were conducted; therefore, no conclusions regarding mycotoxin production or food safety can be drawn. Interactions between yeasts and bacteria during fermentation are known to influence microbial succession and environmental conditions, such as acidification and nutrient availability, which may contribute to shifts in fungal community composition (Payne et al., 2001).

Figure 4

PCR-DGGE profiles of eukaryotic (a) and prokaryotic (b) communities during controlled fermentation after inoculation with starter cultures PB:NP (10:1; 10% v/w) and control treatment of wine-flavored coffee. NP = bacterial inoculum; PB = yeast inoculum; BS = fresh coffee fruit; F1 = 24 h; P5 = 144 h.



2) Variation in prokaryotic microbial population structure

Prokaryotic DGGE profiles were compared based on the post-harvest processing methods used. The total number of visualized prokaryotic DNA bands in natural and controlled fermentation was 12 and 10 bands, respectively (see Fig. 3b and Fig. 4b). In natural fermentation, nine main bands with sharp intensity and thickness were identified (B1–B9). Bands B2, B3, B5, B6, B7, and B8 were identified as *Bacillus cereus*, *Klebsiella oxytoca*, *Lactobacillus fermentum*, *Pantoea agglomerans*, *Lactobacillus brevis*, and *Bacillus subtilis*, respectively. The remaining three main bands were detected only in natural fermentation, namely B1, B4, and B9, which were identified as *Enterobacter aerogenes*, uncultured *Bacillus* sp., and uncultured bacteria, respectively (see Table 1). In natural fermentation, the abundance of prokaryotic microbiota increased during the final day of fermentation and the drying stage. This increase is attributed to microbial succession occurring during the transition from fermentation to drying.

Table 1

Phylogenetic Identification Results of Selected Bands from Fungal DNA DGGE Profiles

Band no[a]	Identified species	Percent identity	GenBank accession [b]		
			Number	Isolate source	Reference
J1	*Pichia kudriavsevii*	100%	MG182349.1	Kefir	Chatraei and Emtiazi (2017)
J2	*Saccharomyces cerevisiae*	99%	AH010212.2	-	Jin et al. (2000)
J3	Uncultured fungus isolate	97%	KX515403.1	Rainwater	Du et al. (2016)
J4	*Fungal* sp. Isolate	99%	MH624516.1	Soil	Anderson et al. (2018)

J5	Uncultured *Candida*	100%	KR905733.1	Fermented soybean condiment	Ezeokoli et al. (2015)
J6	*Candida railenensis*	97%	KF728791.1	Kiwi fruit	Niu et al. (2013)
J7	Uncultured *fungus*	100%	JX388247.1	Soil	Davinic et al. (2012)
J8	*Hanseniaspora* sp.	98%	MG734841.1	Grape fruit	Raymond et al. (2017)
J9	*Pichia kluyveri*	100%	KY580405.1	Fruit skin surface	Lo et al. (2017)
J10	*Pichia anomala*	99%	FJ797686.1	Fermented food	Yu and He (2009)
J11	*Torulaspora delbrueckii*	99%	KM402070.1	Fermented coffee	Evangelista et al. (2014)
J12	*Aspergillus carbonarius*	100%	MF436162.1	Grape wine	Zouaoui et al. (2017)

[a] Bands are numbered according to Fig. 3a
[b] Percent identity is based on the GenBank NCBI database

Table 2
Phylogenetic Identification Results of Selected Bands from Bacterial DNA DGGE Profiles

Band no[a]	Identified species	Percent identity	GenBank accession [b]		
			Number	Isolate source	Reference
B1	*Enterobacter aerogenes*	99%	KY751358.1	Wastewater	Andleeb et al. (2017)
B2	*Bacillus cereus*	98%	KC510021.1	Coffee fruit	Dong et al. (2013)
B3	*Klebsiella oxycota*	100%	MG210668.1	-	Wei (2017)
B4	Uncultured *Bacillus* sp.	99%	JQ437383.1	Coffee fruit	Oliveira et al. (2013)
B5	*Lactobacillus fermentum*	100%	AF329113.1	Whey cheese production	Ercolini et al. (2000)
B6	*Pantoea agglomerans*	99%	JX464166.1	Barley wine	Zhao et al. (2012)
B7	*Lactobacillus brevis*	99%	KX057642.1	Hudy	Zhang (2016)
B8	*Bacillus subtilis*	98%	KY010306.1	Coffee pulp waste	Arimurti et al. (2016)
B9	Uncultured bacterium	100%	GQ979309.1	Gastrointestinal microbiome	Steven and Ward (2009)

[a] Bands are numbered according to Fig. 3b
[b] Percent identity is based on the GenBank NCBI database (2018)

The bacterial population identified during coffee processing was predominantly composed of species from the family *Enterobacteriaceae* (Gram-negative bacteria) and the genus *Bacillus* (Gram-positive bacteria), as shown in Table 2. *K. oxytoca*, *L. fermentum*, *P. agglomerans*, *L. brevis*, and the Gram-positive bacterium *B. subtilis* were identified in fresh coffee fruit prior to fermentation. By the third day of fermentation, additional species such as *E. aerogenes* and *B. cereus* were also detected.

During the drying process, other bacteria, such as uncultured prokaryotes and uncultured bacteria, were identified. Changes in the dominant microbial species may be influenced by variations in environmental conditions, including pH and humidity (Vilela et al., 2010).

Based on the intensity of the visualized DNA bands, *L. brevis* and *L. fermentum* appeared to dominate and remain involved until the end of coffee processing. In addition, *K. oxytoca*, *P. agglomerans*, and *B. subtilis* also persisted until the final stages of drying and hulling. The increased microbial abundance observed at the end of the process may be associated with the resting (storage) phase, which can increase water activity and thereby support microbial growth.

In this study, two *Bacillus* species were identified. These bacteria are commonly found in soil and are capable of sporulation, which allows them to survive under various environmental conditions. *E. aerogenes* and *K. oxytoca* identified in this study have also been reported in the natural processing of *Coffea arabica* in Brazil (Silva et al., 2008). *Bacillus*, *Klebsiella*, and *E. aerogenes* are known to produce pectin lyase, an enzyme involved in degrading pectin compounds in coffee fruit mucilage, thereby accelerating the fermentation process (Coughlan, 1991).

Following the inoculation of starter cultures, microbial abundance in the controlled fermentation was lower than that observed in the uninoculated (control) fermentation. In the controlled fermentation, the community profile indicated that the bacterial inoculum (NP) was already present in fresh fruit, and its abundance increased during fermentation. Bacteria from the genus *Klebsiella* are known to degrade pectin into simpler substrates or precursors that can subsequently be utilized by other microorganisms, such as *Pichia*, for the production of aroma compounds in coffee (Avallone et al., 2001).

The abundance of the bacterial inoculum decreased during the final stages of processing, possibly due to reduced substrate availability to support growth (see Fig. 4b). In contrast, the abundance of yeast inoculum (IR) (see Fig. 4a) remained relatively stable until the final processing stage. Yeasts have been recognized as potential starter cultures in coffee fermentation due to their positive contributions. Silva et al. (2013) demonstrated that yeasts are prominent among other microbiota because of their ability to secrete pectinolytic enzymes and produce volatile compounds that contribute to the final coffee aroma.

The growth of eukaryotic microbiota proceeds in parallel with the consumption of sugar substrates in the pulp (glucose and fructose), resulting in the production of organic acids and a consequent decrease in pH during fermentation. Coffee fermentation involves complex interactions among microbiota, primarily yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). These interactions encompass physical and chemical changes experienced by bacterial and yeast cells throughout fermentation. A decrease in pH, together with reduced oxygen levels (anaerobic fermentation) in the fruit pulp, favors yeast growth, which utilizes carbohydrates to produce ethanol. As fermentation progresses, LAB and AAB populations increase and become dominant, followed by a decline in yeast populations. When conditions shift to semi-anaerobic, LAB growth is promoted during the late fermentation stage and the beginning of drying. Meanwhile, AAB growth is stimulated by aeration and the availability of ethanol produced by yeasts. These microbial activities generate metabolites that can diffuse into coffee beans and influence coffee aroma (Pereira et al., 2016).

C. Microbial Diversity and Dynamic Analysis of DGGE Banding Profiles

1) Clustering Analysis

Clustering analysis based on the intensity and thickness of DNA bands generated UPGMA dendrograms, as shown in

Fig. 5. The dendrogram illustrates the microbiota involved in natural fermentation, which were grouped into two main clades with a similarity level of 52%. Microbiota found in fresh fruit and on the first and second days of fermentation occupied the same clade. The similarity between microbiota in fresh fruit and on the first day of fermentation was 79%, and both shared 62% similarity with microbiota present on the second day of fermentation. The second cluster consisted of microbiota from the third day of fermentation, the drying stages, and post-hulling, with a similarity level of 69%, as shown in Fig. 5a. The most similar community structures were observed between microbiota on drying days 5 and 10 (84%) and between drying day 15 and post-hulling (85%). Certain natural microbiota of coffee fruit and those involved at the end of fermentation were replaced by microbiota associated with the drying process or originating from the drying environment. According to Kim et al. (2008), during coffee drying, humidity decreases, and a transition occurs toward microbial groups commonly found in the environment, particularly soil microbiota.

Microbial clustering analysis of fermentation variations after inoculation with starter cultures is shown in Fig. 5b. Based on UPGMA analysis, the entire microbiota throughout the process showed a similarity level of 51%. The analysis resulted in two main clusters. The first cluster consisted of the total microbiota in fresh fruit samples, samples at 0 h before fermentation immediately after inoculation (F0), and microbiota from the control processing. The absence of changes in population dynamics before fermentation classified the microbial populations at F0 and BS at the same similarity level. Microbiota clusters in the control were grouped together with BS and F0, indicating that no significant changes occurred in the microbiota community dynamics in the control, as no impact of inoculation was observed.

The second cluster consisted of all stages of controlled fermentation and drying following inoculation with both starter cultures, with a similarity level of 70%. Microbial succession from the fermentation stage (F1) to the drying stages (P5 and P10) formed a single cluster, indicating high stability in the controlled process, as reflected by the high similarity levels. In contrast, samples from fermentation (KF1) and drying (KP5 and KP10) without inoculation formed separate clusters, suggesting lower stability in the uncontrolled process. At the end of processing, similarity levels of 82% and 70% were observed for controlled fermentation and control samples, respectively. These findings suggest that inoculum addition may influence community dynamics during wine-coffee processing.

2) Microbial diversity and dinamics analysis

The abundance of total microbiota (prokaryotes and eukaryotes) in natural fermentation was analyzed using the Shannon–Wiener diversity index (see Fig. 6). The results showed higher microbial abundance and diversity at the genus/species level in fresh coffee fruit samples. Diversity decreased during fermentation and subsequently increased during the drying process. According to Odum (1971), in populations with high diversity, the dominance of a single species has less influence compared to populations with lower diversity levels.

Fig. 6 also shows dominance indices at each stage of coffee processing, with values below 0.1. Along with diversity, dominance levels fluctuated throughout coffee processing. According to Odum (1971), higher dominance index values indicate a greater likelihood of certain genera or species dominating an ecosystem. The highest dominance index value, approaching 1, was observed during the fermentation stage, indicating the presence of microbial groups playing a significant role at this stage. In natural fermentation, diversity and dominance fluctuated at each stage, suggesting that no specific microbiota exerted control over the fermentation process until the final stages of processing.

Figure 5

Cluster analysis of total microbial community dynamics based on DGGE profiles of natural fermentation (a) and controlled fermentation with inoculation and control (b). The scale

below the dendrogram indicates percentage similarity based on the UPGMA clustering algorithm. BS = fresh coffee fruit; fermentation F1 = 24 h, F2 = 48 h, F3 = 72 h; drying P5 = 192 h, P10 = 312 h, P15 = 432 h; RH = after resting and hulling; KBS = control of fresh fruit; KF1 = control of 24 h fermentation; KP5 = control of 5 h drying; KP10 = control of 10 h drying.

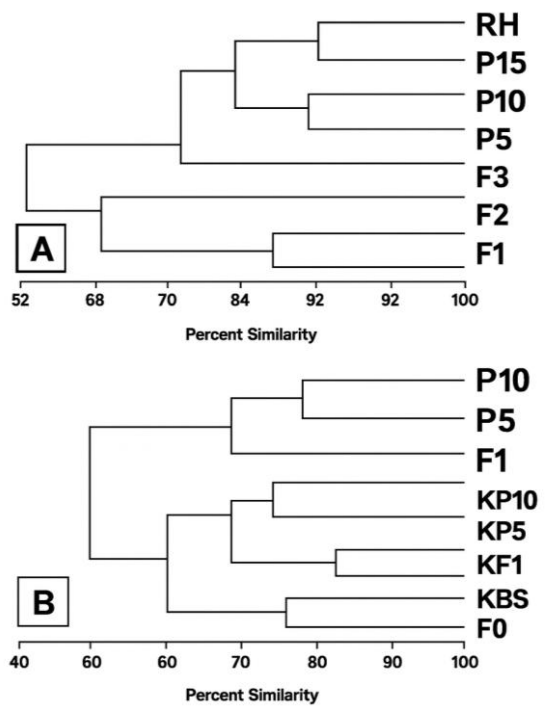


Figure 6

Microbial diversity indices: Shannon–Wiener index (H) and dominance index (D) during natural fermentation of wine coffee. BS = fresh coffee fruit; fermentation F1 = 24 h, F2 = 48 h, F3 = 72 h; drying P5 = 192 h, P10 = 312 h, P15 = 432 h; RH = after resting and hulling.

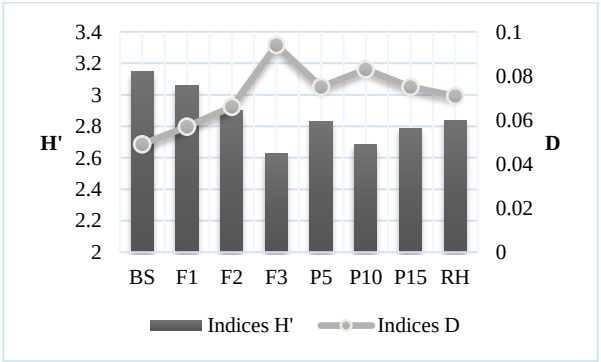
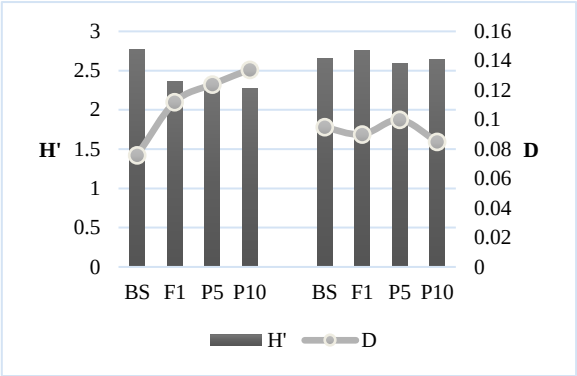


Figure 7

Microbial diversity indices: Shannon–Wiener index (H) and dominance index (D) during controlled fermentation of wine coffee with inoculation of starter cultures (10:1 / 10% v/w). BS = fresh coffee fruit; fermentation F1 = 24 h; drying P5 = 144 h, P10 = 264 h; KBS = control of fresh fruit.



The Shannon–Wiener indices of controlled fermentation (with inoculation) and fermentation without inoculation were compared using a t-test analysis, as shown in Fig. 7. The results indicated significant differences in microbial diversity between inoculated and control processing. Microbial abundance decreased in controlled fermentation following the addition of PB and NP inocula and tended to remain stable ($p = 0.05$) compared to the control. Therefore, the reduction in microbial diversity during coffee processing may be associated with inoculum addition. As shown in Fig. 7, dominance index analysis revealed increasing dominance levels toward the final stages of processing, indicating the presence of dominant species in this ecosystem. In contrast, dominance indices in the control samples remained relatively stable, suggesting that no single species dominated during processing. Based on these results, the yeast inoculum *P. kudriavsevii* and the bacterium *Klebsiella* sp. appeared to control the fermentation process by dominating throughout wine-coffee processing under controlled conditions.

Research by Pereira et al. (2015) also demonstrated that the addition of starter cultures, including *Pichia* yeast, to spontaneous or natural coffee fermentation resulted in dominance of specific microbiota and reduced overall microbial diversity until

the end of processing. Thus, inoculum addition may serve as an alternative approach for achieving controlled fermentation conditions and preventing undesirable microbial growth and the production of unpleasant aromas in the final wine coffee. However, understanding of the impact of starter culture inoculation on final coffee quality remains limited, and further research is needed to comprehensively elucidate the roles of these microorganisms during coffee processing.

CONCLUSION

The results of the present study indicate that the microbial community undergoes a transition from the fermentation stage to the drying stage. Microbial diversity during the fermentation stage was lower than that during the drying stage, with eukaryotic diversity being higher throughout the process, as indicated by 27 DNA bands compared to only 12 visualized DNA bands for the prokaryotic community. Predominant yeasts involved in natural fermentation were identified from the genera *Pichia*, *Saccharomyces*, *Hanseniaspora*, and *Candida*, while predominant bacteria belonged to the genera *Enterobacter*, *Klebsiella*, *Lactobacillus*, *Pantoea*, and *Bacillus*. Inoculation with starter cultures of *P. kudriavsevii* and *Klebsiella* sp. was able to alter the microbial community structure by dominating the process and exerting a significant impact on the establishment of controlled wine-coffee fermentation.

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