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The indigenous Lactic Acid Bacteria from Fermented Cocoa Bean and Its Role in Cocoa Bean Fermentation.

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ABSTRACT

An indigenous lactic acid bacterial strain that was isolated from fermented cocoa bean, was characterized and identified as a member of the *Lactobacillus plantarum* species based on phenotypic characteristics and 16S rRNA gene sequences. Important properties for cocoa bean fermentation, namely ability to ferment of sucrose, glucose, and fructose; pH-, ethanol-, and heat-tolerance, were examined for this isolate. The Quality of fermented cocoa bean was analyzed by cut test and fermentation index. *Lactobacillus plantarum* KSL2 was tolerance to low pH value, high temperature, ethanol, could fermented of glucose, sucrose and fructose, reflecting it is able to adapt to cocoa fermentation environment condition. The addition of *Lactobacillus plantarum* KSL2 strain as a starter for cocoa fermentation, served to increase the quality of cocoa bean. Therefore, *L.plantarum* KSL2 potentially useful for its utilization as starter in cocoa bean fermentation.

Keywords: Lactic acid bacteria (LAB), cocoa bean fermentation, *Lactobacillus plantarum*

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INTRODUCTION

Fermentation process is one of the important in cocoa bean processing that governs the ultimate product quality. Cocoa beans are fermented in order to remove mucilage pulps resulting biochemical changes in the cotyledons and causes cotyledon death [1]. The mucilage pulps surrounding bean were removed by the actions of various microbial species indigenously present in the cocoa beans, such as yeast, lactic acid bacteria (LAB), and acetic acid bacteria (AAB), during the cocoa beans fermentation [2,3]. Complex enzymatic reactions from the microorganisms, simultaneously are initiated in the cocoa beans that are responsible for the formation of the necessary colour and flavor precursors of well-fermented dry cocoa beans. The species of microorganisms involved in the cocoa bean fermentation process vary with the geographical location of the plantation [3].

Natural cocoa fermentation conducted by indigenously microbes are difficult to control. In this context, many studies suggest the use of starter microbial culture as a best approach to improve fermentation process [2,5]. Thus, several microbial cultures, including lactic acid bacteria have been assayed to analyze their potential as starters [6,8]. These assays are mainly to analyze the effect of chosen strains, on the quality of fermented product.

Lactic acid bacteria (LAB) is one of the indigenously present microorganisms that roles in the cocoa bean fermentation. The bean pulp is rich in fermentable sugars such as glucose, fructose and sucrose, and has a low pH of 3.0–3.5, mainly due to the presence of citric acid. These conditions select for the initial growth of yeasts and lactic acid bacteria [3]. The lactic acid bacteria also ferment pulp sugars and utilize citric acid [2,7]. However, the studies related with the isolation, identification and functional role of LAB species involved in cocoa bean fermentation has not been much performed.

The previous study have been identified the contributions of LAB on the cocoa bean fermented at several location in Java, but further research is needed to determine the potential of these species to development of cocoa bean and chocolate quality [3]. Early studies on microbiology of fermented cocoa beans at cocoa farm in South Konawe regency, Southeast Sulawesi has been obtained the indigenously LAB, but its identity and its role in the cocoa fermentation has not been revealed. Therefore, the study of controlled fermentation that integrate physicochemical analyzes is required to better understand how individual species contribute to cocoa bean fermentation and chocolate quality. The objective of this research are to identify the indigenous LAB from Southeast Sulawesi, Indonesia and to determine contribution of the indigenous LAB on cocoa bean quality.

MATERIALS AND METHODS

Materials

Bacterial strain and Media

The KSL2 LAB strain was isolated from fermented cocoa bean that retrieved from South Konawe regency, Southeast Sulawesi, Indonesia. This strain was kept in deMan Rogose Sharpe Agar (MRSA) media for 2 days. The KSL2 strain was cultivated in MRS Broth medium containing 2% (w/v) pulp before it was inoculated in the beginning of the cocoa bean fermentation.

Methods

Identification of LAB strain

The strain KSL2 was identified based on phenotypic and genotypic characterization. Phenotypic characterization was performed by examining morphological properties (cell shape, Gram reaction, motility, and spore forming), biochemical test (catalase, nitrate reduction, hydrolysis of casein, gelatin, indole test, citrate utilization and sugar fermentation) and physiological test (influence of pH, NaCl, ethanol and temperature on growth).

Identification of KSL2 strain based on genotypic characterization was conducted by 16S rRNA gene sequence comparison. DNA extraction was obtained using GES method [9]. Amplification of 16S rDNA by PCR was done using universal bacterial primer 20F (5'–AGTTTGATCCTGGCTC–3') and 1540R (5'–AAGGAGGTGATCCAGCC– 3'). The PCR product was purified using PEG presipitation method (http://www.pebiodocs.com). Purified 16S rRNA gene was sequenced using ABI PRISM 310 big dye terminator cycle sequence reading reaction kit, according to the protocol of the manufacturer (Applied Biosystems). Base sequences were determined in an Applied Biosystems model 310 genetic analyzer.

The 16S rRNA gene sequence of KSL2 strain was aligned with representative the LAB 16S rRNA gene sequences retrieved from in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov) and was edited manually. Pairwise evolutionary similarities and distances were computed by using the DNADIST program in the phylogeny inference package (PHYLIP) versi on 3,5 [10]. The phylogenetic tree was constructed by using the Neighbour-joining algorithm [11]. The root position of the unrooted tree was estimated by using *Bacillus subtilis* DSM 10^T (Gen Bank accession no. AJ 276351) as the outgroup strain.

Fermentation of Cocoa Beans condition and sampling

Laboratory scale cocoa fermentation trials were carried out with 1 kg of fresh cocoa beans (Forastero variety). The cocoa pods were retrieved from cocoa farm in Kolaka regency, Southeast Sulawesi, Indonesia. Ripe cocoa pods were first washed with detergent, dipped in phenol and finally sprayed with 5% thymol prepared in ethanol in a sterilized inoculation chamber to ensure aseptic conditions. The pods, together with all materials for the micro fermentation, were subjected to a final sterilization by u.v irradiation overnight [12]. The cocoa beans were collected in plastic box fermentation. Fermentation was carried out at room temperature for 5 days. The LAB strain was inoculated on the cocoa beans at 10^6 CFU/g of cocoa beans. For comparison, fermentation of beans without inoculums addition (naturally fermentation) was also performed. Sub samples of about 100 g of fermented cocoa beans were taken at the end of different durations of fermentation process. After fermentation, the fermented cocoa beans were dried in sunshine by exposition the beans on plastic and clean surface from 8 am to 5 pm daily until the moisture content reached 7%–8% as commonly recommended in the Indonesian cocoa bean standards [13]. Each samples were used for determination of physical quality of beans and lactic acid content.

Physical Quality Assessment of Fermented Cocoa Beans

The cut test is used for evaluation of sanitary and fermentation quality of beans. A total of ninety (3×30) dried cocoa beans from each treatment were analyzed by the cut-test according to methods previously used by Guehi, et al. [14] and Jamili, et al. [5]. Observations were made and beans were placed in one of the following categories: fully brown (fermented); purple (partly or under-fermented); slaty (unfermented); moldy; or germinated. Slaty beans include rubbery cotyledon, blackish color, and resistance to cutting. Defectives beans are the sum of germinated beans. Results were expressed as a percentage and according to the official standard, a batch of cocoa beans with more than 60% fully brown color beans is considered as good quality product. The fermentation index was measured by spectrophotometry following Kongor et al. [15].

Determination of Acidity

Total acidity was expressed as percent (%) lactic acid. Lactic acid was determined following the method given by Cappucino and Sherman [16] with slight modification. Ten g cocoa powder was added by 100 mL distilled water and the contents were boiled for 1 minute, stir gently to form a suspension that is free of clots. The suspension was filtered and cooled to room temperature (± 27°C) [13]. Five drops phenolphthalein (1%) was added to 10 mL filtrate. The titration with 0,1 N NaOH was carried out until a light pink colour persisted. The percent lactic acid was calculated by using the formula was given below :

$$\text{\% lactic acid} = \frac{Vol.of\ alkali\ used\ X\ normality\ of\ alkali\ X\ 9}{Vol.sample\ taken\ (i.e.\ 10\ mL)}$$

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RESULTS AND DISCUSSION

Identification of Lactic acid bacteria strain

KSL2 strain was an indigenous bacteria that isolated from spontaneous cocoa bean fermentation was carried out at farm in South Konawe regency, Southeast Sulawesi, Indonesia. Strain KSL2 was Gram positive bacteria with rod cell shape. The main characteristics of strain KSL2 was shown at Table 1.

Table 1. The main characteristics of KSL2 strain

Characteristics	*Strain KSL2*
Cell shape	Bacil/Rod
Gram reaction	Positive
Motility	Negative
Catalase	Negative
Endospore	Negative
O_2 required	Facultative anaerobic

Characteristics of strain KSL2 namely Gram-positive bacteria, non-motile, non-spore-forming, catalase negative and facultative anaerobic growth (Table 1), indicated that KSL2 strain was belong to group of lactic acid bacteria (LAB). Khalid [17] reported that Lactic Acid Bacteria are Gram-positive usually non-motile, non-spore-forming, rods or cocci cell shape and produce lactic acid as their major end product.

Cell shape of KSL2 strain was rod (Table 1), indicated that KSL2 strain was a member of *Lactobacillus* genus. Dicks and Endo [18] and Bergey's Manual of Determinative Bacteriology [19] explain that the name *Lactobacillus* pertains to a small rod, cells are Gram-positive, catalase negative, usually non-motile, long and slender (often bent), or short coryneform-like coccobacilli. This result showed that *Lactobacillus* was detected as the indigenously LAB at cocoa fermentation. Ardhana & Fleet [3] was found the predominant LAB in West Java Indonesia cocoa fermentation, was *Lactobacillus.* Lactobacilli were also found to be dominant in Ghanaian and Nigerian cocoa fermentation [20,21].

Identification of KSL2 strain was done based on phenotypic and genotypic characterization. The phenotypic properties of KSL2 strain is summarized in Table 2. Based on phenotypic characteristics at Table 2 showed that KSL2 strain has a similar characteristics with species of *Lactobacillus plantarum*. Table 2 showed that KSL2 strain was survive in the presence of ethanol, did not produce gas from glucose and ability to ferment of manitol. These characters were matched with key characters for *L.plantarum* [18,22]. Hence, the KSL2 strain was identified as a member of species *L.plantarum* based on phenotypic characteristics. Result of identification based on phenotypic characteristics were used for an independent validation of the sequence-based identification.

Based on characteristics at Table 2, it can be also obtained several information about adaptation ability of the KSL2 strain to the cocoa bean fermentation environment. The tolerance of low pH value (4,5), ethanol concentration (5-10%) and the high temperature (40^{o}C) of the KSL2 strain was in accordance with environmental conditions prevailing during cocoa beans fermentation. The higher temperatures, lowest pH value and ethanol concentrations towards the end of a cocoa bean fermentation, due to increased microbial activities, influence survival of cocoa-specific LAB species, as their tolerance towards these factors was variable [3,20]. The limited number of LAB capable to grow at these conditions [23]. KSL2 strain gave significant growth at the ethanol up to 15% (Table 2) and this characteristic indicated that KSL2 strain was similar with *L.plantarum.* Dirck and Endo [18] explained that *L. plantarum* is one of *Lactobacillus* genus that grow well in the presence of ethanol.

KSL2 strain did not grow at 45^{o}C (Table 2) showed that this strain did not contribute to the end cocoa fermentation. On the natural fermentation, Lactic acid bacteria (LAB) was observed in the beginning of fermentation and reached maximum concentration at 2 days fermentation, after that acetic acid bacteria was detected at maximum concentration at 4-5 days fermentation (data not shown). The limited number of LAB capable to grow at 45^{o}C explains the disappearance of the LAB population once ethanol oxidation by acetic acid bacteria, causing a substantial temperature increase during cocoa bean fermentation, has started [20].

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Table 2. Physiological and biochemical characteristics of KSL2 strain and *L. plantarum* strain

Characteristics	KLK4 STRAIN	*L.plantarum* ATCC 14917^{T}[18,22]
Physiological characteristics		
Growth in MRS medium containing NaCl		
4 %	+	+
6 %	+	+
8 %	+	+
10 %	-	-
Growth at pH		
4	+	-
5	+	+
7	+	+
Growth at temperature (°C)		
15	+	+
30	+	+
37	+	+
40	+	+
45	-	-
Growth at MRS medium containing ethanol		
5 %	+	+
10 %	+	+
15 %	+	-
Biochemical characteristics		
Citrate assimilation	-	-
Nitrate reduction	-	-
Gelatin liquefaction	-	-
Production of Indol	-	-
Urea hydrolysis	-	-
Citrate assimilation	-	-
Fermentation Carbohydrate		
Glucose	+	+
Sucrose	+	+
Fructose	+	+
Maltose	+	+
Manitol	+	+
Gas produced from glucose	-	-

+ positive reaction or growth; - no reaction or no growth were observed
ATCC 14917^{T} is a type strain of *L. plantarum*.

KSL2 strain which was identified as *L. plantarum,* was isolated from fermented cocoa bean at fermentation 1 day. This result was supported by Pereira, et al. [23] which explained that *L. plantarum* (facultatively heterofermentative) dominated the LAB community in cocoa fermentation, but after 36 h, *L. fermentum* (strictly heterofermentative) became the dominant LAB.

Genotypic characterization study was carried out to identify KSL2 strain after studying phenotypic characteristics. This study includes 16S rRNA gene analysis, which is considered as a most standard and reliable method for identification of an unknown microorganism. Comparative 16S rRNA gene sequence analyses and the estimation of phylogenetic relationships demonstrated that the closest relative of KSL2 strain was *Lactobacillus plantarum* ATCC 14917 (Fig. 1).

The 16S rRNA gene sequence similarity with the type strain of the species *L. plantarum* ATCC 14917 (AJ 965482) was 99,29 % and differed only by 10 base pairs (bp) for 1416 bp 16S rRNA fragment from *L. plantarum*, clearly indicating that KSL2 belong to this species. Therefore a new strain, name '*L.plantarum* KSL2' was obtained from cocoa fermentation in Southeast Sulawesi.

Several previous studies have found the *Lactobacillus plantarum* from the cocoa bean fermentation process. Ardhana & Fleet [3] has found *L.plantarum* from cocoa bean were fermented at 3 estate in East Java,

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Indonesia. Camu et al. [20] also has found *L.plantarum* from Ghanaian cocoa bean heap fermentation. This is indicates that *L.plantarum* including a specific species of Lactic acid bacteria in the cocoa fermentation.

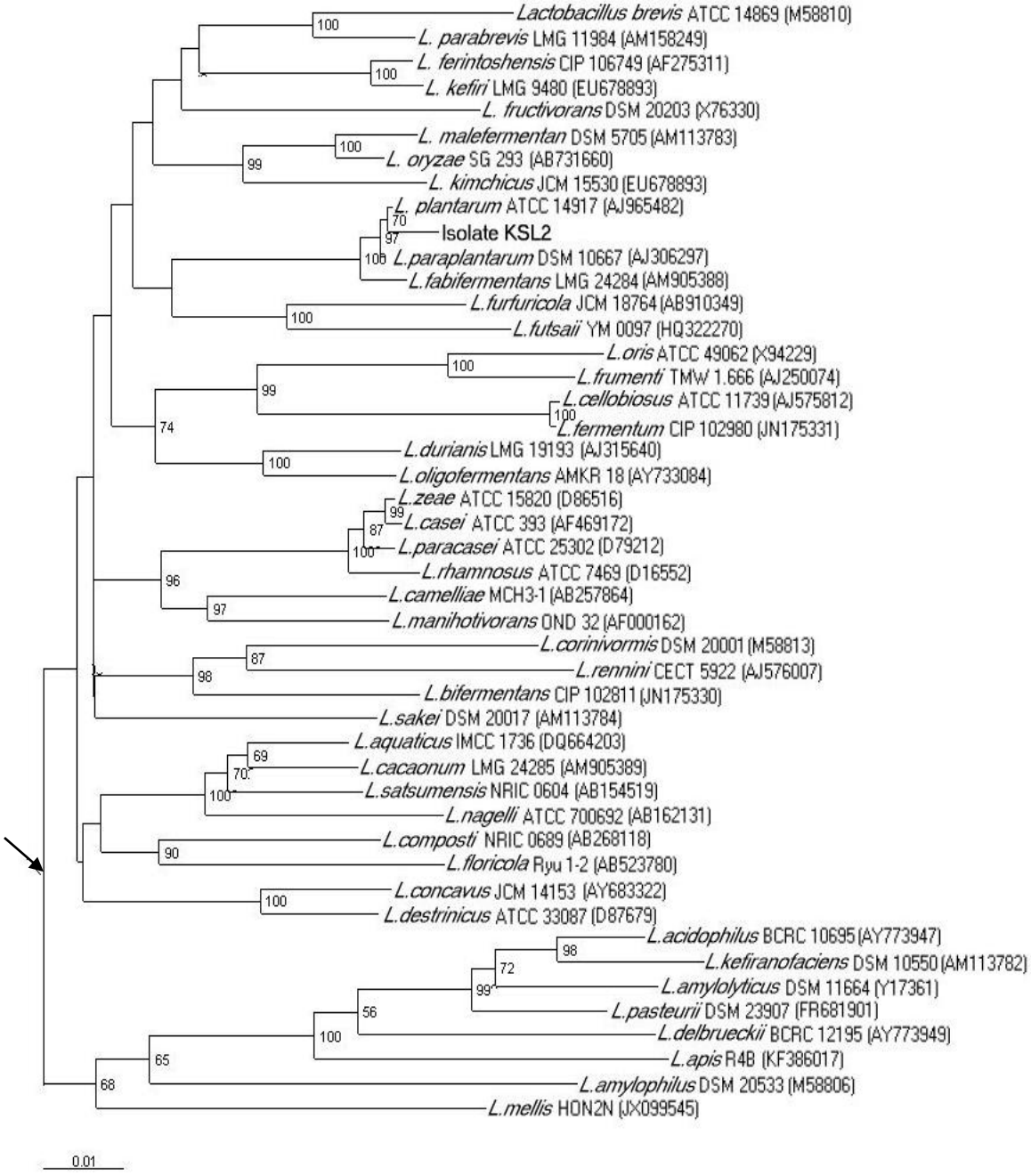

Figure 1. Phylogenetic tree showing relationships between strain KSL2 and representatives of the genus *Lactobacillus* based on 16S rRNA gene sequences. The scale bar indicates 1 nucleotide substitution per 100 nucleotides in 16S rRNA gene sequences. The numbers at nodes indicate the levels of bootstrap support (%) based on a neighbor-joining analysis of 1000 resampled data sets. The arrow indicates the estimated root position of the tree

The role of *Lactobacillus plantarum* KSL2 in Cocoa Beans Fermentation

The role of KSL2 LAB strain on the improvement of cocoa quality was determined based on result of the cut test and lactic acid content of the dry cocoa bean. The quality characteristics of cocoa bean after fermentation is shown in the Table 3.

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Table 3. Quality characteristics of fermented cocoa bean in 5 days fermentation

Experiment regimens	Fermentation time (days)	Characteristics					
		Slaty (%)	Purple (%)	Brown (%)	Moldy (%)	Germinated (%)	Lactic Acid Content (%)
Control*	0	95,00	5,00	0,00	0	0	0,57
	3	20,00	26,00	54,00	0	0	0,59
	5	2,00	11,00	87,00	0	0	0,65
Fermentation with KSL2 strain addition	0	93,00	7,00	0,00	0	0	0,59
	3	15,00	20,00	65,00	0	0	0,62
	5	0,00	8,00	92,00	0	0	0,67

*Control (fermentation without inoculums addition/standard fermentation)

The cut test result and lactic acid content of cocoa bean revealed significant differences in the outcomes of the fermentations (Table 3). Generally, there were increases in brown beans with increasing fermentation time for both of the treatments. The brown beans (fermented beans) increased from 0% to 87% and from 0% to 92% for the control (natural fermentation) and fermentation with KSL2 strain addition for 5 days, respectively, at the end of drying. While slaty beans (unfermented beans) decreased from 95% to 2% and 93% to 0% for the control and fermentation with KSL2 strain addition for 5 days, respectively, at the end of drying. There were also reductions in the purple beans with increasing fermentation time for both treatments. The percentage of purple beans at the end of fermentation for both treatment ranged from 15% to 20%. Results showed that the proportion of purple beans did not exceed 50% for all treatments and this gave an indication that the beans were adequately fermented [14,24]. The enzymatic microbial activities and the pigment degradation led to the internal color changes of nibs from purple to brown reducing considerably the percentage of both purple and slaty beans [8,24]. As slaty beans are an indicator of improperly fermentation of cocoa, slight percentage of such beans demonstrate that our fermentation was processed properly.

Physical analyses at Table 3 showed that the formation of moldy beans was not detected at naturally fermented and seeding experiment with KSL2 LAB strain. There was not obtained germinating beans for both fermentation treatments (Table 3). Mouldy beans and germinating beans that are not detected in both naturally fermented beans and fermentation with the addition of KSL2 strains showed that the fermentation process can improve the quality in accordance with Indonesian cocoa bean standards (SNI 2323 :2008). Indonesian National standards require for the first class quality cocoa beans is a maximum of 2% for the content of the germinated beans and moldy beans [13].

The slight percentage of mouldy beans and germinated beans indicated that the fermentation with KSL2 strain addition could be considered as a good fermentation method as conclude which is expressed by high percentage up 90% of brown cocoa beans. That is indicated cocoa bean that was fermented using KSL2 LAB strain can improve the physical quality of final product.

Table 3 also showed that acid production during cocoa fermentation for both of treatments. Lactic acid content at cocoa beans was fermented using KSL2 LAB strain and control were 0,65% and 0,67% respectively, at the end fermentation. The presence of acid in the fermented cocoa beans can develop of flavour precursors and pigment degradation. Production of acids in the pulp is important in cocoa fermentation as these acids diffuse into the beans and subsequently induce the important biochemical reactions leading to well fermented cocoa beans. However, high acid production in the pulp is detrimental as it leads to excessive acid diffusing into the beans resulting in the production of acidic beans [25].

The completeness of the cocoa bean fermentation is normally measurized by the fermentation index (FI). In this study, the FI was measured daily to know the fermentation progressed. The FI during cocoa bean fermentation shown in the Table 4.

Table 4 showed that FI the beans during fermentation for both treatments increased. FI increased from 0,45 at the start of fermentation to 1,26 for control and for fermentation with KSL LAB strain starter increased from 0,44 at the start of fermentation to 1,41 for 5 days. Table 4 also showed that fermentations were observed to progress at the same pace initially, but later on the fermentation with KSL2 LAB strain addition progressed faster. At day 3, the FI value of the fermentation with KSL2 LAB strain has reached 1 (1.02), while the standard fermentation is still less than 1 (0,92). Thus, indicated that the KSL2 LAB strain

capable of fermenting cocoa bean was better than the standard fermentation. Biehl et al. [26] explained that fermented cocoa beans with FI values below one indicate under fermentation whiles fermented beans with FI values of one and above are considered to be well fermented.

Table 4. Effects of LAB Starter Addition to cocoa bean fermentation index

Time (day)	0	1	2	3	4	5
Control*	0.45±0.00	0.56±0.03	0.73±0.02	0.92±0.06	1.18±0.03	1.26±0.01
Fermentation with KSL2 strain addition	0.44±0.01	0.57±0.02	0.75±0.04	1.02±0.04	1.21±0.06	1.41±0.00

*Control (fermentation without inoculums addition/standard fermentation)

Takrama et al. [27] explained FI is a measure of brownness of cocoa nibs and it is measured to ascertain the degree of fermentation of the beans. Polyphenol compounds such as anthocyanins responsible for the characteristic purple colour of unfermented cocoa beans [28]. Anthocyanins usually disappear rapidly during fermentation process, e.g., 63% were reportedly lost after 6 days fermentation and colour of the beans changes from slaty over purple to brown [28].

The quality of cocoa beans and chocolate depends strongly on the type and characteristics of microbial strains involved in the fermentation. On this basis, the use of starter microbial strains presenting a high fermentative ability is believed to be favourable for cocoa fermentation improvement and producing high quality. Based on result of this study demonstrated that *L. plantarum* KSL2 can be used as starter to cocoa fermentation.

CONCLUSION

Lactobacillus plantarum KSL2 is an indigenous LAB on the cocoa bean fermentation. The addition of *Lactobacillus plantarum* KSL2 strain as a starter for cocoa fermentation can improve cocoa bean quality. However, further studies would be interesting to determine volatile compounds produced from cocoa bean fermentations by LAB strain.

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