The use of starter cultures in lab-scale cacao fermentation and its impact on non-volatile compounds and flavor profile

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Abstract. Indonesian cacao exhibits strong potential as Fine Flavor Cacao (FFC), as demonstrated by its recognition at international events. However, maintaining consistent cacao bean quality remains a major challenge, largely due to spontaneous fermentation processes that result in variable microbial communities. This study aimed to optimize laboratory-scale cacao bean fermentation through the application of defined microbial starter cultures. Fermentation was conducted using either a single yeast starter (*Pichia kudriavzevii*) or a mixed starter culture comprising *P. kudriavzevii*, *Lactiplantibacillus plantarum*, and *Acetobacter tropicalis*. Each treatment utilized 1.2 kg of fresh cacao beans and was performed in duplicate. Following fermentation, the non-volatile compounds composition and flavor profile of the cacao beans were analyzed. The results showed that fermentation with the *Pichia* starter produced cacao beans characterized by more pronounced floral and woody notes, whereas the mixed starter culture resulted in cacao beans with enhanced fresh fruit notes. Both fermentation strategies yielded cacao beans with high-quality flavor profiles. These findings highlight the potential of starter culture–based fermentation as a viable approach for small-scale cacao processing to achieve consistent and tailored flavor characteristics.

1. Introduction

The development of Indonesian cacao, which in recent years has earned international Cacao of Excellence awards [1,2] has revealed the potential for developing Fine Flavor Cacao (FFC). This is supported by Indonesia's natural diversity, stretching from Sabang to Merauke. FFC is an important cacao product whose economic value relies significantly on the quality of fermented cacao beans [3].

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Fermentation is a biological process that transforms the chemical components of cacao beans through the activity of microorganisms, resulting in the formation of precursors for chocolate flavor and aroma. However, the spontaneous fermentation commonly carried out by farmers often produces variable and suboptimal results. Therefore, the application of microbial starter cultures is a solution to improve the quality and efficiency of cocoa fermentation [4,5].

Commonly used starter cultures include *Saccharomyces cerevisiae* [6,7], a yeast that ferments sugar into alcohol and produces CO_2, which aids aeration; *Lactiplantibacillus plantarum*, a lactic acid bacteria that converts sugar into lactic acid, lowering pH and aiding flavor formation; and *Acetobacter aceti,* an acetic acid bacteria that oxidizes alcohol into acetic acid, essential for the development of cacao's distinctive aroma [8,9]. Using this combination of starters can direct fermentation to the desired metabolic pathway, accelerate the fermentation process, and produce more optimal metabolites, including non-volatile compounds that contribute to the development of cocoa flavor [5, 10,11].

Cocoa flavor is the result of the complex interaction of various chemical compounds formed during the fermentation, drying, and roasting processes of cacao beans. These compounds are divided into two main groups: volatile compounds responsible for aroma, and non-volatile compounds responsible for basic tastes such as sour, sweet, bitter, and astringent [12]. These non-volatile compounds are sensed by the tongue and significantly determine the final flavor characteristics of cocoa and chocolate products. For example, isoamyl acetate or ethyl acetate contribute to fruity flavors [13].

This research was conducted on a laboratory scale using microorganism isolates identified in previous studies: *Pichia kudriavzevii, Lactiplantibacillus plantarum, and Acetobacter tropicalis*. These three types of microorganisms were successfully isolated from spontaneous cacao fermentation. Several studies have shown that the use of indigenous starter cultures produces more consistent, safer, and more efficient cocoa products than commercial starter cultures [14, 15]. Indigenous microorganisms are well adapted to the cacao environment, resulting in higher production of non-volatile flavor precursors (e.g., peptides, amino acids, and reducing sugars) and more flavor development than commercial or spontaneous fermentation [15].

The objective of this study was to examine the impact of starter treatments using yeast alone and mixed starter cultures during fermentation to the non-volatile compounds and flavor profiles of fermented cocoa bean in laboratory scale. The novelty of this study is the use of these three types of microorganisms in cocoa fermentation from East Java, Indonesia.

2. Materials and Methods

2.1. Material

Fresh cacao beans from Trawas, East Java, isolates *Pichia kudriavzevii* (in YEPD Broth), *Lactiplantibacillus plantarum* (in MRS Broth), and *Acetobacter tropicalis* (in YED Broth).

2.2. Equipments

A wooden fermentation box ($10 \times 10 \times 20$ cm³) with small perforations on each side was used for cacao fermentation. Incubation was carried out in a Memmert UF110 incubator. Pyrex glassware was used throughout the experiments. Chemical analysis was performed using a Shimadzu LCMS-8040 system (Japan) equipped with a C18 reverse-phase column (2.1 mm × 100 mm, particle size 1.7–3 µm) and an MS-integrated detector with a flow rate range of 0.01–2.0 mL/min. LC–MS analysis was conducted using ethanol as the mobile phase, a column temperature of 40 °C, a flow rate of 0.3 mL/min, an injection volume of 5 µL, and a total analysis time of 30 min.

2.3. Preparation, sampling, and data collection

Wet cacao beans were removed from the shell aseptically and sorted. Cacao beans with white pulp, good and fresh were put in a wooden box of up to 1.2 kg and the starter was given 1% (v/w) then covered with banana leaves. Incubation was carried out with a temperature profile like spontaneous fermentation by adjusting the incubator temperature and controlling the inside cacao temperature in the incubator. Fermentation was carried out for 108 hours by the release of black liquid when the split and the bean flesh formed cavities. Drying of cacao beans using sunlight was carried out after the fermentation process until the moisture content was below 8%.

This experimental study compared two starter culture treatments consisting of *Pichia kudriavzevii* alone (PS) and a mixed culture (MC) consist of *Pichia kudriavzevii* (yeast), *Lactiplantibacillus plantarum* (LAB), and *Acetobacter tropicalis* (AAB) against a non-inoculated control. Cacao beans from each treatment (replicated twice) were dried and analyzed via expert sensory flavor evaluation and liquid chromatography-mass spectrometry (LC-MS). The flavor profiles was conducted by six expert panelists from the Indonesian Coffee Cocoa Research Institute (Puslitkoka/ICCRI). The sensory analysis were conducted based on the descriptive tests on cacao using a standardized procedure of based on the Caobisco/The European Cocoa Association (ECA)/the Federation of Cocoa Commerce (FCC) method (Caobisco/ECA/FCC [16].

2.4. Data analysis

Statistical analysis was performed using analysis of variance (ANOVA), while multivariate patterns were explored using principal component analysis (PCA).

3. Result and Discussion

Fermentation was carried out with 1.2 kg of fresh cacao beans for each fermentation box, which were covered banana leaves. Although each treatment was performed in duplicate (n = 2), consistent trends were observed across replicates. Due to limited replication, further studies with larger sample sizes are required to validate this findings. The temperature profile was controlled by performing the fermentation in an incubator set to simulate spontaneous fermentation, based on preliminary experiments. The temperature was maintained at 25 °C at the onset of fermentation, increased to 30 °C after 24 h, raised to 35 °C at 30 h, 40 °C at 42 h, and 45 °C at 48 h, and further elevated to 47 °C from 60 to 72 h. Thereafter, the temperature was gradually reduced until the fifth day of fermentation, at which point the beans exhibited a dark brown to blackish coloration upon cutting, accompanied by a noticeable decrease in acetic acid odor. The incubator temperature was adjusted to replicate the temperature profile of spontaneous cacao fermentation. To verify that the internal fermentation conditions were representative of natural processes, the temperature within the cacao bean mass was measured using a calibrated thermometer throughout the fermentation period. This monitoring ensured that the internal mass temperature followed the desired profile, thereby reflecting natural fermentation dynamics

3.1. Flavor Profile of Dried Fermented Beans Using *Pichia* Starter (PS) and Mixed Starter (MS)

The sensory evaluation showed that cacao beans fermented with the *Pichia kudriavzevii* starter (PS) exhibited increased woody and nutty notes compared with the non-inoculated control. A qualitative shift in floral perception was also observed, changing from a jasmine-like note in the control to a kenanga-like note in the PS-fermented beans. Table 1 was a sensory flavor profiles of cacao beans from Control, Pichia starter (PS), and Mixed starter (MS) fermentations evaluated by trained and internationally certified panelists (descriptive trends without statistical analysis). The intensity of the woody note increased from 1.75 in the control to 2.50 in the PS treatment, indicating a clear enhancement of this sensory attribute. This increase in woody perception may be attributed to the metabolic activity of *Pichia* yeast during fermentation. *Pichia* species are known to produce aroma active compounds such as phenols, ester, aldehydes, and higher alcohols, which contribute to woody and nutty sensory characteristics. In addition, *Pichia* may facilitate the degradation of complex phenolic compounds in cacao beans into more volatile derivatives, thereby enhancing the perception of woody notes [17]. The use of *Pichia* as a starter culture may also enrich the overall aroma spectrum through interaction among aroma compounds, ultimately intensifying woody perceptions [18]. A reduction in the intensity of browned fruit notes, from 2.00 to 1.00, was also observed. This degradation may have contributed to the increased prominence of woody aromas by reducing competing sensory attributes, although it did not result in a substantial change in the overall flavor balance.

Table 1. Sensory flavor profiles of cacao beans from Control, Pichia starter (PS), and Mixed starter (MS) fermentations

Description	Control	Fermented Beans with PS	Fermented Beans with MS
Cocoa	6.00	6.00	5.50
Acidity	1.00	1.00	2.00
Bitterness	5.00	5.00	4.50
Astrigency	4.75	4.75	4.50
Fresh Fruit	0.50	0.50	2.00
Browned Fruit	2.00	1.00	1.00
Floral	2.00	2.00	1.00
Vegetal	1.25	1.00	1.00
Spicy	0.00	0.00	0.00
Woody	1.75	2.50	1.50
Nutty	1.00	2.00	2.00
Sweet	0.00	0.00	0.00
Roasted	5.50	5.50	5.50
Overall	7.13	7.13	6.00

The nutty flavor attribute increased significantly with the *Pichia* starter treatment. This was consistent with the previous research that found *Pichia* spp. produced nutty flavor volatiles, including 2-acetylpyrrole, 2,3-butanedione (diacetyl), and pyrazine derivatives. Those compounds contribute directly to nutty, roasted, or grainy aromas [18]. *Pichia* yeast catabolizes leucine and valine via Strecker degradation, producing characteristic nutty fragrance aldehydes such as 2-methylbutanal (from isoleucine/leucine), 3-methylbutanal (from leucine), and isovaleraldehyde (from valine). [19]. Compounds resulting from *Pichia* fermentation can increase reactivity in the Maillard reaction during roasting which is strengthen the nutty aroma through the formation of compounds such as furfural or pyrazine during roasting [19][20].

The floral characteristics of the control treatment resemble jasmine (light, fresh, and soft). Meanwhile, the *Pichia* starter resembles kenanga flavor (stronger, more exotic, and tends to be slightly sweet and pungent). *Pichia* is known to produce floral compounds, but the types can differ compared to spontaneous fermentation (control). It could be associated that *Pichia* can convert aroma precursors (such as terpenoids from cacao beans or pulp) into different compounds through enzymes like alcohol acetyltransferase and esterase. These enzymes can transform floral aromas into more exotic or intense versions [18]. Floral aromas are often influenced by the presence of other compounds. Reducing the intensity of dried fruit or vegetation in the *Pichia* starter may contribute to the heavier floral aromas (kenanga floral) more prominent than the lighter ones (jasmine floral).

MS-fermented cocoa beans had reduced cocoa, bitterness and astringency taste compared to that of control. However, there was an increase in acidity, fresh fruit and nutty notes. These differences in the results compared to that of PS might be associated with the activity of other microorganisms such as lactic acid and acetic acid bacteria. These bacteria were known to produce various organic acids and other metabolites. Hence, some of the metabolites may contribute to increased acidity and fresh fruit notes (which are related to the acidity). Furthermore, decreased perception of bitterness and astringency may help to increase the perception of acidity in the cocoa beans [21]. This was in-line with the result showed by the non-volatiles compound analysis.

3.2. Non-Volatile Compound Profiles of Cacao Beans Determined by LC–MS

The LCMS test examined compounds found in dried fermented cacao beans, particularly non-volatile compounds. These compounds significantly influence cocoa flavor. On the other hand, volatile compounds primarily influence cocoa aroma. Flavor and aroma influence the overall flavor. Non-volatile compound testing revealed several increases and decreases in group compound composition with the addition of starter. Bean flavor, influenced by several amino acids, such as leucine and valine, both experienced increases in LCMS results (detailed data per compound is not presented in this article) for control cocoa beans (2.73% area; 2.51% area), with starter (3.35% area; 3.12%area), as well as vanillic acid in the control (1.43%area) and with Pichia starter (1.49%area).

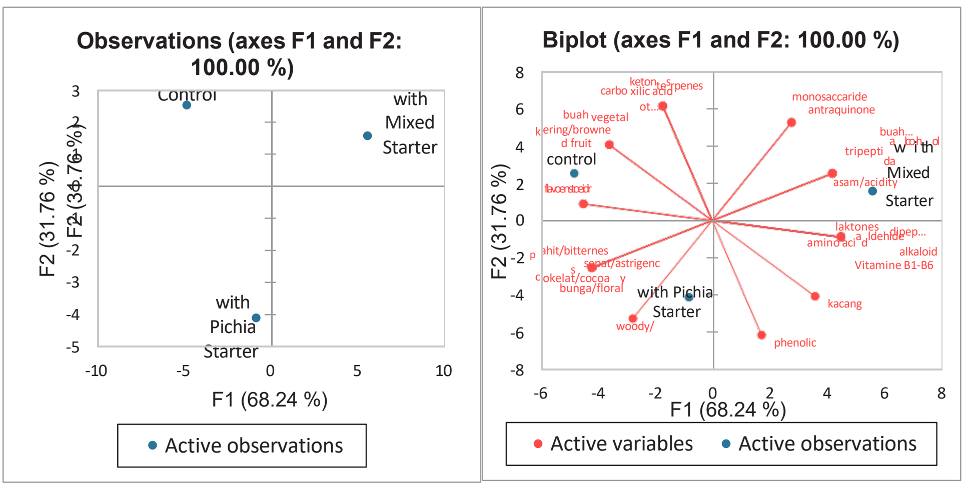
Table 2. Non-volatile compound groups (% area) in dried cacao beans fermented with *Pichia* starter (PS) and mixed starter (MS)

Compound Group	Control	Fermented Beans with PS	Fermented Beans with MS
Aldehide	0.3166	1.2027	2.2844
Alkaloid	4.4365	6.9959	7.629
Antraquinone	1.4424	1.0370	1.5843
Amino acid	25.9069	37.6917	40.7052
Carboxilic acid	8.3908	5.0208	5.9843
Dipeptide	1.6784	3.4660	5.5966
Ester	4.5047	4.1828	3.2311
Phenolic	4.3417	5.6221	4.5233
Flavonoid	40.6628	26.9017	16.0852
Ketones	2.3693	2.1233	2.2424
Others	3.0041	0.7280	1.8759
Laktones	0.4059	1.4786	2.6748
Monosaccaride	1.2702	0.8312	1.2724
Terpenes	0.7650	0.7073	0.7470
Vitamine B1-B6	0.5047	2.0109	2.1237
Tripeptide	0	0	0.4961
Alcohol	0	0	0.7339

Fermented beans with MS showed an increase in aldehyde, alkaloid, lactone, amino acid and peptide composition (amino acids, dipeptides, and tripeptides), contributing to the resulting flavor complexity. The presence of acidic compounds, such as carboxylic acid, ester, aldehyde, ketones, lactones may contribute to the increased sour taste observed in Table 1. Other non-volatile compounds that appear dominant compared to the test results of non-volatile control compounds and fermented beans with MS are the composition of 3 type of alcohol compounds and this is likely what strengthens the fresh fruit taste in Table 1. This result is similar with [22] was research on volatile compounds of fermented bean with *Pichia kudriasevii* and *Hanseniaspora*. Table 2 also shows that the composition of several vitamins B1 to B6 appears and this provides added value to the fermentation results in terms of nutritional content.

3.3. Principal Component Analysis (PCA) of Flavor Attributes and Non-Volatile Compounds in Control, Pichia Starter (PS), and Mixed Starter (MS) Fermentations

The control is located in the upper left quadrant, far from the two starter treatments. The metabolite composition of dried fermented cacao beans with *Pichia* (*Pichia* Starter) and the control starter mixture of yeast, lactic acid bacteria and acetic acid bacteria (Mixed Starter), as well as the PCA analysis of the flavor results, is shown in the figure below.



A B

Fig 1. Principal Component Analysis (A:Scatter Plot, B:Biplot) of flavor and non-volatile compounds of cocoa beans obtained from different treatments

The starter with *Pichia* and the mixed starter are significantly separated from the control, and also slightly separated from each other, with the mixed starter in the upper right quadrant and the starter *Pichia* in the lower left quadrant (Figure 1A). This indicates that the chemical composition (likely metabolites from fermentation) differs significantly between the control and the two starter treatments. The starter with *Pichia* and the mixed starter also differ, although not as significantly as compared to the control.

Figure 1B shows that the fermented cacao beans with the control treatment are predominantly browned fruit and floral, the fermented cacao beans with the addition of 1% Pichia starter are in the bottom left showing dominance related to the phenolic compound group and woody flavor. The fermented cocoa beans with the addition of mixed starter are in the top right showing dominance related to fresh fruit flavor, nuts and acidity.

4. Conclusion

This study showed that the application of *Pichia kudriavzevii* as a single starter (PS) and in mixed culture (MS) significantly influenced the sensory properties and non-volatile compound profiles of fermented cacao beans. PS enhanced woody and nutty attributes, while MS increased acidity and fresh fruit notes with reduced bitterness and astringency. Yeast-based starter cultures improved overall flavor complexity. However, high bitterness was still observed. In the current state, the use of mixed starter is more appropriate. Further studies are required to optimize yeast-based cultures to reduce bitterness and achieve cacao bean quality comparable to fine- flavor cacao

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