Inhibition the growth of fungi and improving the quality of cocoa beans through fermentation using lactic acid bacteria

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Abstract. The addition of lactic acid bacteria (LAB) improved the fermentation performance of cocoa bean. Uncontrolled fermentation causes the mycotoxin contamination in cocoa which is produced by fungi. Lactic Acid Bacteria that have been shown to inhibit fungi are from the species Lactobacillus plantarum and fermentum. Therefore, the purpose of this study was to determine the ability of Lactobacillus plantarum and fermentum to increase the quality and inhibit mold growth of cocoa beans. This research was carried out using Completely Randomized Design. We examined one variable, namely the use of dry starter culture (with Lactobacillus plantarum, with Lactobacillus fermentum, with mixed culture of Lactobacillus plantarum, Saccharomyces cerevisiae and Acetobacter aceti, and without starter culture). We found that the addition of Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus plantarum in combination with Saccharomyces cerevisiae and Acetobacter aceti could provide suitable temperature and pH conditions for a good fermentation process of cocoa beans and suppress the growth of fungus. Fermented cocoa beans produced by those starter cultures could meet the quality requirements of Indonesian National Standard 2323:2008/Amd1:2010.

1. Introduction
Cocoa (Theobroma cacao L.) is one of the leading commodities in the plantation sector in Indonesia. Indonesia is the world's sixth largest cocoa bean producing country after Nigeria with annual production reaching 200,000 tons, while Côte d'Ivoire and Ghana respectively occupy the first and second positions with production of 2,180,000 and 850,000 tons [1]. The top five importing countries for Indonesian cocoa are Malaysia, America, China, India, and the Netherlands. Yogyakarta Special Region (DIY) as one of the cocoa-producing provinces in Indonesia, in 2018 and 2019 had a cocoa plantation area of 5,630 and 5,050 hectares, respectively, with a production of 1,362 and 1,498 tonnes [2]. One of the cocoa productions centers in DIY, namely in Patuk District, Gunungkidul Regency, in 2018 and 2019 had an area of 1,403.8 and 972.30 hectares and produced 407.10 and 715.90 tons [3]. In addition to increasingly open export opportunities, the domestic cocoa bean market is still quite large, including for the cocoa processing industry in Java [4].
Fermentation is one of critical stage in the handling of cocoa beans which mycotoxin-producing fungal contamination may occur [5–7]. During the fermentation process, several types of microbial growth occur, such as yeast, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). These microorganisms have their respective roles in the fermentation process of cocoa beans [8]. When the conditions of fermentation are not controlled, fungal contamination will occur which causes damage and even produces toxins.

Aspergillus sp., Fusarium sp., Trichoderma sp. and Penicillium sp. produce mycotoxins and contaminate the uncontrolled fermentation [9]. Various fungal genera have been identified both during fermentation (including Aspergillus, Paecilomyces, Talaromyces, and Penicillium) [7]. Several mycotoxin-producing fungi found in dry cocoa beans in Indonesia due to poor fermentation and drying, namely Aspergillus flavus, A. niger, A. clavatus, A. wentii, A. ochraceus with a population of $2.3 \times 10^4$ – $7.2 \times 10^6$ CFU/ g [9]. Several fungus including Aspergillus niger, A. fumigatus, Penicillium sp., Fusarium sp, Trichoderma sp., Rhizopus sp., Mucor sp. and Verticillium sp have been found on dry cocoa beans at farmers’ levels. At the traders’ level, A. flavus, A. niger, Penicillium sp., Fusarium sp., Trichoderma sp. and Mucor sp. have been identified. The total population of mycotoxigenic fungi at the farmers’ and traders’ levels were $1.4 \times 10^6$ CFU/ ml and $6.0 \times 10^5$ CFU/ ml respectively [10]. In Yogyakarta, Aspergillus niger YAC-9, an ochratoxin A-producing fungus in cocoa beans, had been detected [11].

Research on inhibition of fungi and mycotoxins has been widely carried out. Lactic acid bacteria have been proven to have the power to inhibit fungi from organic acid production are from the species Lactobacillus plantarum and Lactobacillus fermentum, while the ability to inhibit fungi from the production of protein compounds is Saccharomyces cerevisiae and Candida ethanolica [12]. One of the antifungals mechanisms in Lactobacillus plantarum is through production 4-hydroxyphenyllactic acid which is a type of polylactic acid [13]. The addition of Lactobacillus plantarum to the fermentation of cocoa beans, increases the production of lactic acid and decreases the acidity which then suppresses the growth of unwanted microbes [14].

Mycotoxin-producing fungi can be inhibited by the addition of Lactobacillus plantarum HL-15 culture, which is isolated from cocoa beans during fermentation [15]. L. plantarum HL-15 can inhibit fungal growth and synthesis of ochratoxin A in cocoa beans during fermentation and drying [16]. The addition of L. plantarum HL-15 as a culture starter in old and new fermentation boxes had a lower level of fungi contamination as compared to the treatment without L. plantarum HL-15 [17].

Application of dry culture of Lactobacillus fermentum in the fermentation process, produces good quality cocoa which is free of mycotoxins [18]. Adding a starter of lactic acid bacteria to the fermentation of cocoa beans can modify the microbial composition during fermentation so that the formation of metabolic products such as ethanol, lactic acid and acetic acid were more intensive [19]. The fermentation process, with the addition of lactic acid bacteria shows an increase in the fermentation index, which indicates that the fermentation process can be accelerated [20]. Based on the previous explanation, this research was aimed to determine the ability of the dry starter of Lactobacillus plantarum HL-15, Lactobacillus fermentum, and mixed culture of S. cerevisiae, L. plantarum, and A. acetii to inhibit mold growth during fermentation of cocoa beans.

2. Material and methods

2.1. Cocoa beans and starter culture preparation

2.1.1. Cocoa beans preparation. Healthy pods were collected from smallholder plantations in Gunungkidul Yogyakarta, Indonesia. The selected pods were then taken to the Ngudi Raharjo II processing unit at Gunungkidul Yogyakarta for. Fresh cocoa fruits were carefully broken down using a wooden beater or by hitting one fruit with another fruit. Cocoa beans were removed from the fruit and separated from the placenta attached to the seeds. Afterwards, sorting of cocoa beans was done by
separating the fully ripe cocoa beans from clustered and deformed seeds due to pests, fungi, or sprouting. The procedure used to obtain high quality wet cocoa beans was according to the Indonesian National Standard 2323:2008/Amd1:2010 [21].

2.1.2. Starter culture preparation. Lactobacillus plantarum HL-15 was isolated from the cocoa bean fermentation process in Yogyakarta [15]. Dry starter culture of Lactobacillus plantarum HL-15 was produced at Laboratory of Microbiology, Food and Nutrition Studies Centre, UGM, Yogyakarta, Indonesia [22], while Lactobacillus fermentum dried culture was prepared by Indonesian Coffee and Cocoa Research Institute, Jember, Indonesia [18], meanwhile dry mixed culture of Lactobacillus plantarum, Saccharomyces cerevisiae and Acetobacter aceti was obtained from Center for Agricultural Postharvest Research and Development, Bogor, Indonesia [23].

2.2. Cocoa beans fermentation
The research was carried out on a field scale with a 40 kg capacity tiered fermentation in wooden boxes [21,24]. The fermentation of cocoa bean, using a starter culture, refers to the modification method performed by [18,22–24]. The fermentation was carried out for five days. On the third day, upside down turning was carried out so that the seeds were evenly fermented. On day five, cocoa beans were removed from the fermentation box, soaked and washed prior to drying. Drying was done by a combination of sun drying and cabinet drying at 50-60°C. This research was carried out using a Completely Randomized Design, with 3 replications. The fermentation treatments were S1 = with a dry starter of Lactobacillus plantarum HL-15, S2 = with a dry starter of Lactobacillus fermentum, S3 = with a dry starter of mixed culture of Lactobacillus plantarum, Saccharomyces cerevisiae and Acetobacter aceti, and S4 = without dry starter culture.

2.3. Physical analyses of cocoa beans
Measurement of the pH and temperature of cocoa beans were carried out once a day during five days of fermentation. pH and temperature measurements were carried out on 3 parts of the pile of cocoa beans in the fermentation box, namely the top layer, middle layer and bottom layer using a pH meter and thermometer.

2.4. Microbiological analysis
The microbiological analysis includes yeast, lactic acid bacteria, acetic acid bacteria, and fungal populations by the Total Plate Count (TPC) method [25]. The media used Malt Extract Agar (MEA), Peptone Glucose Yeast Extract Agar (PGYA), de Man Rogosa Sharpe Agar (MRSA), and Dichloran Rose Bengal Chloramphenicol Agar (DRBCA) [26]. Samples were taken as much as 40 g from fermenting mass and were put into a plastic bag. Samples were added with 60 mL of 0.85% sterile NaCl solution and then crushed using a stomacher. The crushed samples were put into a sterile beaker, then added 300 mL of 0.85% NaCl solution and stirred using a shaker. A total of 1 mL of sample extract was diluted in 0.85% sterile NaCl to 10−9 dilution. The analysis was using the pour plate method. Incubation was carried out at 37 °C for 48 h for lactic acid bacteria growth evaluation and 5 days at room temperature for fungal growth evaluation. Enumeration of the colony was performed using the Quebec Colony Counter.

3. Results and discussion

3.1. The degree of acidity (pH) during fermentation
During the cocoa beans fermentation process, there was a change in pH in all treatments, with the initial pH of the outer layer of cocoa beans was 3-4 and the final pH 4-7 (figure 1). Changes in the pH value are in accordance with cocoa beans fermentation research by Triyadi (2017), Kustyawati and Setyani (2008), Ni’matuzahra (2018), Marwati et al. (2018) with initial pH 2-3 and final 4-7 [16,27–29]. Cocoa bean pulp contains 2-2.5% (w/w) of citric acid, which causes acidic properties [30]. This acidity causes
a low pH. This is also supported by [14] that the addition of \textit{L. plantarum} inoculum significantly reduces pH. In addition, on study Copetti MV \textit{et al.} (2014), also reported that lactic acid and citric acid produced during fermentation played an important role in decreasing the pH value [31].

The pH slightly decreased on day two for all treatments, in line with the study Ho \textit{et al.} (2013) [8]. The increase in pH value of the outer layer of cocoa beans during the fermentation is thought to be caused by acids produced during fermentation that diffused inside the cocoa beans [16]. Organic acids and heat are the results of biochemical changes that occur in cocoa beans during the fermentation period. The organic acids then encourage the diffusion of the fermented products into the cocoa beans [29,32].

![Figure 1](image)

\textbf{Figure 1.} The degree of acidity (pH) of cocoa beans during fermentation on the four of fermentation treatments, namely S1= with dry starter of \textit{Lactobacillus plantarum} HL-15; S2= with dry starter of \textit{Lactobacillus fermentum}; S3= with dry starter of mixed culture of \textit{Lactobacillus plantarum}, \textit{Saccharomyces cerevisiae} and \textit{Acetobacter aceti}, and S4 = without dry starter culture.

3.2. Temperature during fermentation

There was a change in temperature during cocoa fermentation in all treatments (figure 2), and reached the highest levels on the third day of fermentation, the profile agrees with the reported one [33]. The temperature then decreased until the end of the process. The highest temperature was achieved in fermentation with a dry starter of \textit{Lactobacillus fermentum} (45.09 °C). According to Visintin S \textit{et al.} (2016), the temperature (45 °C) corresponds to the growth of yeast, LAB, and AAB [34]. The temperature increase caused by the oxidation of alcohol to acetic acid [35]. The higher the amount of acid present, the higher the heat produced (exothermic reaction), where the exothermic reaction can produce 118.6 cal or 497 KJ of heat per mole of oxidized ethanol [32,36,37]. At the end of fermentation, all cocoa beans reached temperatures above 40 °C. This is in line with the results of research by Papalexandratou \textit{et al.} (2011) [38]. Variations in the component content in the substrate and aeration will affect temperature changes during the fermentation process [39]. The addition of a starter and the total microbes present in each treatment is also contributed to the difference in temperature profiles [16]. Changes in fermentation temperature in all treatments (figure 2) influenced by the growth pattern of acetic acid bacteria in figure 4. After the third day, there was a decrease in fermentation temperature (figure 2), in line with a decrease in the population of acetic acid bacteria (figure 6).
3.3. Microbial population

Total microbes on cocoa beans by four fermentation treatments for five days showed changes in the population of microbes. Microbial population decreased in fermented cocoa beans with the addition of the three starter cultures, and conversely increased in fermentation without the addition of starter cultures. The highest percentage decline in microbial population was on fermentation with the addition of *Lactobacillus fermentum* of 32.1%, followed by the addition of mixed culture of *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Acetobacter aceti* (15.1%) and *Lactobacillus plantarum* HL-15 (12.1%) (figure 3).

Changes in the diversity of the microbial community were influenced by the addition of an inoculum of *Lactobacillus plantarum* HL-15, *Lactobacillus fermentum*, and mixed culture of *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Acetobacter aceti* during fermentation (figure 3). This is in line with the opinion that the structure of the microbial community is influenced by the fermentation method and the fermentation system that has an impact on yeast and lactic acid bacteria populations [40,41]. The addition of *L. plantarum* inoculum efficiently suppresses unwanted bacteria and reduces the diversity of the microbial community [14].
3.4. Yeast populations

The highest yeast population occurred in fermentation with dry starter of mixed culture of *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Acetobacter aceti* (figure 4). In the fermentation with the addition of dry starter of *Lactobacillus plantarum* HL-15 and *Lactobacillus fermentum*, a decrease in yeast population occurred after the second day and continued until the fifth day of fermentation (end of fermentation). This result is in line with the statement by [16,30,42].

The decrease in the number of yeast populations was due to the aeration of the pulp and the formation of acetic acid. Aeration occurs because yeast secretes pectinase so that the viscosity of the pulp decreases. As the yeast is growing, enough acids and aeration will be formed. Aeration will encourage the growth of acetic acid bacteria, which will suppress the yeast growth rate so that the yeast population decreased from the second day to the end of fermentation [16,30,43].
3.5. Lactic Acid Bacteria populations

In the fermentation of cocoa beans with the addition of dry starter of *Lactobacillus plantarum* HL-15, *Lactobacillus fermentum*, and of mixed culture of *Lactobacillus plantarum*, *Saccharomyces cerevisiae*, and *Acetobacter aceti*, the population of lactic acid bacteria was higher than without the addition of culture (figure 5). On the second day for all fermentation treatments, the population of lactic acid bacteria was quite high (7 log CFU/g). The growth of lactic acid bacteria began to decline on the third day of fermentation and encouraged acetic acid bacteria to grow [16]. While at the end of fermentation (day five) the population decreased. A similar situation was also found by other studies Triyadi R (2017) and Marwati T *et al.* (2017), which reported that the total population growth of lactic acid bacteria during 5 days of fermentation is naturally in the range between 6.0-8.0 Log CFU/g [15,16].

![Figure 5](image_url)

*Figure 5.* The population of Lactic Acid Bacteria in 4 methods fermentation, namely, S1= with dry starter of *Lactobacillus plantarum* HL-15; S2 = with dry starter of *Lactobacillus fermentum*; S3 = with dry starter of mixed culture of *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Acetobacter aceti*, and S4 = without dry starter culture.

3.6. Acetic acid bacteria populations

The population of lactic acid bacteria increased on the first day in all fermentation methods with the highest population in the method with the addition of mixed cultures of *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Acetobacter aceti* (figure 6). After the third day, the growth slightly decreases until the fifth day of fermentation, in line with Ni’matuzahra (2018) [28].

The population numbers of acetic acid bacteria at the end of fermentation in each of the variations of fermentation codes S1, S2, S3 and S4 were 7.85; 7.41; 7.17 and 7.53 7 CFU / g, respectively. This condition is in accordance with Papalexandratou *et al.* (2011), which stated that the population of acetic acid bacteria increased to 8.0 log CFU / g on days 4 to 5 of fermentation [38]. This is also in line with the statement Djiaafar *et al.* (2017) that the growth of acetic acid bacteria over a period of one to five days is in the range of 6.0 to 8.0 Log CFU/g [42].

The presence of acetic acid bacteria in the fermentation process will affect the organoleptic properties and the death process of cocoa beans [16].
Figure 6. The population of Acetic Acid Bacteria in 4 methods fermentation, namely, S1 = with dry starter of *Lactobacillus plantarum* HL-15; S2 = with dry starter of *Lactobacillus fermentum*; S3 = with dry starter of mixed culture of *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Acetobacter aceti*, and S4 = without dry starter culture.

3.7. Fungal populations

The presence of fungus was detected at the beginning of fermentation. In the fermentation with starter cultures of *Lactobacillus plantarum* HL-15 (S1), fungal growth was inhibited on the first day, whereas with starter cultures of *Lactobacillus fermentum* (S2) and mixed cultures of *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Acetobacter aceti* (S3) fungal growth was inhibited on the third day. In the fermentation of cocoa beans without the addition of starter culture (S4), there was an increase in the number of fungal populations until the fifth day of fermentation (table 1). This shows that the addition of starter cultures of *Lactobacillus plantarum* and *lactobacillus fermentum* during the fermentation of cocoa beans can suppress the growth of fungal populations, as reported by Ruggirello *et al.* (2019) and Marwati *et al.* (2017) [12,15]. *Lactobacillus plantarum* HL-15 is the isolate that has the most effective antifungal properties, in line with the results of previous antifungal tests [15].

The decline in fungal population during fermentation occurs due to the role of lactic acid bacteria added at the beginning of fermentation. Some of the metabolites that inhibit fungal growth produced by lactic acid bacteria include several metabolites such as organic acids (lactic acid, acetic acid, and acid. propionate), carbon dioxide, ethanol, hydrogen peroxide, diacetyl, reuterin, the production of protein compounds or peptides with small molecular weights that cause lactic acid bacteria to have the ability to inhibit fungal growth [35,44,45]. One of the antifungals in *Lactobacillus plantarum* is through production 4-hydroxyphenyllactic acid which is a type of polyactic acid [13]. The addition of *Lactobacillus plantarum* to the fermentation of cocoa beans, increases the production of lactic acid and decreases the acidity which then suppresses the growth of unwanted microbes [14].
Table 1. The population of fungal during fermentation on 4 treatments.

| Fermentation method | Total fungal contamination (log CFU/g) during fermentation |
|---------------------|----------------------------------------------------------|
|                     | 0 Day | 1 Day | 2 Days | 3 Days | 4 Days | 5 Days |
| S1                  | < 10^1 | ND    | ND     | ND     | ND     | ND     |
| S2                  | 2.0 x 10^1 | < 10^1 | < 10^1 | ND     | ND     | ND     |
| S3                  | < 10^1 | 1.0 x 10^1 | < 10^1 | ND     | ND     | ND     |
| S4                  | 2.0 x 10^1 | 2.0 x 10^1 | 2.0 x 10^1 | 3.0 x 10^1 | 3.0 x 10^1 | 3.0 x 10^1 |

Remarks: S1 = with dry starter of Lactobacillus plantarum HL-15; S2 = with dry starter of Lactobacillus fermentum; S3 = with dry starter of mixed culture of Lactobacillus plantarum, Saccharomyces cerevisiae and Acetobacter aceti, and S4 = without dry starter culture.

3.8. The quality of dry cocoa beans

The results of the quality analysis of cocoa beans which included the percentage of mouldy, unfermented beans (slaty), germinated, and insect damaged seeds, as well as broken and flat beans can be seen in Table 2. In the fermentation with the addition of starter cultures of Lactobacillus plantarum HL-15 (S1), Lactobacillus fermentum (S2) and mixed cultures of Lactobacillus plantarum, Saccharomyces cerevisiae and Acetobacter aceti (S3) did not produce mouldy seeds, whereas in fermentation without starter culture, there were mouldy seeds (0.67%) (Table 2), this is in line with the data in table 1.

In all samples of cocoa beans, the number of germinated and insect-damaged seed showed 0% level. This means that no sample had been germinated or contaminated by insects. Seed germination is undesirable in dried cocoa bean products because germination shows that the seeds were alive during fermentation. Bean death is one of the hallmarks of fermentation that enable the formation of flavor precursor. The seeds that are contaminated by insects are also undesirable because their quality decrease, especially physically. The moisture content of cocoa beans for all fermentation method treatments had met the standard, which was below 7.0%. The standard moisture content of export quality cocoa beans is a maximum of 7.5%, if it is higher than this value, cocoa beans are prone to mould growth during prolonged storage. However, if the water content is too low, cocoa beans tend to be fragile [46].

From all parameters being analyzed, the values obtained were compared with Indonesian standard for cocoa beans. Fermented cocoa beans produced with and without adding starter culture could meet the quality requirements of Indonesian National Standard 2323:2008/Amd1:2010, where the maximum percentage of moldy seeds was 2%; the maximum percentage of slaty seeds was 3%; the percentage of insect containing seeds was maximally 1%, the maximum percentage of impurities was 1.5%, the percentage of germinating seeds was maximally 2% and moisture content maximum 7.5%.

Table 2. The quality of cocoa beans on four fermentation treatments.

| Fermentation method | Analysis results |
|---------------------|------------------|
|                     | Number of seeds / 100 grams | Moldy seeds (%) | unfermented beans (slaty) (%) | Germinated seeds (%) | Insect containing seeds (%) | Broken seeds (%) | Flat beans (%) | Moisture Content |
| S1                  | 115               | 0               | 0                             | 0                   | 0                   | 0               | 0               | 3.25 |
| S2                  | 107               | 0               | 0                             | 0                   | 0                   | 0               | 0               | 7.01 |
| S3                  | 117               | 0               | 0                             | 0                   | 0.33                | 0               | 1.0             | 3.29 |
| S4                  | 122               | 0.67            | 0                             | 0                   | 0                   | 1.0             | 0               | 3.89 |

Remarks: S1 = with dry starter of Lactobacillus plantarum HL-15; S2 = with dry starter of Lactobacillus fermentum; S3 = with dry starter of mixed culture of Lactobacillus plantarum, Saccharomyces cerevisiae and Acetobacter aceti, and S4 = without dry starter culture.
4. Conclusions
From this study, it is revealed that the addition of dry starter of *Lactobacillus plantarum* HL-15, *Lactobacillus fermentum* and of mixed cultures of *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Acetobacter aceti* on fermented cocoa beans can suppress fungal growth, improve the quality of cocoa beans and fit the standard set by Indonesian National Standard 2323: 2008 / Amd1: 2010.

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