

Exploring Civet Fecal Microbiota from Toraja and Simalungun as a Starter Culture for Improving Coffee Fermentation

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ABSTRACT

Civet coffee is a globally renowned specialty beverage, yet its production is often associated with inconsistent quality and ethical concerns due to the involvement of civet animals and the use of its feces in post-harvest processing. To address these challenges, this study explores the potential of microbial starters derived from civet feces to improve coffee fermentation outcomes while offering a scalable, ethical, and halal-compliant alternative to traditional civet coffee. Microbes were isolated from digested coffee beans taken from civets' fresh feces in Toraja and Simalungun, Indonesia. From 21 isolates, ten strains exhibiting high enzymatic activity (amylase, protease, pectinase, and cellulase) were selected and evaluated in wet fermentation trials. Three isolates produced superior fermentation outcomes: two yeasts (SY.Y4 and TF.Y1) identified as *Kluyveromyces marxianus* and one bacterial isolate (TM.B1) identified only to the family level (Enterobacteriaceae). These isolates produced fermented beans with final pH values around 3.9 and cupping scores of 85.0 (SY.Y4), 85.0 (TF.Y1), and 85.5 (TM.B1), exceeding both control treatments, namely sugar water (C1: 83.25) and water only (C2: 82.25), by as much as 3.25 points according to the SCAA Cupping Protocol. This study demonstrates the feasibility of using civet-derived microbiota as starter cultures in specialty coffee fermentation, providing a reproducible and animal-free method to achieve high-quality flavor while addressing concerns related to animal welfare, food safety, and religious acceptability.

Key words: Civet coffee; enzymatic activity; *Kluyveromyces marxianus*; microbial starter; sensory analysis.

1 INTRODUCTION

Coffee is among the most globally traded commodities, with Indonesia playing a vital role as a producer and exporter. The coffee sector contributes significantly to Indonesia's foreign exchange earnings, accounting for an average of 36.08% of total agricultural exports during 2020-2024 (Statistics Indonesia, 2025). A critical post-harvest stage in coffee production is the fermentation process. Fermentation, an essential step for mucilage removal, involves sugar breakdown under aerobic or anaerobic conditions. The mucilage mainly contains pectin, cellulose, and starch. Microbial-driven fermentation not only aids in mucilage removal but also enhances coffee aroma and flavor through the formation of bioactive precursors (da Silva Vale et al., 2021; Haile & Kang, 2019c).

Traditionally, fermentation is done spontaneously, relying on indigenous microbiota found on the coffee fruit. While this approach is cost-effective, it is inconsistent, often leading to under- or over-fermentation and undesirable flavors (Elhalis, Cox & Zhao, 2023). Controlled fermentation using defined microbial starter cultures has been proposed as a strategy to improve reproducibility, reduce spoilage, and enhance sensory quality (Watanabe et al., 2020).

Microbial starters can originate from various sources, including the surrounding environment, directly impacting how coffee ferments (Elhalis, Cox & Zhao, 2023). The fermentation process of animal digestion is one of the microbial starting

culture sources that can yield specialty coffee at high prices. The most well-known example is civet coffee, or *Kopi Luwak* in Indonesia, which is made from the droppings of Asian palm civets (*Paradoxurus hermaphroditus*) (Ngamnok et al., 2023).

Civet coffee is a specialty product made from beans that have passed through the gastrointestinal tract of *P. hermaphroditus*. During digestion, microbial and enzymatic processes alter the bean's chemical composition, especially affecting amino acid and sugar profiles, which are thought to contribute to its distinct sensory characteristics. Among the key enzymatic activities involved are amylolytic enzymes, which hydrolyze complex carbohydrates such as starch into glucose and simpler sugars (Rokhmah & Mulyani, 2021). Recent studies have shown that certain microbes, particularly *Gluconobacter* spp., are dominant and functionally unique within civet feces. These microbes possess genes for cell motility and sulfur metabolism, which may influence the fermentation of coffee beans in the civet gut and contribute to the smooth, low-bitterness flavor profile of civet coffee (Watanabe et al., 2020).

However, rising demand for civet coffee has led to concerning practices involving the caging and force-feeding of civets, raising serious animal welfare concerns (Adadi et al., 2024). In addition, the origin of the product, which is civet feces, poses religious and hygienic challenges. The halal status of civet coffee has been debated, with many Muslim consumers reluctant to consume a product derived from

animal excreta (Rokhmah & Mulyani, 2021). To address these concerns, recent research has explored *in vitro* wet fermentation methods for producing synthetic civet coffee using a microbial starter culture derived from civets. This approach has also been applied by Indonesian researchers, such as Towaha and Rubiyo (2016), who used yeasts isolated from the civet's small intestine and caecum. They combined these probiotics for a 6-day fermentation, resulting in coffee with a cupping score of 81.44, which is 2.19 points higher than the original civet coffee control. However, their research did not specify the source of the civet, whether it was from a caged or wild civet, or the region it came from. Additionally, the fermentation lasted about a week, longer than the typical 6 to 72 hours for wet fermentation in the field (Elhalis, Cox & Zhao, 2023). Likewise, Darwin et al. (2023) performed wet coffee fermentation using a mixed-microbial starter culture derived directly from civet feces collected in Central Aceh. They found that the starter culture selectively enriched with starch exhibited a higher sugar-conversion rate than the culture enriched with glucose. While this indicates that starch-enriched mixed cultures may be suitable for *in vitro* coffee fermentation, the study did not identify the specific microbial isolates, as the civet feces were applied directly to the starch or glucose enrichment media without prior isolation.

Therefore, this study aims to develop a microbial starter for a synthetic wet-processing method of civet coffee using isolates from civet feces with the highest enzymatic activity. In this study, we isolated and characterized microbial communities from civet feces collected in Toraja and Simalungun, Indonesia. These two regions are renowned for producing high-quality Arabica coffee with Geographical Indication (GI) status (Harding et al., 2025). We hypothesize that the unique terroir and diet of civets in these regions support distinct microbial communities that may offer superior fermentation potential. To our knowledge, this is the first study to investigate civet-associated microbiota from these specific GI areas. Our goal is to identify enzymatically active microbes suitable as starter cultures for wet processing, thereby replicating the flavor-enhancing effects of civet digestion without ethical compromise. This work introduces a sustainable, halal-compliant, and field-scalable approach to civet-style coffee fermentation.

2 MATERIAL AND METHODS

2.1 Civet feces collection

Fresh civet feces that were still moist and had not visibly dried at the time of collection were obtained from two regions in Indonesia, Toraja and Simalungun, using aseptic sampling techniques. For each region, a tube containing 15-20 grams of fresh civet feces was collected into sterile tubes as the

total sample and transported to the lab. Toraja's sample was collected from a traditional farm that kept wild civets, while Simalungun's sample was collected from wild civets found in a coffee plantation near the forest.

2.2 Isolation and characterization of microbiota

Microbial isolation was performed by adding 1 gram of civet feces to 9 mL sterile physiological saline solution 0.85% (w/v) and then serially diluting up to 10^6 . From each dilution, 20 μ L was inoculated following the single plate-serial dilution spotting (SP-SDS) method on nutrient agar (NA) as a basal medium, yeast glucose chloramphenicol agar (YGCA), De Man–Rogosa–Sharpe Agar (MRSA), and fumarase media as a selective medium (Takata et al., 1979; Thomas et al., 2015). Samples were incubated at room temperature (25 ± 2 °C) for 24 to 48 hours. Subsequently, bacterial and yeast isolates were purified on the same medium following the four-way streak method. The inoculated medium was incubated at room temperature for 24 to 48 hours. Colonies that grew were observed macroscopically and microscopically. Microbial isolates were then stored for further analysis.

2.3 Enzymatic assays

The presence of amylase, protease, pectinase, and cellulase activity was assayed through semi-quantitative enzymatic activity using the well-diffusion method. Bacterial amylase activity assay was carried out using Luria Bertani (LB) agar media with the following composition: yeast extract 5 g/L, NaCl 10 g/L, tryptone 10 g/L, starch 10 g/L and agar 15 g/L (Pinjari & Kotari, 2018), while screening yeast isolates carried out using media with the following composition: peptone 15 g/L, yeast extract 10 g/L, starch 10 g/L and agar 20 g/L (Taufik et al., 2024). Bacterial protease activity assay was carried out using LB agar media with a composition consisting of 5 g/L yeast extract, NaCl 10 g/L, tryptone 10 g/L, skim milk 28 g/L and agar 15 g/L; while yeast isolation was carried out using media that had the following composition: peptone 15 g/L, yeast extract 10 g/L, skim milk 28 g/L, and agar 20 g/L (Taufik et al., 2024).

Pectinase-producing bacteria and yeast were assayed using mineral pectin-5 (MP-5) media. MP-5 medium comprises of 15 g/500 mL agar solution, 250 mL basal medium (consisting of 6 grams Na_2HPO_4 , 5 grams orange pectin, 4 grams KH_2PO_4 , 2 grams NH_4SO_4 , and 1 gram yeast extract), as well as 250 mL mineral solution (1 mL 0.1% FeSO_4 solution, 1 mL 20% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mL 0.1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mL 0.001% H_3BO_3 , 1 mL 0.001% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1 mL $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.007%, 1 mL $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.005%, and 1 mL MoO_3 0.001%) (Atlas, 2010). Bacterial cellulase activity was assayed using carboxymethyl cellulose (CMC) media with the following composition: yeast extract 1 g/L, CMC 5 g/L, and agar 20 g/L. Whereas yeast cellulase activity was carried out using media

with the following composition: peptone 3 g/L, yeast extract 3 g/L, CMC 10 g/L, and agar 20 g/L (Taufik et al., 2024).

In general, assays were done by inoculating 10 µL of approximately 10⁶ cells/mL of bacteria or yeast into a 5 mm well in solid growth media, followed by incubation for 48 hours at 37 °C for bacteria and 72 hours at room temperature (25-28 °C) for yeast (Carrasco-Palafox et al., 2018). Following incubation, the amylase and pectinase assay media were stained with Lugol's dye around the well. For the cellulase assay, 2 g/L Congo Red solution was used and left for 10 minutes, then rinsed using 1 M NaCl solution for 15 minutes. The clear zone that formed was observed, and its diameter was measured. The enzyme index (EI) was estimated by dividing the clear zone's diameter by colony growth, as shown in Equation 1 (Carvalho et al., 2021).

$$\text{Enzyme index (EI)} = \frac{\text{diameter of clear zone}}{\text{diameter of the colony growth}} \quad (1)$$

2.4 Inoculum preparation

Selected bacteria were grown in nutrient broth (NB), whereas yeast was grown in potato dextrose broth (PDB), both under 150 rpm of agitation at room temperature (25-28 °C) for 24 hours. The cells were harvested using centrifugation, followed by viability measurement.

2.5 Laboratory-scale fermentation

Ripe Arabica coffee fruits were sourced from the Wanoja Farmers Cooperative, Bandung Regency, West Java, Indonesia. The cherries consisted of a mixture of two commonly cultivated local varieties, Ateng and Lini S, which are representative of the cooperative's production. Cherry ripeness was standardized, with an average soluble solid content of 18 °Brix, measured using a handheld refractometer to ensure uniform sugar levels before fermentation. Prior to inoculation, freshly harvested cherries were depulped using a mechanical pulper and thoroughly washed with clean water to remove surface contaminants while retaining the natural mucilage layer. The fermentation process was done by adding 1.5 kg de-pulped coffee, water, a starter culture of 0.15% (v/w) single bacteria, a single yeast, and a control without additional starter culture (Cassimiro et al., 2022). Fermentation was carried out at room temperature (25 ± 2 °C) for 12 hours. Fermentation was terminated at 12 hours based on the observation that mucilage degradation had reached completion, indicated by the absence of sticky pectin residues on the bean surface. During fermentation, pH and temperature measurements were carried out every 6 hours with two types of controls: sugar water (C1) and water (C2). Then, coffee was washed with clean water and dried until the moisture content was 11-12%.

2.6 Sensory analysis based on cupping test

The cupping test was carried out on all fermentation trial samples by a certified Q grader at Catur Coffee

Company, Jakarta, Indonesia, following the SCAA Cupping Protocols, which consider the parameters of fragrance/aroma, flavor, aftertaste, acidity, mouthfeel, balance, uniformity, clean cups, sweetness, overalls, and defects. The scale per parameter is points 1 to 10. The sum of all parameter values will be accumulated into a cupping score (Specialty Coffee Association - SCA, 2018).

2.7 Identification of potential microbial isolates through DNA sequencing

The microbes with the best cupping score results were then cultured on appropriate media using the streak method. The agar plates were then incubated for 24 hours before finally being sent for sequencing. DNA sequencing was conducted by Macrogen Singapore, and sequences obtained were analyzed with BioEdit (Hall, 1999) and MEGA11 (Tamura, Stecher & Kumar, 2021). Typically, sequence references were obtained using BLAST (Altschul et al., 1990) against the ITS and 16S rRNA databases for yeast and bacteria, respectively. Multiple sequence alignment was performed using MUSCLE, and a phylogenetic tree was constructed using Maximum Likelihood within MEGA11 (Tamura, Stecher & Kumar, 2021).

2.8 Statistical analysis

Enzyme index measurement data were analyzed using Microsoft Excel. One-way analysis of variance (ANOVA) and Tukey HSD test were performed to determine significant differences, and values were considered statistically significant if $p < 0.05$.

3 RESULTS

3.1 Isolation and characterization of microbial population in civet feces coffee beans

The results of the isolation and data on the macroscopic and microscopic characteristics of the microbial population in the feces of the civet coffee beans are presented in Table 1.

3.2 Enzymatic assays

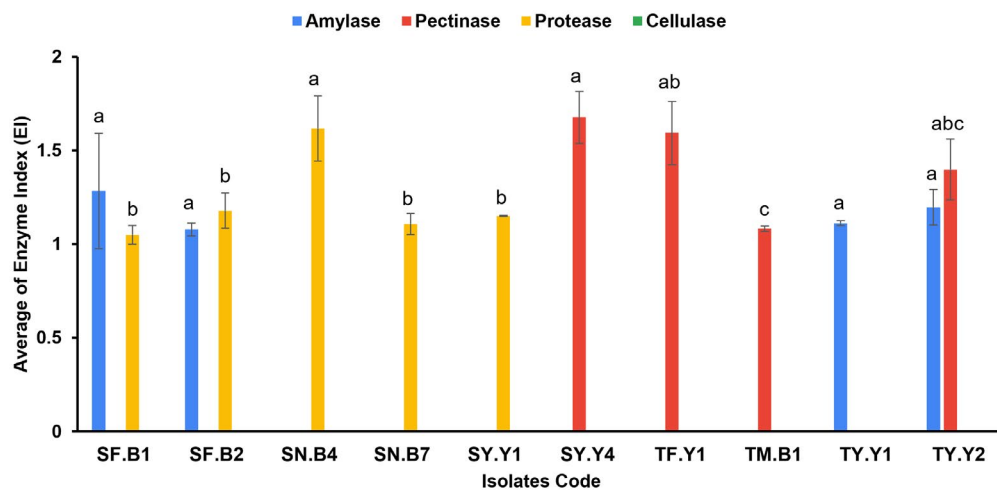
The enzyme indices for amylase, protease, pectinase, and cellulase in the isolated microbes exhibited varying levels of activity. Of the 21 isolates tested, only ten displayed detectable enzymatic activity, as illustrated in Figure 1. The assays were conducted using enzymatic assay plates, as shown in Figure 2.

3.3 Laboratory-scale fermentation

pH changes during laboratory-scale coffee fermentation at 0, 6, and 12 hours with two types of controls in the form of sugar water (C1) and water (C2) can be seen in Figure 3.

Table 1: Characteristics of microbes isolated from civet feces from Simalungun (S) and Toraja (T).

No.	Isolates code	Isolates type	Cell shape	Gram stain	Colony shape	Colony size	Colony margin	Colony elevation	Colony color	Optical property
1	SF.B1	Bacteria	Bacilli	+	Irregular	Large	Filamentous	Flat	White	Opaque
2	SF.B2	Bacteria	Bacilli	+	Circular	Large	Undulate	Flat	White	Opaque
3	SF.B3	Bacteria	Coccobacilli	+	Circular	Small	Entire	Flat	White	Translucent
4	SM.B1	Bacteria	Spiral	+	Circular	Small	Entire	Raised	White	Opaque
5	SN.B1	Bacteria	Bacilli	+	Circular	Large	Entire	Flat	White	Opaque
6	SN.B2	Bacteria	Bacilli	+	Circular	Large	Entire	Flat	White	Opaque
7	SN.B3	Bacteria	Bacilli	-	Circular	Small	Entire	Flat	White	Opaque
8	SN.B4	Bacteria	Bacilli	+	Circular	Large	Undulate	Flat	White	Opaque
9	SN.B5	Bacteria	Bacilli	-	Circular	Large	Entire	Flat	White	Opaque
10	SN.B6	Bacteria	Bacilli	-	Circular	Medium	Entire	Flat	Creamy white	Translucent
11	SN.B7	Bacteria	Bacilli	+	Circular	Large	Filamentous	Flat	White	Opaque
12	SY.Y1	Yeast	Ovoid	N/A	Circular	Large	Entire	Convex	Creamy white	Opaque
13	SY.Y2	Yeast	Elongated	N/A	Circular	Large	Undulate	Umbonate	White	Opaque
14	SY.Y3	Yeast	Elongated	N/A	Irregular	Large	Undulate	Flat	White	Opaque
15	SY.Y4	Yeast	Ovoid	N/A	Circular	Large	Entire	Convex	Creamy white	Opaque
16	TF.Y1	Yeast	Ovoid	N/A	Circular	Punctiform	Entire	Convex	Cream	Opaque
17	TM.B1	Bacteria	Bacilli	-	Circular	Small	Entire	Convex	Cream	Translucent
18	TM.Y1	Yeast	Elongated	N/A	Circular	Medium	Entire	Flat	White	Opaque
19	TN.B1	Bacteria	Bacilli	-	Circular	Punctiform	Entire	Flat	Cream	Translucent
20	TY.Y1	Yeast	Elongated	N/A	Circular	Medium	Entire	Umbonate	Cream	Opaque
21	TY.Y2	Yeast	Elongated	N/A	Circular	Small	Entire	Convex	Cream	Opaque

**Figure 1:** Average enzyme index of 10 selected microbes. Value represented by mean $\pm$ SD (N = 3). The bar with the same letter shows that the enzyme index is not significantly different in the same enzyme category based on the Tukey HSD test ($p > 0.05$).

3.4 Cupping test evaluation and molecular identification of potential microbial isolates

Cupping score and sensory characteristics of coffee assessed in this study were carried out by considering the parameters of fragrance/aroma, flavor, aftertaste, acidity,

mouthfeel, balance, uniformity, clean cups, sweetness, overall, and defects. Table 2 shows the assessment of each attribute.

The results of the identification of potential microbial isolates through DNA sequencing using the ITS and 16S rRNA databases for yeast and bacteria can be seen in Figures 4 and 5, respectively.

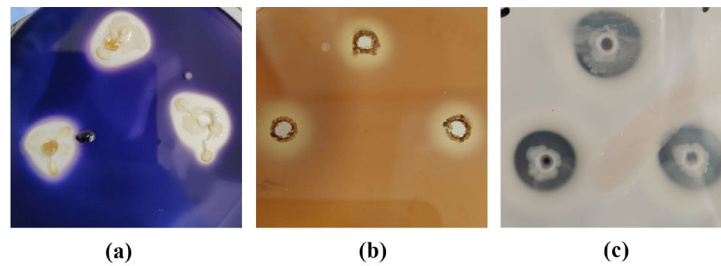


Figure 2: Samples of enzymatic assay plate for amylase (a), pectinase (b), and protease (c).

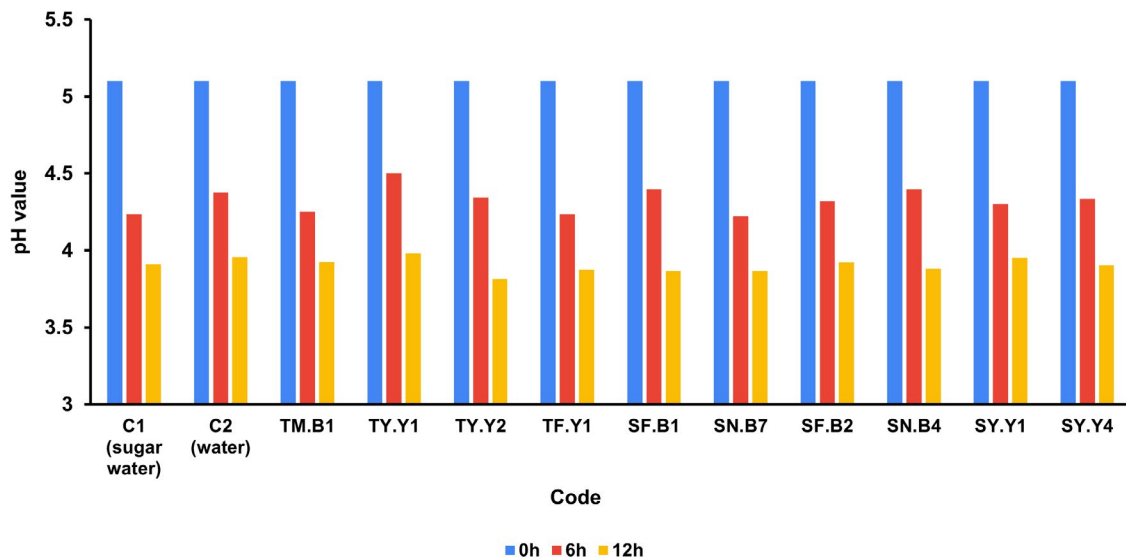


Figure 3: pH changes during laboratory-scale coffee fermentation.

Table 2: Total cupping score and note for all variations.

Variation	Total cupping score	Notes
Control 1 (Sugar water)	83.25	Sweet, has hints of citrus, brown spices, and black tea combined, but slightly murky at the same time. Got better as it cools. Good balance of bold body, citrusy acidity.
Control 2 (Water)	82.25	Sweet, citrusy, but lack of complexity and murky. Short aftertaste.
SF.B1	82.75	Clean, tea-like with citrusy and herbal notes. A bit lacking in complexity and dry aftertaste.
SF.B2	83	Black tea- like with hints of citrus, herbs. Sweet but has a slightly dry nutty-like finish.
SN.B4	83.25	Citrusy layered with black tea, herbs, agave sweetness, but slightly dry on the aftertaste.
SN.B7	82.75	Black tea-like, layered with herbs, but lacking sweetness and acidity.
SY.Y1	83	Round mouthfeel, hints of citrus, brown spices, and also herb-like bitterness at the end. It gets worse as it cools.
SY.Y4	85	Sweet, juicy, balanced. Has hints of yellow fruits layered with herbs and spices. Candy-like sweetness, just a bit murky.
TF.Y1	85	Sweet, bright, tea-like with a nutty and dry finish like white wine. Slightly short on the aftertaste. Got better as it cools, more sweetness.
TM.B1	85.5	Sweet, bright, complex acidity, has hints of nectarine and floral.
TY.Y1	82.75	Tea-like, layered with herbs, citrus, and hints of jasmine. Good acidity but slightly unbalanced, lacking body and structure.
TY.Y2	82	A bit muted, herbal tea-like. Still has some acidity but not the good quality one. Powdery mouthfeel, slightly rough.

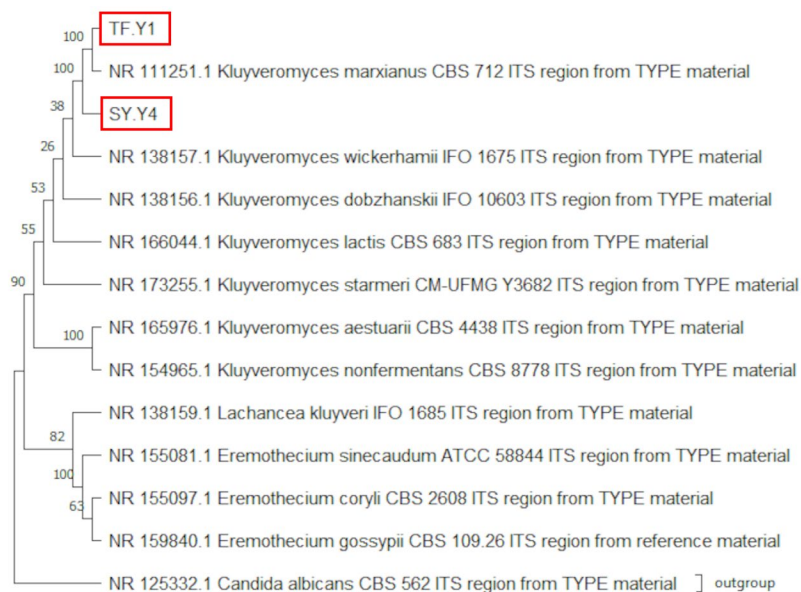


Figure 4: Phylogenetic tree of TF.Y1 & SY.Y4 constructed from ITS gene sequences of yeast with Maximum Likelihood algorithm. Bootstrap values (%), derived from 100 replications, are indicated by numerals on the nodes. Isolates utilized in this study are marked by red boxes. *Candida albicans* was chosen as the outgroup for yeast.

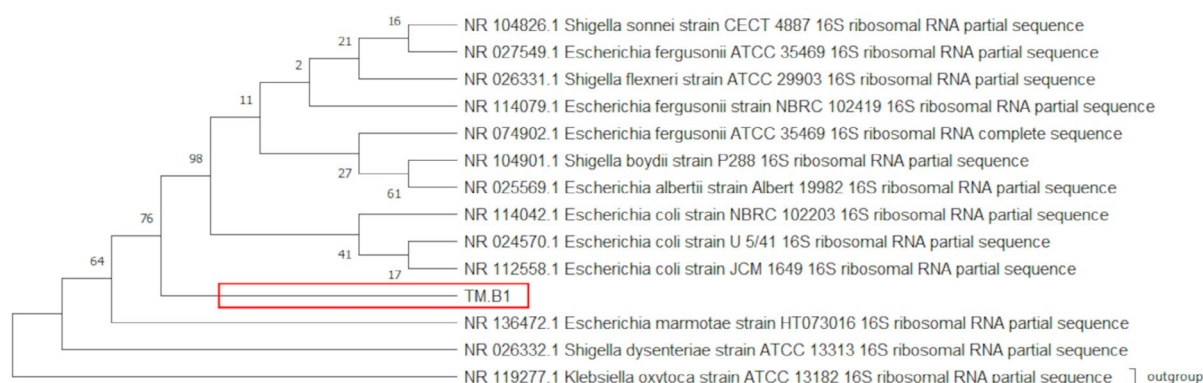


Figure 5: Phylogenetic tree of TM.B1 constructed from 16S rRNA gene sequences of bacteria with Maximum Likelihood algorithm. Bootstrap values (%), derived from 100 replications, are indicated by numerals on the nodes. Isolates utilized in this study are marked by red boxes. *Klebsiella oxytoca* was chosen as the outgroup for bacteria.

4 DISCUSSION

4.1 Field-Adaptable fermentation workflow and overview of microbial findings

In contrast to the previous study by Darwin et al. (2025), who used a temperature of 37 ± 0.5 °C during inoculum preparation and fermentation to mimic the civet's body temperature, our study applied room temperature (25 ± 2 °C) for both processes instead. The use of room temperature during microbial isolation and laboratory-scale fermentation was intentional and not aimed at mimicking the civet's gut environment. Our objective in these steps was to obtain stable *ex-situ* cultures and evaluate their performance under practical fermentation conditions commonly used by coffee processors.

Conducting fermentation at 37 °C would require controlled bioreactors or heating systems, which are generally not feasible in field-relevant settings. Previous study conducted by Hadiipernata and Nugraha (2018) utilized lactic acid bacteria isolated from the civet's small intestine to ferment coffee in a 5-L laboratory-scale bioreactor. Similarly, Wirawati and Winarto (2025) used a low-cost improvised bioreactor made from styrofoam and plastic containers, with civet feces serving as the culture initiator. Both bioreactor systems were designed to mimic the civet's digestive process by including acidification and neutralization stages and operating at a controlled temperature of 25 - 35 °C. However, the need for HCl acidification, NaOH neutralization, and continuous temperature regulation increases the operational complexity and energy demand. The energy cost of temperature-controlled

fermentation was demonstrated by Sagita et al. (2025), who applied ohmic heating to ferment 500 grams of Robusta green beans. Maintaining a temperature of 40 °C for 24 hours at an optimal voltage gradient of 8 V/cm resulted in an energy consumption of 335.41 ± 47.38 kJ. This energy requirement would increase proportionally with larger coffee volumes. Therefore, maintaining controlled temperatures during coffee fermentation presents significant challenges for scaling up under field conditions.

In this study, we successfully isolated 21 microbial strains consisting of 13 bacteria and eight yeasts from civet feces collected in Toraja and Simalungun, as shown in Table 1. Culturable microbes were isolated from wild civets (Simalungun), yielding 7 bacterial isolates on NA, 1 bacterial isolate on MRSA, and 4 yeast isolates YGCA. In contrast, caged civets (Toraja) produced 1 bacterial isolate on NA, 2 bacterial isolates on MRSA, and 2 yeast isolates on YGCA. Additionally, we used a fumaric acid-enriched medium to isolate microbes with potential fumarase activity, as fumarase catalyzes the conversion of fumaric acid into malic acid. Malic acid, along with other organic acids in coffee, such as citric acid, quinic acid, and chlorogenic acid, contributes to the unique profile of civet coffee (Jumhawan et al., 2013). Using this medium, we isolated three bacterial cultures from the Simalungun sample and one yeast from the Toraja sample. Bacteria and yeast growing on the same selective medium were differentiated through Gram staining.

4.2 Enzymatic capacity and selection of microbial starters

Enzymatic screening revealed key differences in amylase, pectinase, and protease production across isolates, as shown in Figure 1. In this study, these enzymes were selected as key indicators for evaluating each isolate's potential as a microbial starter, as their corresponding substrates comprise the major components of coffee mucilage. In general, coffee mucilage consists of approximately 35.8% pectic substances, 30% reducing sugars, 20% non-reducing sugars, 17% cellulose, and 8.9% protein (dry weight) (Elhalis, Cox & Zhao, 2023).

Three isolates, including SF.B1, SY.Y4, and SN.B4, demonstrated superior enzymatic indices, supporting their capacity to hydrolyze complex substrates in coffee mucilage. SF.B1 bacteria isolate produced the highest amylase enzyme index (1.284 ± 0.308^a) among the four microbes capable of producing amylase. Microbes that secrete exoenzymes in the form of α -amylase can diffuse into the medium containing starch. The α -amylase enzyme hydrolyzes the α -1,4-glycosidic bond in starch to produce several glucose subunits that can later be transported into cells and converted into energy through glycolysis. Detection of amylum hydrolysis in the medium was done by dripping Lugol's dye (iodine)

solution (Cappuccino & Sherman, 2020; Fatoni & Zusfahair, 2012; Lal & Cheeptham, 2012). Starch, which has a chain with a spiral conformation, can form a complex with an iodine reagent. Furthermore, triiodide ions in iodine can enter the spiral structure so that the starch-iodine complex will turn blue. Meanwhile, the α -amylase enzyme can destroy the spiral conformation of the complex. As a result, the blue color formed will disappear and cause a clear zone because iodine cannot create a colored complex with the glucose subunit, as shown in Figure 2a.

From pectinase enzyme test results, SY.Y4 yeast produced the highest pectinase enzyme index, 1.677 ± 0.138^a . Pectin substance is a complex colloidal acid polysaccharide with a long galacturonic acid chain accompanied by glycosidic bonds. The pectin compound chain comprises seven polysaccharides and 17 monosaccharides, such as d-glucuronic acid, l-fucose, d-glucose, d-mannose, and d-xylose (Haile, Masi & Tafesse, 2022). Like the amylase production test, pectin degradation was detected using Lugol's dye solution. Lugol's can bind to pectin to form a yellowish-brown complex (Haile & Kang, 2019a; Jayani, Saxena & Gupta, 2005; Soares, Da Silva & Gomes, 1999). Pectinase enzymes, especially endopolygalacturonase (endo-PG), can randomly break the 1,4- α -glycosidic bonds in the galacturonic acid chain, thereby degrading the pectin and breaking the complex formed with Lugol's. This causes clear zone formation due to Lugol's inability to form colored complexes with galacturonic acid subunits or glucose, as shown in Figure 2b.

SN.B4 bacteria produced the highest enzymatic index in the protease enzyme test results, 1.619 ± 0.174^a . This test employs a skim milk medium that contains high levels of casein. Casein comprises amino acid subunits connected by peptide bonds (CO-NH). To be assimilated by cells, casein macromolecules must be broken down by extracellular protease enzymes, by proteolysis that breaks casein into peptone, polypeptides, dipeptides, and amino acids. Protease hydrolyzes casein into amino acids that can be transported through the cell membrane for the cells' building blocks. The protein in skim milk is a colloidal suspension that provides color and opacity due to its light-deflection properties. Microbes capable of producing extracellular proteases will degrade milk proteins, causing the medium's opacity to disappear due to the formation of soluble amino acids with non-colloidal properties. Therefore, a positive result of the casein test is characterized by a clear zone, as shown in Figure 2c. On the other hand, a negative result of the casein test is characterized by a medium with a fixed opacity (Cappuccino & Sherman, 2020). Meanwhile, none of the microbes tested produced cellulase. These enzymatic properties are key, not only for mucilage breakdown, but also for producing volatile compounds that shape coffee flavor.

4.3 Fermentation dynamics and physicochemical trends

This study used a wet processing method for laboratory-scale fermentation. The wet process was selected over others because it closely mirrors the acidification and fermentation process of gastrointestinal civet fermentation (Marcone, 2004). The wet method is also commonly used by farmers and coffee processing units, especially in Indonesia. Laboratory-scale fermentation was carried out for ten single microbial isolates with the best index enzyme, accompanied by controls with and without adding sugar (C1 and C2, respectively).

During the coffee fermentation process, physicochemical parameters such as pH were monitored (Figure 3). All inoculated batches exhibited a consistent pH decline to 3.9 within 12 hours, indicating active microbial metabolism and organic acid production. This acidification trend is consistent with previous studies on both *in vivo* and *in vitro* civet fermentation systems, where microbial activity leads to the formation of lactic, acetic, and butyric acids (Darwin et al., 2023). Microorganisms metabolize simple sugars to sustain cellular processes and generate metabolites, including organic acids and alcohols that contribute to pH reduction (Elhalis et al., 2021; Hatiningsih, Antara & Gunam, 2018). A decrease in pH is a key indicator used to determine the endpoint of coffee fermentation, which typically occurs at around pH 4 (Wei et al., 2015). Additionally, the appearance of a rough bean surface, which indicates that the sticky mucilage layer has been fully degraded, provides further confirmation that fermentation can be terminated (Velmourougane, 2013). The fermentation process was stopped by washing and drying under the sun for two hours, then in a drying room for three days until the moisture content of the green beans reached 11–12% w/w.

4.4 Sensory outcomes and microbial influence on flavor

Three isolates, including TF.Y1, TM.B1, and SY.Y4 produced coffee with superior cupping scores (≥ 85), outperforming controls by over two points. These three isolates produced high pectinase enzyme indices of $1.594 \pm 0.169^{\text{ab}}$, $1.083 \pm 0.015^{\text{c}}$, and $1.677 \pm 0.138^{\text{a}}$, respectively, as shown in Figure 1. In contrast, the remaining isolates produced either amylase or protease enzymes, except for the TY.Y2 isolate, which can produce both amylase and pectinase enzymes simultaneously. All these isolates had cupping scores equal to or lower than those of the control samples. This might be due to over-fermentation occurring during the process.

Coffee fruits naturally contain endogenous pectinase that degrades mucilage, and microbial pectinase can further accelerate this process. Pectin degradation is also more effective under acidic conditions, where pectin becomes more

soluble and accessible to enzymatic breakdown (Elhalis, Cox & Zhao, 2023). Starter cultures with strong amylolytic activity can rapidly degrade non-pectin carbohydrates into simple sugars, which are subsequently fermented by other microbes into organic acids. Meanwhile, starter cultures with proteolytic activity can break down proteins into amino acids that support the growth of other microbes, such as lactic acid bacteria that also produce organic acids (Cassimiro et al., 2023). The combined activity of these enzymes can accelerate the decline in pH and increase the rate of mucilage degradation. Therefore, under the same 12-hour fermentation duration, treatments supplemented with starter cultures capable of producing amylase and protease are more likely to undergo over-fermentation compared with cultures that mainly produce pectinase. Over-fermentation leads to excessive accumulation of organic acids, including acetic, butanoic, and propionic acids, which contribute to off-flavors (Elhalis et al., 2020) and ultimately result in lower cupping scores compared with the control samples. In contrast, treatments containing starter cultures that produce only pectinase are more likely to achieve effective mucilage degradation and remain well-suited to the 12-hour fermentation period.

From the DNA sequencing results in the ITS region, TF.Y1 and SY.Y4 were identified as *Kluyveromyces marxianus* (Figure 4). *K. marxianus* is a yeast that can be found in a wide variety of habitats, ranging from dairy products, plants, to coffee wet-processing wastewater. This yeast is classified as a food-grade and has been commonly utilized in biotechnology and the food industry. *K. marxianus* can produce various enzymes, including inulinase, β -galactosidase, lipase, and pectinase, especially endo-PG. The ability of *K. marxianus* to produce endo-PG enzymes has been well recognized in the mucilage removal process of coffee and cocoa fruit fermentation (Haile & Ayele, 2022; Karim, Gerliani & Aider, 2020). Consistent with these findings, TF.Y1 and SY.Y4 were confirmed to be endo-PG-producing yeasts.

In coffee fermentation, *K. marxianus* contributes not only to mucilage degradation through pectinase activity but also to the synthesis of key metabolites involved in flavor formation (Haile & Kang, 2019a). Phylogenetic analysis showed that TF.Y1 and SY.Y4 are distinct strains within *K. marxianus*, which corresponds with the differing sensory profiles they produced. These strain-specific flavor notes can be plausibly attributed to variations in the key metabolites typically generated by *K. marxianus* during coffee fermentation. The mucilage-rich environment provides abundant sucrose, glucose, and fructose (Silva et al., 2024), which *K. marxianus* efficiently converts into alcohols and organic acids that subsequently form esters, major contributors to fruity and sweet aromas (Medeiros et al., 2001; Perpetuini et al., 2024). This species is well documented for producing esters such as ethyl acetate, isoamyl acetate, and phenethyl acetate (Adame

et al., 2023), compounds widely associated with sweet, juicy, candy-like, and fruity attributes. Such metabolites are consistent with the sweet, juicy, yellow-fruit, candy-like notes observed in SY.Y4. Controlled fermentation studies further show that elevated ester formation, particularly short-chain fatty acid esters, correlates with enhanced sweetness, aroma intensity, and overall cup quality (Shen et al., 2025), supporting the bright and fruit-forward characteristics found in this strain.

In contrast, TF.Y1 exhibited sweet, bright, tea-like, nutty, and white-wine-like notes, which may be associated with *K. marxianus* production of higher alcohols such as 2-phenylethanol, known for imparting floral, tea-like, and honeyed aromas. Subtle wine-like acidity may also arise from strain-dependent variation in organic acid metabolism, which influences the balance between fruity esters and acidic precursors. Importantly, strain-level differentiation among yeasts is known to cause significant divergence in volatile profiles and precursor metabolites (Yu et al., 2025). Metabolomic studies across yeast strains, including *Saccharomyces*, show that even closely related isolates can generate distinct patterns of alcohols, esters, flavonoids, and fermentation by-products with clear sensory consequences (Meeampun et al., 2024). This evidence strengthens the interpretation that the contrasting sensory profiles between TF.Y1 and SY.Y4 arise not merely from species-level traits but from strain-specific metabolic capacities, particularly in the synthesis of esters, higher alcohols, and related flavor-active intermediates.

On the other hand, based on 16S rRNA information, TM.B1 bacteria was identified as belonging to the Enterobacteriaceae family with a phylogenetic tree as shown in Figure 5. However, further information is needed to determine its exact species. Enterobacteriaceae is one of the bacterial families with the highest relative abundance found in civet feces, especially from masked palm civets (*Paguma larvata*) (Ishida et al., 2020). There are several dominant genera from the Enterobacteriaceae family obtained from *P. hermaphroditus* feces, including the genera *Citrobacter*, *Enterobacter*, *Escherichia/Shigella*, *Raoultella*, *Klebsiella*, *Salmonella*, and *Plesiomonas* (Watanabe et al., 2020). TM.B1 bacteria were isolated from MRS agar, a selective medium for growing LAB. A lower pH, about 5.4, is typically used to produce MRS agar's selectivity, favoring LAB's growth over other bacteria. Nevertheless, some members of the diverse family Enterobacteriaceae can withstand a certain amount of acidity, and some of them might still be able to thrive on MRS agar. Furthermore, some Enterobacteriaceae species might have defenses against MRS agar's inhibitory effects, such as the amino acid-dependent acid resistance system mechanism (Li, Huang & Gu, 2024). Interestingly, TM.B1, a bacterial isolate from Toraja, showed strong fermentation potential despite belonging to the Enterobacteriaceae, a group with

both beneficial and opportunistic microbes. While preliminary results are promising, further genomic and safety analyses are warranted to evaluate its use in food-grade fermentation systems.

Although TM.B1 was only identified at the family level (Enterobacteriaceae), several genera within this group are known to participate in the early stages of coffee fermentation and influence flavor development. Members of Enterobacteriaceae such as *Enterobacter*, *Pantoea*, *Erwinia*, and *Klebsiella* metabolize the abundant sugars in coffee mucilage through mixed-acid fermentation pathways, producing compounds such as acetic acid, lactic acid, succinic acid, citric acid, and ethanol (da Silva Vale et al., 2022; Vale et al., 2024). These metabolites have been reported to contribute to acidity and enhanced sweetness when present at moderate concentrations. Notably, low levels of acetic acid can impart a clean and sweet sensory impression, supporting the “sweet” attributes observed in TM.B1 (Elhalis, Cox & Zhao, 2023).

Studies also show that Enterobacteriaceae in early fermentation can interact with indigenous yeasts to modulate the formation of esters and higher alcohols, particularly when sugar availability is high in the first hours of fermentation (de Oliveira Junqueira et al., 2019). Such interactions can generate fruity and floral notes, which are consistent with the nectarine-like and floral characteristics identified in the TM.B1 cup profile. Furthermore, controlled accumulations of organic acids such as lactic, acetic, and succinic acids have been associated with improved balance between sweetness and lively fruit-floral aromatics in fermented coffees (Fardenan, Suroto & Supriyadi, 2024). Taken together, these metabolite patterns provide a plausible explanation for the sweet, bright acidity, and fruit-floral nuances detected in coffees fermented with TM.B1, while acknowledging that further species-level identification is required to confirm the specific metabolic pathways involved. Furthermore, a metabolomics approach will be essential to elucidate the key precursor compounds that drive the flavor and aroma profiles of coffees fermented with defined starter cultures.

4.5 Toward ethical, halal-compliant, and scalable civet coffee

The use of microbial starters from civet feces allows for the replication of desirable fermentation effects without animal exploitation. This addresses global concerns around the inhumane treatment of caged civets in commercial civet coffee production (Haile & Kang, 2019b). Additionally, using only microbial isolates that are not directly from feces may improve the perception of safety and halal compliance in Muslim-majority markets, which are currently hesitant about civet-derived products (Rokhmah & Mulyani, 2021).

Moreover, isolating, characterizing, and applying civet-associated microbes for fermentation opens the door to

scalable starter culture production, as the microbes identified in this study are cultivated under laboratory-controlled conditions and do not rely on wild civet sourcing. This controlled cultivation enables consistent, replicable production processes and simplifies scale-up for industrial applications, aligning with current industry trends toward standardized and traceable fermentation systems (Elhalis, Cox & Zhao, 2023; da Silva Vale et al., 2021). This research lays the groundwork for developing ethical, reproducible, and potentially halal-certified alternatives to civet coffee, one that preserves its mystique without compromise.

Despite these promising findings, several limitations of this study should be acknowledged. First, the fermentation and cupping test stages were conducted without replication, limiting our ability to validate the results and draw inferences using robust statistical analyses. Second, one of the promising bacterial isolates, TM.B1, could only be identified to the Enterobacteriaceae family level. Re-sequencing of this isolate is necessary to determine its genus and species, particularly to address safety considerations prior to any future application. Based on the limitations and outcomes of this study, we propose future research to explore the use of mixed cultures in coffee fermentation, as microbial consortia frequently offer complementary biochemical activities that may enhance flavor complexity. For instance, Todhanakasem et al. (2024) reported diverse microbial communities during natural wet processing of Arabica coffee, where dominant yeasts and lactic acid bacteria showed strong correlations with desirable flavor and aroma compounds. Similarly, Nakyinsige et al. (2025) demonstrated the advantage of mixed cultures. A consortium consisting of two bacteria and two yeasts reduced the fermentation time by 30% compared with the control and achieved a cupping score of 84, outperforming the control and all single-, two-, and three-starter treatments. Their results support the idea that mixed-culture fermentation is a promising path for improving coffee quality since they show that multi-taxa consortia, not simply single strains, generate flavor complexity. Even though the current study concentrated on single-strain inoculations to determine baseline performance, these findings offer a basis for developing future mixed-culture or consortium-based systems after strain compatibility, safety, and the best inoculation techniques have been thoroughly examined.

5 CONCLUSIONS

This study successfully isolated and characterized microbial strains from civet feces collected in Toraja and Simalungun and identified two yeast isolates as *Kluyveromyces marxianus* and one bacterial isolate belonging to the Enterobacteriaceae family. These strains demonstrated relevant enzymatic activities and contributed to positive changes in fermentation outcomes under the conditions tested. *K.*

marxianus isolates, in particular, showed consistent potential for enhancing wet-processed Arabica fermentation and therefore represent promising candidates for further development of ethical, animal-free approaches to civet-style coffee processing.

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7 AUTHORS' CONTRIBUTIONS

Conceptual idea: Taufik, I.; Renggaman, A.; Methodology design: Taufik, I.; Putri, N.; Salsabilla, S.; Data collection: Taufik, I.; Putri, N.; Salsabilla, S.; Data analysis and interpretation: Putri, N.; Salsabilla, S.; Writing and editing: Taufik, I.; Renggaman, A.; Putri, N.; Salsabilla, S.

8 DATA AVAILABILITY STATEMENT

Data available upon request to authors

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