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Pulp Reduction and Addition of Indigenous Microorganisms as Starter: Effects on Fermented Cocoa Bean Characteristics

Anna Fajariyah[1], Endang Sutriswati Rahayu[2*], Titiek Farianti Djaafar[1], Tyas Utami[2], Dian Anggraini Suroto[2], Tri Marwati[1]

[1]Research Center for Food Technology and Processing, Jalan Jogja-Wonosari Km. 31.5, Gading, Playen, Gunungkidul, Yogyakarta 55861, Indonesia
[2]Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Jl. Flora No. 1, Bulaksumur, Yogyakarta 55281, Indonesia
[*]Corresponding author: Endang Sutriswati Rahayu, Email: endangsrahayu@ugm.ac.id

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ABSTRACT

Indonesian cocoa smallholder plantations frequently produced low-quality dry beans due to poor post-harvest handling, necessitating improvements through controlled fermentation. This study evaluated pulp reduction and local microorganism starter addition in farmer-level cocoa fermentation on the microbiological, physical, and chemical properties of cocoa beans. Using a Factorial Randomized Block Design, the research included four experimental units: 1) spontaneous fermentation, 2) reduced-pulp fermentation, 3) starter-added fermentation, and 4) reduced-pulp and starter-added fermentation. Pulp was reduced by approximately 35%, and microorganisms utilized were *Candida famata* HY-37, *Lactiplantibacillus plantarum* subsp. *plantarum* HL-15, and *Acetobacter* sp. HA-37. The five-day fermentation resulted in the growth of yeast, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). This was indicated by a peak fermentation temperature of 45 °C, a decrease in fermentation pH to 3.7, and a decrease in bean pH to 4.7, in line with total titratable acidity increasing. Over the course of two days, the Fermentation Index aboved 1.0. Pulp reduction raised fermentation temperatures and resulted in an earlier peak by the third day. The use of a starter can suppress fungal growth during fermentation. The total fungal population is lower in combining pulp reduction with starter addition fermentation cocoa beans. The physical properties met the Indonesian National Standard (SNI) 2323:2008/Amd I:2010. The chemical characteristics showed that the Fermentation Index was above 1.0, the bean pH values were from 5.16 to 5.36, and the reducing sugar contents were from 1.50 to 1.69 %. Incorporating starter treatment effectively inhibited fungal growth during fermentation. The combination of reducing pulp and adding starter inhibited fungal growth in fermented cocoa beans without affecting their physical and chemical properties.

Keywords: *Acetobacter* sp HA-37; *Candida famata* HY-37; cocoa fermentation; *Lactiplantibacillus plantarum* subsp. *plantarum* HL-15; pulp

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INTRODUCTION

According to global statistics, Indonesia was ranked sixth in the world for cocoa (*Theobroma cacao* L) production in 2019 and the highest producer in Asia and Oceania (ICCO, 2023). Most of this production is obtained from smallholder plantations (Badan Pusat Statistik, 2020). However, despite high production levels, the quality of fermented cocoa beans remains low, reducing their market competitiveness. Many cocoa beans are either fermented, have a bitter taste, are too sour, or contain free amino acids that form a lower taste compared to products from other countries. Additionally, the presence of many fungi and mycotoxins is a significant issue, thereby leading to contamination (Sabahannur et al., 2016; Caligiani et al., 2016; Nugroho et al., 2013). This condition is partly caused by farmers not carrying out cocoa bean fermentation properly.

During the post-harvest cocoa phase, fermentation is an important factor to be put into consideration because it can affect the quality of the chocolate produced. Cocoa pulp is hydrolyzed by microorganisms into organic acids and other metabolic end products. These acids enter cocoa beans and initiate enzymatic reactions that contribute to the development of flavor and color precursors, while also effectively preventing bean germination. This process plays a vital role in enhancing the characteristics of cocoa. The microorganisms involved in this process consist of yeast, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) (De Vuyst and Leroy, 2020).

In fermentation, each of the microorganisms has a specific role. Yeast metabolizes carbohydrates in pulp to generate carbon dioxide, ethanol, and heat. It also released pectinase enzymes to degrade pectin in the pulp. Furthermore, yeast can produce glycerol, succinic acid, acetic acid, acetoin, and esters as flavor components in fermented cocoa beans. Fructophilic LAB that grow with yeast ferment fructose and produce lactic acid, acetic acid, and pyruvate metabolites. Pulp citric acid is also converted by LAB, resulting in acetic acid and lactic acid. Heterofermentative LAB metabolizes glucose into lactic acid, acetic acid, ethanol, carbon dioxide, or mannitol. AAB oxidizes ethanol to acetic acid, producing heat and lactic acid to acetoin and acetic acid (De Vuyst and Leroy, 2020). Yeast and LAB are also known to be able to inhibit fungal growth during fermentation (Ruggirello et al., 2019).

The microorganisms' growth pattern, fermentation time, and acidity of fermented cocoa beans can be influenced by the content of cocoa pulp. Forastero cocoa, which is widely grown in Indonesia, has a thicker pulp compared to other varieties. When fermented without prior treatment, it tends to develop excessive sourness and requires a longer fermentation period (Sumanto et al., 2015). The reduction of some of the pulp leads to a decrease in sugar that can be fermented by microorganisms. Furthermore, the acidity and sourness of cocoa beans can be reduced (Afoakwa et al., 2012). Fermentation time can also be reduced, thereby making it more efficient in the production of fermented cocoa beans (Sumanto et al., 2015). In addition, pulp by-products can be processed into various food products, herbicide ethanol, and composting activators (Purwaningsih et al., 2021).

Fermentation can occur spontaneously after the cocoa pod is opened, but the quality of the results is inconsistent, making it challenging for the industry to formulate high-quality cocoa products. To obtain better quality cocoa beans, controlled fermentation can be carried out by reducing temperature and adding a culture starter.

Starter cultures are used to improve dried cocoa beans and chocolate qualities further, accelerate fermentation, and prevent fungal growth and mycotoxin production. Mixed cultures successfully added to cocoa fermentation experiments include *Saccharomyces* cerevisiae, *Lactiplantibacillus plantarum subsp. plantarum* (updated nomenclature of *Lactobacillus plantarum*) and *Acetobacter aceti* (Saunshi et al., 2020), as well as *S. cerevisiae, L. plantarum, and A. pasteurianus* (Magalhães da Veiga Moreira et al., 2017). In another study, *Candida famata* HY-37, *Lactiplantibacillus plantarum* subsp. *plantarum* HL-15 and *Acetobacter* sp. HA-37 cocktail was used by Rahayu et al., (2021). These microorganisms are locally isolated from cocoa fermentation in Gunungkidul Regency, Yogyakarta, Indonesia (Djaafar et al., 2017). In laboratory-scale experiments, during fermentation and drying, *L. plantarum* HL-15, alone or combined with *Candida famata* HY-37 and *Acetobacter* sp. HA-37 effectively inhibits *Aspergillus niger* YAC-9 growth and ochratoxin A production (Rahayu et al., 2021).

Consider the importance of the role of yeast, BAL, and AAB added in controlled fermentation, and the benefits of pulp reduction that can be obtained. A study is needed on cocoa bean fermentation that involves a combination of pulp reduction and the addition of local microorganism starters: *Candida famata* HY-37, *Lactiplantibacillus plantarum* subsp. *plantarum* HL-15, and *Acetobacter* sp. HA-37. This research should be conducted on a large scale in cocoa processing units owned by farmers, with a capacity of 40 kg of cocoa beans. An investigation is needed to analyze the microbiological, physical, and chemical characteristics of fermented cocoa beans. The aim is to produce high-quality beans with minimal fungal contamination that meet the Indonesian National Standard (SNI) and have strong flavor potential.

METHODS

Materials

The main materials used include (1) cocoa fruit from farmer plantations in Gunungkidul, Bantul, and Kulon Progo Regencies, Yogyakarta, Indonesia, and (2) pure cultures of *Candida famata* HY-37, *Lactiplantibacillus plantarum subsp. plantarum* HL-15, and *Acetobacter* sp. HA-37 acquired from the Food Nutrition Culture Collection, Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta, Indonesia.

The media used in microbiological analysis included de Man, Rogosa, and Sharpe (MRS) broth (Merck Darmstadt, Germany), Dichloran Rose Bengal Chloramphenicol Agar (DRBCA) (Merck Darmstadt, Germany), Bacteriological Peptone (Oxoid LP0037 Basingstoke, UK), D(+)-glucose (Merck Darmstadt, Germany), yeast extract (Oxoid LP0021 Basingstoke, UK), $CaCO_3$ (Merck Darmstadt, Germany), 0.85% NaCl solution, and distilled water. For chemical analysis, hydrochloric acid (Merck Darmstadt, Germany), methanol (Merck Darmstadt, Germany), anhydrous glucose, Nelson's reagent, arsenomolybdate reagent, NaOH (Merck Darmstadt, Germany), OPA solution (orthophalaldehyde) (Merck Darmstadt, Germany), Na acetate (Merck Darmstadt, Germany), and tetrahydrofuran (THF) (Merck Darmstadt, Germany), and distilled water were used.

Method

This study used a Factorial Randomized Block Design with two influential factors, namely pulp reduction and starter addition. The experimental units were 1) spontaneous fermentation (F1), 2) reduced-pulp fermentation (F2), 3) starter-added fermentation (F3), and 4) reduced-pulp and starter-added fermentation (F4), with the experiment repeated three times.

Preparation of Cocoa Fermentation Starter Culture

The media for yeast growth is a liquid medium composition containing peptone (4.5 g/L), glucose (20 g/L), and yeast extract (7.5 g/L). The growth medium for *L. plantarum* HL-15 used MRS broth (52 g/L), while *Acetobacter* sp. HA-37 used peptone (4.5 g/L), glucose (20 g/L), and yeast extract (4.5 g/L). Culture involved adding 0.1 mL of stock culture to a test tube with 5 mL of media, then incubating at 30°C for yeast and AA and 37°C for LAB for 24 h. After transferring 5 mL of the culture into an Erlenmeyer flask with 50 mL of medium, the incubation process was carried out as before. Fifty mL of culture was transferred into a 750 mL of medium in an Erlenmeyer flask, then incubated as previously described. The cells were then centrifuged to obtain cell supernatant as a liquid starter with a concentration of 10^{10} CFU/mL (Anantama, 2020).

Cocoa Pulp Reduction

Cocoa pulp reduction was prepared by reducing cocoa bean pulp by about 35% of pulp weight, using a mechanical depulper machine (Honda, Japan). The mechanical depulper machine is a horizontal cylindrical stainless steel machine, with a capacity of 500 kg, driven by a 5.5 HP gasoline engine and a speed of 370-450 rpm (Sumanto et al., 2015).

Cocoa Fermentation Implementation

Cocoa fermentation was conducted in a cocoa fermentation room using a wooden fermentation box (size 40x40x40 cm) with a capacity of 40 kg. As much as 36 kg of sorted cocoa beans were placed in the box. The box was surrounded by banana leaves and covered with banana leaves and burlap on the top of the cocoa bean pile before being closed with a wooden lid. Fermentation was carried out for 5 days at room temperature with stirring and turning every 2 days. After the fermentation process was completed, the cocoa beans were put into water and soaked for 15 min, then washed, drained, and dried using a semi-mechanical dryer until the water content in the beans reached approximately 7% (Marwati et al., 2021). The analysis was carried out every day of fermentation, including measuring temperature, fermentation pH, bean pH, total titrated bean acid, Fermentation Index, and total microorganisms. A bar thermometer was used to measure temperature at three different locations throughout the fermentation box. The pH of the fermented mass was determined using a pH meter.

Enumeration of Microorganism Population

Microorganisms analyzed included the total population of yeast, LAB, AAB, and fungi. Forty grams of fermented cocoa beans were added with 360 mL of sterile 0.85% NaCl solution to make a slurry using a stomacher. The slurry was then diluted into 7 series, and the last three dilution series were plated using dilution plating. After incubating at 30 °C for 48 h for yeast and AAB, and at 37 °C for 48 h for LAB, the population was counted (Marwati et al., 2021).

Fungal enumeration by direct plating began with taking 60 g of cocoa beans from the fermentation box. Furthermore, 15 cocoa beans were disinfected on the bean surface before plating and then placed on DRBC media. Incubation was completed for 5 days at 30 °C (Rahayu et al., 2014). The total weight of 15 cocoa beans was approximately 40 g. The samples taken were almost the same as cocoa bean samples used for microorganism enumeration. The total fungal enumeration was performed by calculating the percentage of beans attacked by fungi (Rahayu et al., 2014). This calculation was the number of beans attacked by fungi divided by 15 cocoa beans, then multiplied by 100%.

Measurement of pH of Fermented Cocoa Beans and Total Titratable Acidity

A total of 5 g dry cocoa bean powder was dissolved in 45 mL of ±90 °C aquadest, then filtered by using number 4 Whatman paper. The filtrate obtained was cooled to room temperature. Furthermore, 5 mL of filtrate was taken to measure pH. Another 5 mL of filtrate was used for titratable acidity measurement by adding three drops of phenolphthalein indicator and then titrated with 0.1 N NaOH solution. Titratable acidity was calculated as a percentage of acetic acid (Saunshi et al., 2020).

Cocoa Bean Fermentation Index Analysis

The determination of the Fermentation Index was carried out according to Gourieva & Tserevitinov (1979) in Ooi et al. (2020). 50 mL of a mixture of methanol and hydrochloric acid (97:3) (v/v) extracted a total of 0.5 g of dry cocoa bean powder at 8 °C for 16–19 h. Furthermore, the suspension was vacuum-filtered using number 1 Whatman paper. The absorbance of the clear extract was measured using a UV-Vis spectrophotometer (Shimadzu, Maryland, USA) at wavelengths of 460 nm and 530 nm. The Fermentation Index was derived by comparing absorbance values at both wavelengths.

Analysis of Physical and Chemical Properties of Fermented Dry Cocoa Beans

Physical and chemical properties of fermented dry cocoa beans observed consisted of the presence of slaty/unfermented cocoa beans, germinated cocoa beans, moldy cocoa beans, moisture content referring to SNI 2323 2008/Amd I:2010 (Badan Standardisasi Nasional, 2010), water activity (Aw), reducing sugar and amino acid content, pH, total titratable acidity and Fermentation Index.

Water content analysis was carried out thermogravimetrically. Water activity was analyzed using the Conway cup method and saturated salt standards NaCl (0.7532) and KCl (0.8432) (Islam et al., 2016). Quantitative analysis of reducing sugar content used the Somogyi-Nelson method (1944). The optical density of the sample solution and the glucose standard curve were measured with a spectrophotometer at 540 nm to determine the reducing sugar.

Amino acid analysis used High-Performance Liquid Chromatography (HPLC). A 10 µL sample was injected and, at room temperature, passed through a 100 RP-18 column (5 µm) Li-Chrospher. The detector used was Thermo Ultimate 3000 RS Fluorescence. The mobile phase consisted of phase A [methanol:50 mM sodium acetate: tetrahydrofuran (THF) (2:96:2)] at pH 6.8 and phase B (65% methanol) with a 1.5 mL/min flow rate. The wavelength of excitation was 300 nm, the emission was 500 nm, and the linear gradient program was set for 30 min.

RESULTS AND DISCUSSION

Total Yeast Population during Cocoa Bean Fermentation

The total yeast population during fermentation is presented in Figure 1. In all treatments: F1 (spontaneous fermentation), F2 (reduced-pulp fermentation), F3 (starter-added fermentation), and F4 (reduced-pulp and starter-added fermentation), the total yeast population increased on the first day and then gradually decreased until the fifth day of fermentation. The initial total yeast population ranged from 6.79 to 7.36 log CFU/g, then increased on the first day with a population ranging from 6.97 to 7.60 log CFU/g and decreased to the lowest population, ranging from 3.06 to 5.98 log CFU/g.

The total initial yeast population in all treatments was around 7 log CFU/g, following (Marwati et al., 2024) and Marwati et al. (2021) results. The initial population in the F2 and F4 experiments that experienced pulp content reduction was lower compared to F1 and F3. From analysis of variance (ANOVA), it was found that pulp reduction significantly affected yeast population at the beginning of fermentation ($p \leq 0.05$).

In this study, the process of breaking the fruit to obtain wet cocoa beans for fermentation, using four fermentation boxes per experimental repetition, took approximately 10 h. The possibility of yeast population increased to reach about 7 log CFU/g. Furthermore, the starter culture prepared also ranged from 7 log CFU/g (based on several references), therefore the treatment of adding *Candida famata* HY-37 starter, the initial population was not significantly different.

The increase in yeast population reached its peak population on the first day in F1, F2, F3, and F4 with values of 0.29, 0.16, 0.24, and 0.18 log cycles, respectively. These results indicate that the increase in yeast population in fermentation with reduced pulp is lower than in fermentation with full pulp. The pulp reduction treatment was then carried out by reducing the pulp content in cocoa beans; this reduces the sugar

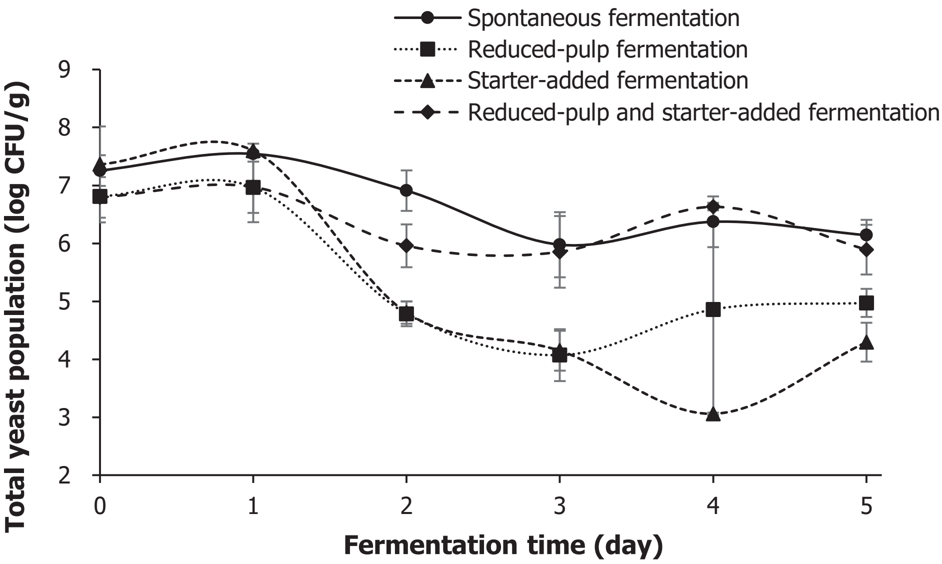

Figure 1. Total yeast population during fermentation

content, which is the major substrate for yeast, and hence impacts its population (Kongor et al., 2016). During the first day of fermentation, when the yeast population was at its peak, pulp reduction and starter addition had no statistically significant influence on yeast population. Yeast could still thrive in cocoa fermentation after pulp reduction and starter addition. These findings align with the findings of Marwati et al. (2024) and Rahayu et al. (2021).

Glucose is a substrate for yeast in fermentation that produces ethanol and CO_2, and the process conditions are exothermic; hence, fermentation temperature increases. The total yeast population then decreased after the first day of fermentation due to decreased glucose availability, decreased anaerobic conditions, and increased fermentation temperature. Anaerobic conditions are reduced due to the activity of depectination by the yeast pectinase enzyme, which causes pulp viscosity to decrease and becomes a pathway for air to enter the fermentation mass (De Vuyst and Leroy, 2020).

The total yeast population in pulp reduction and starter addition treatments was in line with other treatments. Yeast was still able to grow on one day of fermentation and then experienced a decrease in population until the end of fermentation, and this shows that a 35% pulp reduction does not affect yeast growth. Although the sugar substrate is reduced, its availability is still sufficient for yeast growth with the same range as yeast population in other treatments where pulp is not reduced.

The yeast population increased again on the fifth day. This condition is similar to Chagas Junior et al. (2021) study. Sucrose converted into glucose and fructose by the invertase enzyme (De Vuyst & Weckx, 2016). Glucose is then used by yeast for its growth.

Total Population of Lactic Acid Bacteria (LAB) during Cocoa Bean Fermentation

Figure 2 depicts the total LAB population throughout cocoa fermentation. The LAB grew on the first day, then decreased until the fourth day before increasing again on the fifth day. The total LAB population at the start of fermentation was between 6.20 and 6.99 log CFU/g. On the peak of growth, occured on the first day, the population ranged from 7.16 to 7.93 log CFU/g before decreasing to 4.91 to 5.91 log CFU/g. Saunshi et al. (2020) found that during 5 days of fermentation, the total LAB population ranged from 4-8 log CFU/g, which is consistent with our findings.

The increase in the total LAB population occurred on the fourth and fifth days in treatments F1 (spontaneous fermentation), F2 (reduced-pulp fermentation), and F4 (reduced-pulp and starter-added fermentation). Meanwhile, in F3 (starter-added fermentation), there was no increase in the population on the same day because there was a possibility of fluctuation at a slower time. The increase in the LAB population is in line with the rise in yeast population at the end of fermentation. This is possible because the activity of the invertase enzyme is still ongoing (Chagas Junior et al., 2021). This enzyme converts sucrose into glucose and fructose (De Vuyst & Weckx, 2016). Fructose is metabolized by LAB for its regrowth.

In this study, both yeast and LAB can grow at the beginning of fermentation, which indicates that there is

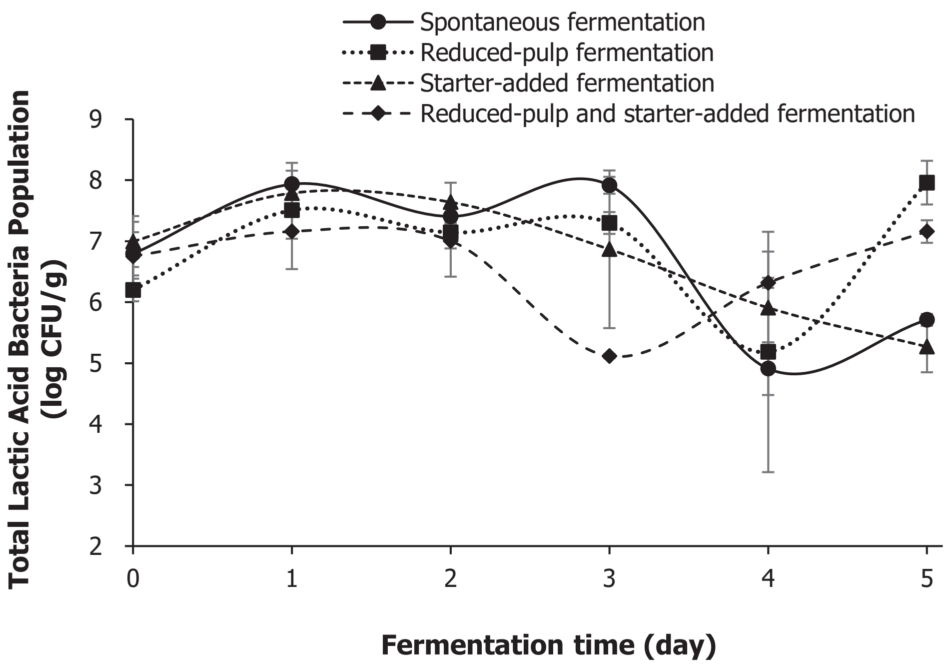

Figure 2. Total Lactic Acid Bacteria population during fermentation

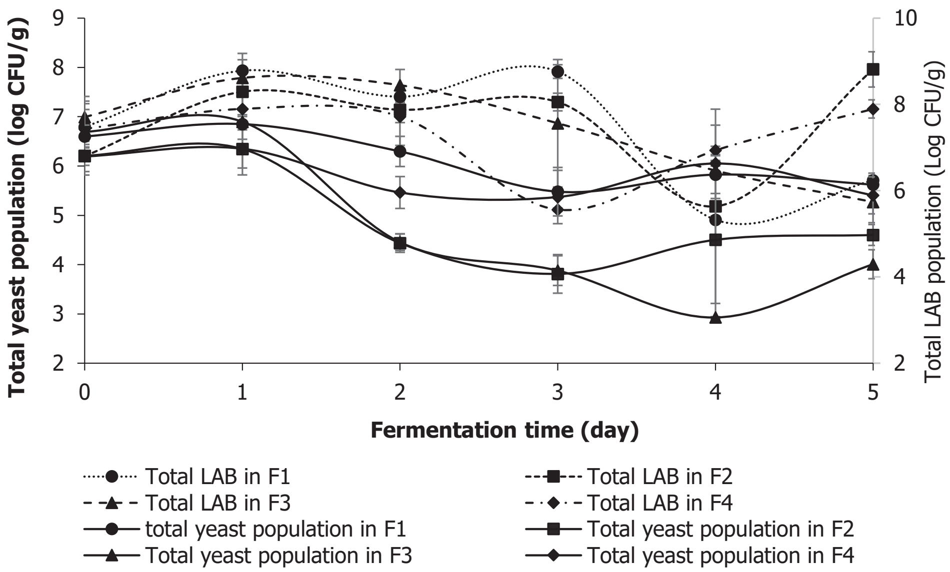

Figure 3. Interaction between yeast and lactic acid bacteria during cocoa fermentation; F1: Spontaneous fermentation; F2: Reduced-pulp fermentation; F3: Starter-added fermentation; F4: Reduced-pulp and starter-added fermentation

no competition between yeast and LAB in carrying out metabolism, namely by utilizing the available substrate (Figure 3). This condition is by the statement of Figueroa-Hernández et al. (2019) that both microorganisms can grow simultaneously in the first 24-36 h of fermentation, under anaerobic conditions, temperatures of 25-30 °C, acidic environments, and rich in glucose and fructose. Yeast can grow by prioritizing available glucose to convert it into pyruvate through glycolysis, and producing ethanol and carbon dioxide. Meanwhile, LAB that grows at the beginning of fermentation is likely to be fructophilic, namely prioritizing fructose consumption and converting citric acid in cocoa pulp (De Vuyst & Leroy, 2020).

This is reviewed from several pieces of literature stating that among LAB, there is a group of fructophilic LAB. This group of bacteria encompasses all species within the genera *Fructobacillus* and *Apilactobacillus*, including *Apilactobacillus kunkeei*, *Fructolactobacillus florum*, *Levilactobacillus brevis*, and *L. plantarum.* These bacteria specifically metabolize fructose as their carbon source and exhibit limited or delayed growth when utilizing glucose as a carbon source (Akihito Endo et al., 2018; Gustaw et al., 2018; Viesser et al., 2020; Zheng et al., 2020).

Statistical analysis results indicate that pulp reduction significantly affected the LAB population on fermentation days 0 and 1 ($p \leq 0.05$). During fermentation with pulp reduction (F2 and F4), the LAB population was lower compared to fermentation without pulp reduction (F1 and F3). This reduction may be due to the lower availability of fructose and citric acid in beans with reduced pulp, which are necessary for fermentation by LAB (Kongor et al., 2016).

The LAB population increased on the first day of fermentation by 1.14, 1.31, 0.79, and 0.41 log cycles for the F1, F2, F3, and F4 treatments, respectively. This increase demonstrates that LAB can thrive during fermentation. Additionally, the treatments for pulp reduction and starter addition did not disrupt LAB growth or the sustainability of cocoa fermentation. These findings are consistent with the study conducted by Marwati et al. (2024) and Rahayu et al. (2021).

Total Population of Acetic Acid Bacteria (AAB) during Cocoa Bean Fermentation

Figure 4 shows that at the beginning of fermentation in the four treatments, the total AAB population ranged from 6.91 to 7.38 log CFU/g, then experienced peak growth on the first day, ranging from 7.38 to 7.69 Log CFU/g. Subsequently, from the second to the fourth day of fermentation, the population tended to decrease, with a population range from 5.08 to 5.47 log CFU/g, and increased on the fifth day of fermentation.

This condition is in line with the statement of Figueroa-Hernández et al. (2019) that AAB have been present since the beginning of the fermentation process and can survive in anaerobic conditions when yeast and LAB grow. After about 48 h of fermentation and "drying" or depolymerization of pulp by the pectinase enzyme from yeast, which produces pulp liquid, the aerobic phase begins. In this phase, AAB can grow and metabolize the ethanol produced by yeast and produce heat.

In all treatments, the anaerobic phase likely ended in less than 48 h and then immediately entered the aerobic phase. This can be observed from the total AAB population, which tended to be stable in the first 2 days. This condition is in line with the growth of total yeast and total BAL in the anaerobic phase, which ended before 2 days of fermentation.

The fluctuation in AAB population on the fifth day of fermentation may be attributed to the simultaneous increase in yeast population. As yeast grows, it metabolizes nutrients and produces ethanol, which serves as a substrate for AAB. Consequently, the total AAB population also rises toward the end of fermentation.

The population increase reached the peak of the AAB population in F1, F2, F3, and F4 in this study, which was 0.33, 0.47, 0.30, and 0.46 log cycles, and the values did not differ much between treatments. These conditions show the growth of AAB; hence, it

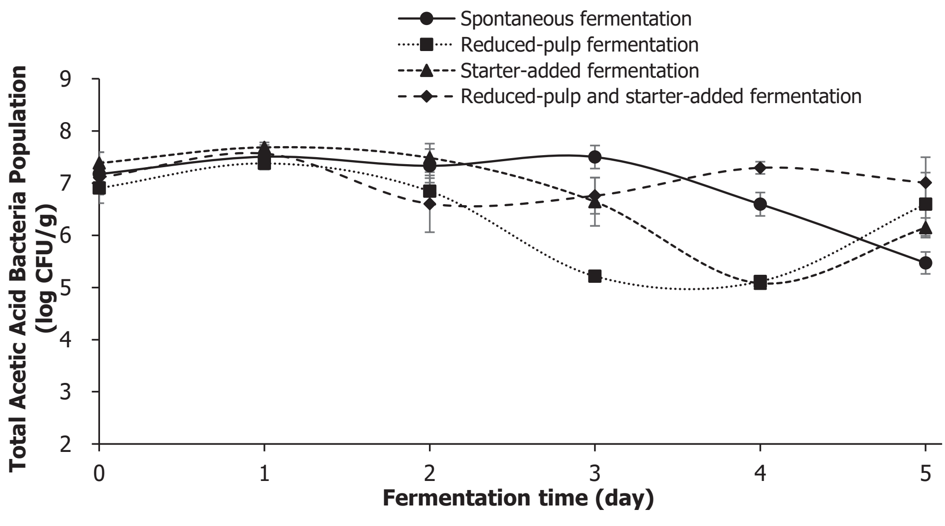

Figure 4. Total acetic acid bacteria total population during fermentation

can be stated that pulp reduction treatment and the addition of a starter did not interfere with the growth of AAB in the cocoa fermentation process. These results are in line with the study by Marwati et al. (2024) and Rahayu et al. (2021).

Total Fungal Population during Cocoa Bean Fermentation

This study also found the presence of fungi on cocoa beans during five days of fermentation, as shown in Figure 5. The total population of fungi was highest at the beginning of fermentation and then tended to decrease until the end of fermentation. The fungi population on each day of observation in all treatments tended to decrease.

Yeasts and LAB involved in cocoa fermentation can inhibit the growth of mycotoxin-producing fungi and the production of their mycotoxins. This antifungal capability mainly results from the organic acids produced by LAB and protein compounds by yeasts, as well as their synergistic effects (Ruggirello et al., 2019). An analysis of diversity (using ANOVA) revealed that neither the pulp reduction treatment nor the addition of starter nor the interaction between the two treatments had a significant effect on the total fungal population during fermentation ($p \geq 0.05$).

In fermentation with reduced pulp (F2 and F4), at the end of fermentation, the fungal population remained relatively high. In cocoa with thicker pulp, LAB produced a higher concentration of metabolites that inhibit fungal growth compared to cocoa with thinner pulp (Marwati et al., 2019). Therefore, thicker pulp results in increased activity of microorganisms in producing acids that can suppress fungal growth.

Changes in Temperature and Fermentation pH during Cocoa Bean Fermentation

The profile of temperature and fermentation pH change during five days of fermentation is shown in Figure 6. In all four treatments, the initial fermentation temperature ranged from 28 °C, then increased to a maximum temperature of 45 °C and decreased, similar to other studies (Visintin et al., 2017; Marwati et al., 2021). Pulp reduction treatment significantly affected the temperature during the first three days ($p \leq 0.05$). In cocoa beans with pulp reduction, the temperature was higher than in beans without pulp reduction. The maximum temperature can also be achieved on the third day of fermentation, which is faster than in spontaneous fermentation and fermentation with the addition of a starter (day 4). This condition is in line with the study of Sumanto et al. (2015).

This study shows that in fermentation with low pulp content, the anaerobic phase becomes shorter and changes to the aerobic phase more quickly. At high pulp volumes, oxygen penetration into the fermented cocoa bean mass will be inhibited, thereby prolonging the anaerobic phase. The heat produced in this anaerobic phase is not as large as the heat produced in the aerobic phase (Haryadi dan Supriyanto, 2017).

The temperature rise is important because it causes cocoa beans to lose their ability to germinate, which is crucial for the development of certain chocolate aromas and flavor precursors (Visintin et al., 2017). The changes observed in the structure of cocoa bean cells depend on temperature, acidity, and fat content. Proteins are degraded at a temperature range of 40-45 °C before lipid vacuole fusion occurs at a temperature of

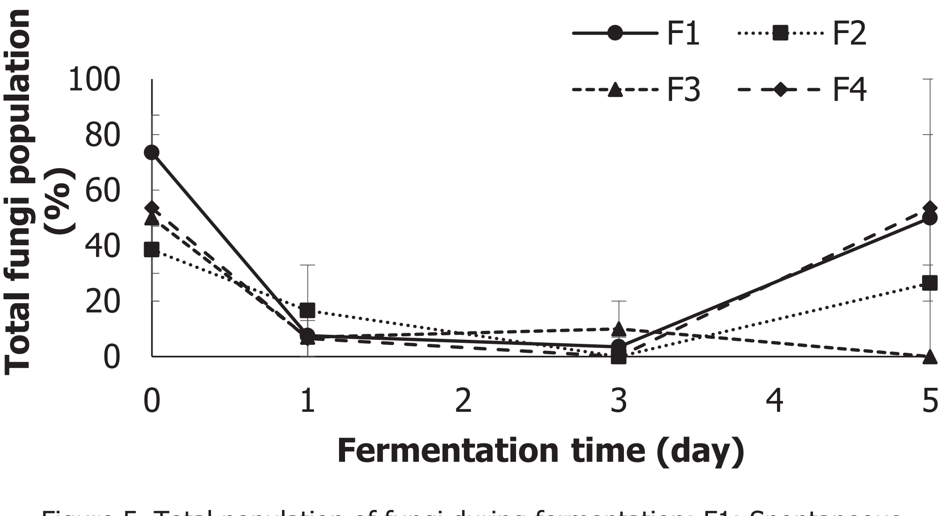

Figure 5. Total population of fungi during fermentation; F1: Spontaneous fermentation; F2: Reduced-pulp Fermentation; F3: Starter-added Fermentation; F4: Reduced-pulp and Starter-added Fermentation

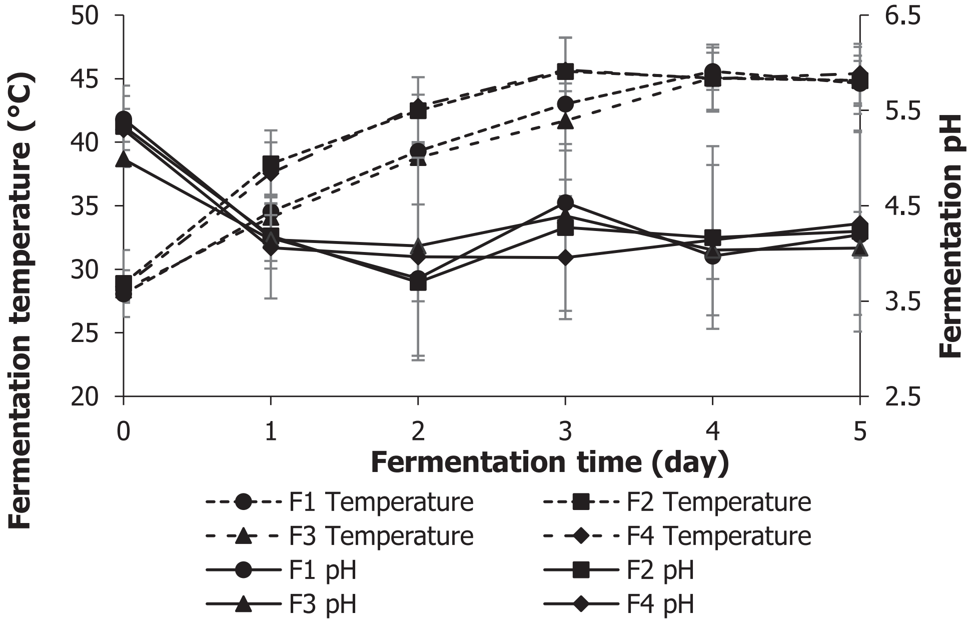

Figure 6. Changes in fermentation temperature and pH during cocoa bean fermentation; F1: Spontaneous Fermentation; F2: Reduced-pulp Fermentation; F3: Starter-added Fermentation; F4: Reduced-pulp and Starter-added Fermentation

50 °C (Santander Muñoz et al., 2020). This temperature range can be achieved in this study; hence, pulp reduction treatment and the addition of a starter cause fermentation to run as it should.

The pH of the fermentation mass was measured on the surface of cocoa beans. Except for the third day of fermentation, there was no significant impact from the pulp reduction treatment, the addition of a starter, or the interaction between these two treatments on the pH of the cocoa mass in any of the fermentation units. On the third day of fermentation, however, the pulp reduction treatment had a significant effect ($p \leq 0.05$). The initial pH was approximately 5, indicating an acidic condition due to the presence of organic acids in the cocoa pulp. The pH decreased slightly on days 1 and 2, then tended to increase from days 3 to 5. This pattern aligns with the results of Sumanto et al. (2015) and Rahayu et al. (2021) studies.

While a subsequent rise in pH denotes a decrease in acidity due to acid diffusion into cocoa beans, a fall in pH indicates the production of acid during fermentation. Cocoa pulp's glucose is fermented by yeast to produce ethanol, which also involves pectinolysis and the creation of flavoring chemicals such organic acids, aldehydes, alcohols, and esters. Lactic acid bacteria (LAB) create a microbiologically stable fermentation environment by fermenting the glucose, fructose, and citric acid in the cocoa pulp to produce lactic acid, acetic acid, mannitol, and pyruvate. Lactate, another byproduct of this process, is a crucial source of carbon for the growth of AAB. AAB then oxidized the ethanol that the yeast produces into acetic acid (De Vuyst & Leroy, 2020).

Changes in pH and Total Titratable Acidity in Cocoa Beans during Cocoa Bean Fermentation

The pH value of cocoa beans tends to decrease during fermentation, in contrast to the Total Titratable Acidity value (as % acetic acid) in cocoa beans, which tends to increase (Figure 7). During the fermentation process, neither the pulp reduction treatment nor the addition of a starter significantly affected the total titratable acidity ($p \geq 0.05$). However, the pulp reduction treatment had a significant impact on pH levels on the fourth and fifth days of fermentation, with a significance level of $p \leq 0.05$. Additionally, the addition of the starter also significantly influenced pH on the fourth day of fermentation ($p \leq 0.05$)

The presence of organic acids in cocoa beans, as shown by a pH decrease, can occur as a result of the accumulation of acetic acid and lactic acid due to diffusion from pulp through the bean and cotyledon tissues. The pH value of cocoa beans, influenced by the acids produced during fermentation, is crucial for enzyme activity. Enzymes such as aspartate protease and carboxypeptidase are essential for the flavor precursors. When the bean pH is between 4.5 and 4, the resulting cocoa beans have little flavor potential. Moderate acidification (pH 5.5-5.0) promotes selective

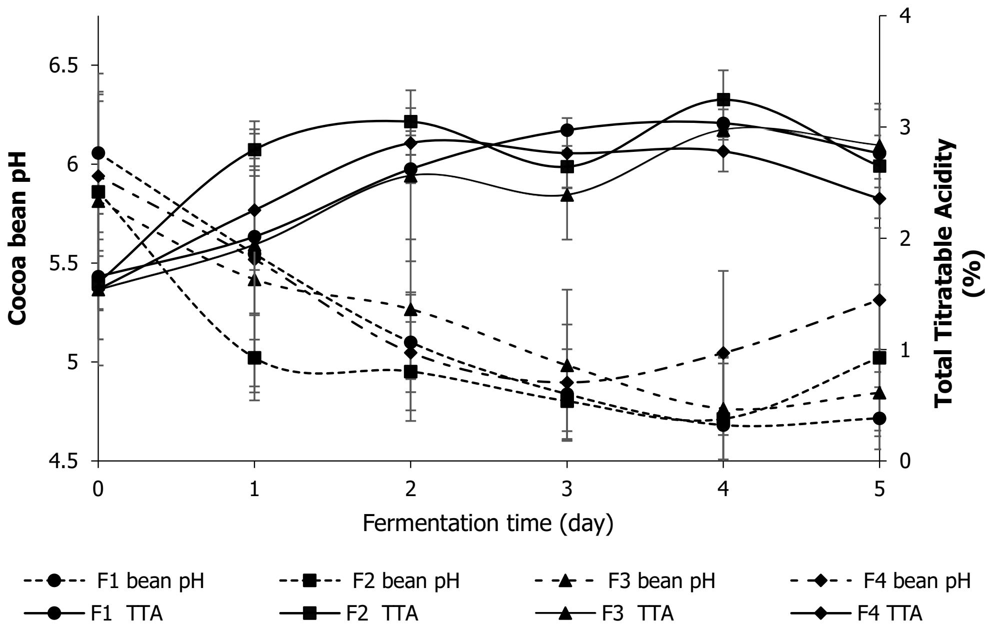

Figure 7. Changes in pH and Total Titrated Acid of cocoa beans during fermentation; F1: Spontaneous fermentation; F2: Reduced-pulp Fermentation; F3: Starter-added Fermentation; F4: Reduced-pulp and Starter-added Fermentation.

protein breakdown, resulting in the synthesis of more hydrophobic amino acids and hydrophilic peptides (Voigt et al. 1994).

Polyphenol oxidase (PPO) enzyme is active at an optimal pH of 5.4. This enzyme produces quinones from catechins through oxidation, and then quinones react and polymerize with nitrogen components to produce condensed tannins in the form of brown pigments in cocoa beans (Spence, 2015 and Hue et al., 2016). Invertase, which has optimal activity at a pH of 4.5, plays a crucial role at the beginning of fermentation. It hydrolyzes sucrose into reducing sugars, specifically glucose and fructose, which serve as chocolate flavor precursors. Glycosidase, on the other hand, is most effective at a pH range of 4 to 4.5. This enzyme breaks down sugars found in anthocyanins, converting them into anthocyanidins (De Vuyst and Leroy, 2020).

In all four treatments, the beans' pHs were in a pH range that could activate the enzymes polyphenol oxidase, invertase, and protease. Therefore, pulp reduction treatment and the addition of a starter did not inhibit the fermentation process.

Changes in Cocoa Bean Fermentation Index during Cocoa Bean Fermentation

Figure 8 illustrates the rise in the Fermentation Index throughout the fermentation process. The Fermentation Index measures cocoa bean fermentation success by browning intensity. An index above 1.6 indicates over-fermentation, while below 1.0 signifies underfermentation (Bariah, 2014). After two days, all treatments produced fully fermented cocoa beans. The Fermentation Index is linked to the breakdown of polyphenols like anthocyanins. Polyphenol oxidase is crucial for the oxidation process, converting o-diphenol into o-quinone. This reaction breaks down colored compounds and forms insoluble substances that give cocoa beans their brown color (Hernández et al., 2016). This reaction is usually accelerates between 42 and 45 °C, which typically occurs on the third day of fermentation.

These conditions align with the observations in all treatments of this study, where the required temperature range was successfully achieved. In this study, fermentation with pulp reduction reached a temperature of 42-45 °C after two days of fermentation and three days of fermentation with other treatments.

In general, there is no significant effect of pulp reduction and inoculum addition on the Fermentation Index of cocoa beans. However, pulp reduction does show a significant impact on the Fermentation Index on the third and fifth days of fermentation. The Fermentation Index values can reach more than 1 and less than 1.6. It has been reported that pulp reduction and the addition of a starter culture do not disrupt the cocoa fermentation process. With pulp reduction, the Fermentation Index reaches 1 more

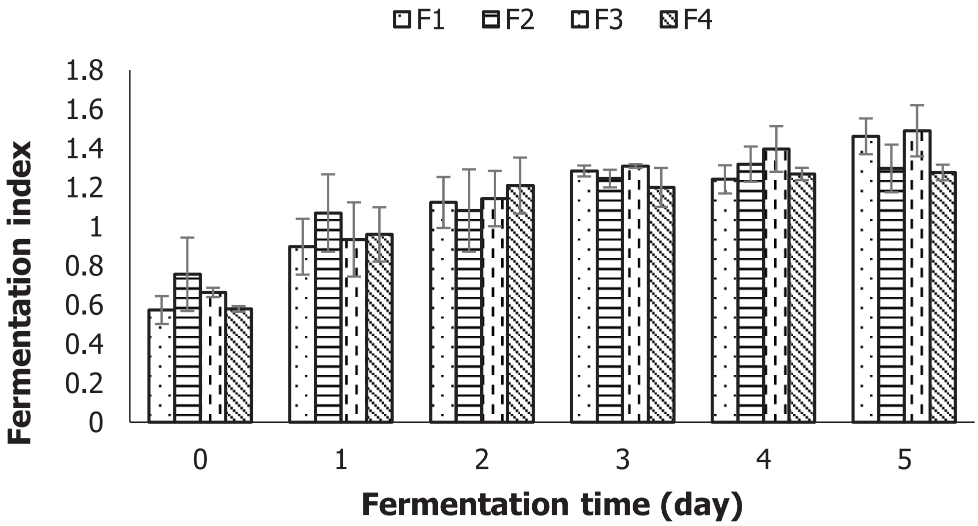

Figure 8. Changes in Fermentation Index during fermentation; F1: Spontaneous fermentation; F2: Reduced-pulp Fermentation; F3: Starter-added Fermentation; F4: Reduced-pulp and Starter-added Fermentation

quickly than with other methods, even though the overall fermentation duration remains the same. The addition of a starter culture is known to accelerate the Fermentation Index achievement in the range of 1 to 1.6 due to it speeds up the cocoa fermentation process. These findings are consistent with the study conducted by Gunam et al. (2021).

The Effect of Pulp Reduction and Inoculum Addition on Physical, Chemical, and Microbiological Properties of Fermented Dry Cocoa Beans

This study investigates the properties of fermented dry cocoa beans, presented in Table 1. The ANOVA analysis found that pulp reduction and starter culture addition, as well as their interaction, had no significant effect ($p \geq 0.05$) on the beans' physical, chemical, and microbiological properties.

According to SNI 2323:2008/Amd I:2010, the maximum allowable percentage of unfermented or slaty cocoa beans is set at 3%, 8%, and 20% for grades I, II, and III, respectively. As shown in Table 1, cocoa beans met grade I with approximately 2-3% unfermented beans. During fermentation, cocoa beans change color from purple to brown as polyphenol compounds oxidize with the enzyme polyphenol oxidase and air. While fermented cotyledons still retain a high polyphenol content, certain varieties do not produce a brown color. While fermented cotyledons still retain a high polyphenol content, certain varieties do not produce a brown color.

In SNI, the maximum percentage of germinated cocoa beans for grades I, II, and III is 2%, 3% and 3%, respectively. Based on Table 1, this study found that all fermentation treatments resulted in the presence of germinated beans within the range of 2-3%. This indicates that they fall into grade II. It is believed that the formation of acetic acid is caused by AAB that diffuses into the cotyledons. Additionally, an increase in temperature within the fermentation substrate contributes to bean death (De Vuyst and Leroy, 2020). For moldy beans, the maximum permissible limits are 2%, 4%, and 4% for grades I, II, and III. According to Table 1, the fermentation treatment in this study did not yield any moldy beans; hence, dry cocoa beans meet the requirements of grade I. Both spontaneous fermentation and fermentation using a starter culture effectively produce alcohol that acts as a disinfectant against mold. Additionally, it is known that in cocoa bean fermentation, LAB growth suppresses fungal growth (Rahayu et al., 2021).

Dry cocoa beans' physical properties are assessed using the method outlined in SNI 2323:2008/AmdI:2010. This involves slicing dry cocoa beans and directly observing them for visible fungi or mycelia while the microbiological properties of fungi are determined through the plating method.

The results of measuring the moisture content of cocoa beans also meet the requirements, remaining below 7.5%. According to Haryadi and Supriyanto (2017), the cocoa bean fermentation process aims to break down the pulp, which facilitates the removal of water from the beans during the drying process. During fermentation, beans also undergo death, which damages their permeability and further aids in the expulsion of water during the drying.

Table 1. Average values of physical, chemical, and microbiological properties of fermented dry cocoa beans

Bean properties	Mean value/experimental unit			
	F1	F2	F3	F4
Unfermented bean (%)	3.00 ± 0.00ª	2.11 ± 0.83ª	2.28 ± 0.55ª	2.90 ± 0.34ª
Germinated bean (%)	2.33 ± 0.13ª	2.39 ± 0.34ª	2.72 ± 0.08ª	2.11 ± 0.44ª
Moldy bean (%)	0	0	0	0
Moisture content (%)	5.03 ± 0.45ª	4.97 ± 0.97ª	4.85 ± 0.88ª	4.32 ± 0.67ª
Water activity/Aw	0.70 ± 0.09ª	0.83 ± 0.12ª	0.76 ± 0.12ª	0.69 ± 0.14ª
pH	5.20 ± 0.19ª	5.36 ± 0.03ª	5.16 ± 0.12ª	5.32 ± 0.17ª
Total titratable acidity (as % acetic acid)	1.56 ± 0.08ª	1.84 ± 0.28ª	1.49 ± 0.24ª	1.68 ± 0.00ª
Fermentation index	1.86 ± 0.21ª	1.46 ± 0.04ª	1.53 ± 0.08ª	1.49 ± 0.02ª
Amino acids (ppm)				
Alanine	39.54 ± 10.31	27.04 ± 19.60	34.83 ± 7.25	39.47 ± 8.95
Valine	39.35 ± 17.75	45.89 ± 23.03	38.06 ± 16.10	44.91 ± 20.40
Phenylalanine	41.71 ± 14.66	42.13 ± 17.01	38.66 ± 11.81	38.77 ± 10.50
Ileucine	28.60 ± 13.40	27.01 ± 14.77	27.45 ± 10.86	31.56 ± 13.40
Leucine	51.97 ± 19.49	50.15 ± 20.67	46.78 ± 15.44	53.43 ± 18.23
Methionine	10.09 ± 1.05	10.84 ± 0.01	10.74 ± 0.35	11.43 ± 0.37
Tyrosine	56.06 ± 19.51	55.60 ± 23.39	50.01 ± 15.11	58.46 ±19.97
Total	268.49 ± 98.89ª	258.00 ± 119.25ª	246.53±76.85ª	278.03 ± 91.82ª
Reducing sugar (%)	1.65 ± 0.12ª	1.60 ± 0.27ª	1.69 ± 0.26ª	1.50 ± 0.10ª
Total fungi (%)	16.5 ± 16.5ª	23.5 ± 23.5ª	43.5 ± 23.5ª	13 0.0ª

Description:
F1: Spontaneous fermentation; F2: Reduced-pulp Fermentation; F3: Starter-added Fermentation; F4: Reduced-pulp and Starter-added Fermentation
Data were taken from 3 experimental replicates.
Numbers on the same row, followed by the same lowercase letter, indicate that there is no significant difference due to the given influence, based on the ANOVA test with a confidence level of 5% ($p \geq 0.05$).

Water activity is one of the most essential intrinsic properties for forecasting microorganisms' survival in food, since it directly impacts product quality and stability. The water activity of dry cocoa beans from all fermentation units ranged from 0.69 to 0.83. Within this range of water activity values, the growth of *mycotoxigenic Aspergillus* is still possible (Fontana, 2020). This potential is further supported by the presence of a significant amount of fungi observed in dry cocoa beans through direct plating analysis.

There is a positive correlation between pH value and total titratable acidity in dry fermented cocoa beans. The pH and bean acidity are interrelated; as the pH value decreases, the acidity value of beans increases. For cocoa processing to yield high-quality cocoa butter, the pH of the beans has to be between 5.2 and 5.8 to produce quality cocoa butter. In this study, the pH of dry cocoa beans ranged from 5.16 to 5.36, indicating that the results obtained meet the market demand. Additionally, the Fermentation Index value for all treatments was above 1.0, demonstrating that the fermentation process was successful (Bariah, 2014).

The activity of peptidase enzyme is shown by the presence of amino acids in fermented cocoa beans. Beans are killed by the heat and acid produced by microorganisms within the cocoa fermentation. Furthermore, peptidases interact with proteins to create amino acids, which serve as flavor precursors. The primary precursors derived include hydrophobic amino acids, such as methionine, leucine, isoleucine, alanine, phenylalanine, valine, and tyrosine (Sari et al., 2023).

During fermentation, the invertase enzyme converts sucrose into reducing sugars, primarily glucose and fructose, which help degrade amino acids and contribute to aroma formation (Haryadi and Supriyanto, 2017). These reducing sugars and peptide amino acids generate flavor precursors that undergo the Maillard reaction during roasting, resulting in distinct flavors (De Vuyst & Weckx, 2016). This reaction starts with the condensation of reducing sugars and amino acids, producing aromatic compounds like ketones, aldehydes, pyrazines, and esters that define chocolate's aroma.

During the cocoa fermentation, the average total fungal count resulting from a combination of pulp reduction treatment and the addition of starter/F4 was the lowest compared to other treatments. These results indicate that this combination can suppress fungal growth during drying, ultimately leading to the production of fermented dry cocoa.

The total fungal analysis of dry cocoa beans differed from that observed during fermentation. On the fifth day of fermentation, the total fungal population was lowest when the starter was added. However, in the case of dry cocoa beans, those treated with the starter exhibited the highest total fungal population. This discrepancy may be due to the variations in solar heat intensity during drying, as it is conducted in farmers's yards where the placement of drying equipment can affect sunlight exposure. To achieve a moisture content of 7%, varying drying times are required, which can create an opportunity for fungal growth in the field.

CONCLUSION

In conclusion, the addition of local microorganism starters-*Candida famata* HY-37, *Lactiplantibacillus plantarum* subsp. *plantarum* HL-15, and *Acetobacter* sp. HA-37 can effectively suppress fungal growth during the cocoa bean fermentation process. Combining a 35% pulp reduction treatment with these local microorganism starters can inhibit fungal growth in dry cocoa beans without affecting their physical and chemical properties of fermented dry cocoa beans.

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CONFLICT OF INTEREST

The authors declare no conflict of interest between any parties in writing this article.

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