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Physicochemical, microbiological, and sensory properties of probiotic chocolate bar Dad-13 made from cocoa beans fermented with *Lactiplantibacillus plantarum* HL-15 during storage

Titiek Farianti DJAAFAR[1*], Endang Sutriswati RAHAYU[2,3], Atika ATIKA[2], Fiametta Ayu PURWANDARI[2], Tyas UTAMI[2,3], Tri MARWATI[1], Retno Utami HATMI[1]

Abstract

Good-quality chocolate can be produced only from good-quality cocoa beans. The cocoa bean quality can be improved using indigenous *Lactiplantibacillus plantarum* HL-15 as a starter culture in fermentation. The functionality of chocolate can be enhanced by the addition of probiotic *L. plantarum* Dad-13. This research aimed to evaluate the physicochemical, microbiological, and sensory characteristics of the probiotic chocolate bar Dad 13 made from fermented cocoa beans *L. plantarum* HL-15 during storage at different temperatures. The research was conducted in three stages. The first stage was the cocoa bean fermentation with and without *L. plantarum* HL-15, the second stage was probiotic chocolate bar Dad-13 production, and the third stage was the storage of probiotic chocolate bar Dad-13 at temperatures of 4 and 26°C. This study found that adding *L. plantarum* HL-15 to the fermentation could produce good-quality cocoa beans, prevent the growth of fungi presented by the pH and Aw of cocoa beans, and form a probiotic chocolate bar during storage. Storage at 4°C maintained the viability of *L. plantarum* Dad-13 and minimized fat breakdown. The organoleptic attributes of the probiotic chocolate bar Dad-13 during storage at 4 and 26°C were not significantly different ($p > 0.05$).

Keywords: characteristic; chocolate; organoleptic; storage time.

Practical Application: Storing probiotic chocolate bars at a temperature of 4°C can maintain the viability of L. plantarum Dad-13 and minimize fat breakdown.

1 INTRODUCTION

Indonesian cocoa has been highly criticized for its inferior quality, mainly due to its high proportion of unfermented cocoa beans and fungal contamination. The quality of fermented cocoa beans is related to the microorganism diversity during their fermentation process. Important microorganisms in cocoa fermentation include yeast, lactic acid bacteria (LAB), acetic acid bacteria, and the genus *Bacillaceae* (Figueroa-Hernández et al., 2019; Kouame et al., 2015a; 2015b). The activity of these microorganisms induces various chemical changes in cocoa beans, including the reduction of phenolic and the formation of flavors and flavor precursors. Previous studies showed that the LAB could be added as the starter culture to produce good-quality fermented cocoa beans (De Vuyst & Weckx, 2016; Ho et al., 2018; Miguel et al., 2017; Viesser et al., 2021). They could prevent the growth of mycotoxin-producing fungi (Marwati et al., 2020; Romanens et al., 2019; Ruggirello et al., 2019). The application of *Lactiplantibacillus plantarum* HL-15 in cocoa beans fermentation inhibited the growth of *Aspergillus niger* YAC-9 and ochratoxin A synthesis (Rahayu et al., 2021c). A detailed study on the quality of cocoa beans obtained from such a process has not been reported yet. Further study needs to be done to better understand the effect of *L. plantarum* HL-15 addition in fermentation on the physicochemical properties of the beans.

Chocolate is one of the most popular products of cocoa processing, which is generally consumed by various ages. Chocolate has unique characteristics of smoothness, melting and flow properties, taste, and aroma; likewise, it has become one of the foods favored by the public (Nafingah et al., 2019). The quality of chocolate is influenced by various factors, including the origin and cocoa variety, fermentation, and roasting (Abballe et al., 2021; Acierno et al., 2019; Żyżelewicz et al., 2018). There is an increasing interest in producing chocolate with high functional properties. The addition of functional food ingredients to chocolate products has been done with mung bean flour (5–15%) and moringa leaf flour (5–15%) (Muhammad et al., 2021). Chocolate can also be

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[1]*National Research and Innovation Agency, Research Center for Food Technology and Processing, Gunungkidul, Yogyakarta, Indonesia.*
[2]*Universitas Gadjah Mada, Department of Food and Agricultural Product Technology, Sleman, Yogyakarta, Indonesia.*
[3]*Universitas Gadjah Mada, Centre for Food and Nutrition Studies, Sleman, Yogyakarta, Indonesia.*
*Corresponding author: titi033@brin.go.id
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enriched with probiotics to develop probiotic chocolate with improved functionality (Cielecka-Piontek et al., 2020; Konar et al., 2016, 2018; Silva et al., 2017; Succi et al., 2017). Research on enriching chocolate using local probiotics in Indonesia has not been carried out. Probiotic affects the composition of gut microbiota resulting in improved metabolism, chronic intestinal inflammatory and functional disorders, and prevention of infections and allergy. The quality of probiotic chocolate is related to its sensory properties and the availability of the probiotics upon consumption. This needs to be studied better to ensure the feasibility of the product in the market. The sensory properties for consumption purposes should be focused, ensuring probiotic availability during the storage of the products.

The *L. plantarum* Dad-13 isolated from *"dadih"* (fermented buffalo milk) has been tested for its ability as a probiotic (Purwandhani et al., 2017; Rahayu et al., 2015, 2019; 2021a; 2021c). *L. plantarum* Dad-13 is a commercial safe-to-consume probiotic. It was reported to provide various health benefits (Purwandhani et al., 2017; Rahayu et al., 2019; 2021b; Utami et al., 2020). Studies on the application of the probiotic *L. plantarum* Dad-13 have been carried out on fermented milk and other fermented products (Meidistria et al., 2020; Purwandhani et al., 2017). However, its application to chocolate has not been done. The development of new functional food using indigenous probiotics must be carried out. Chocolate is a good matrix for incorporating and delivering probiotics due to its sensory characteristics that attract the consumer (Konar et al., 2016). This research aimed to evaluate the quality properties of fermented cocoa beans obtained from the fermentation with *L. plantarum* HL-15 as raw material for probiotic chocolate production. Furthermore, this study also evaluated the viability of probiotic, physicochemical, and sensory characteristics of the chocolate bar enriched with *L. plantarum* Dad-13 as a probiotic during storage in different conditions.

2 MATERIALS AND METHODS

2.1 Materials

The cocoa beans used in this study were of *Lindak* (bulk) variety, which were obtained from a farmer group in Gunungkidul, Yogyakarta. *L. plantarum* HL-15 culture and *L. plantarum* Dad-13 powder were obtained from the Food and Nutrition Culture Collection (FNCC), Centre for Food and Nutrition Studies, Universitas Gadjah Mada Yogyakarta. The material for producing the probiotic chocolate bars was cocoa butter, which was obtained from the Agricultural Technology Park (ATP) Nglanggeran, Gunungkidul, Yogyakarta. Fine granulated sugar, full cream powdered milk, soybean lecithin, baking soda, and vanilla flavor were obtained from a local market in Yogyakarta and were of food grade. The chemicals and culture media used were of analytical grade.

2.2 Equipment

Equipment for cocoa bean fermentation included a wooden fermentation box (40 kg capacity), a washing bucket, and a woven bamboo platform (*rigen*) for drying. The equipment used for chocolate production was a steamer (capacity of 20 kg/batch), roaster (capacity of 100 kg/h), desheller (capacity of 100 kg/h), grinder (capacity of 1 kg/batch), ball mill machines for mixing and refining (capacity of 10 kg/batch), conching machine (capacity of 10 kg/batch), tempering machine (capacity of 10 kg/batch), blender BL-102GL (capacity of 250 mL/batch), aluminum basin, thermometer, plastic molds, and chiller.

2.3 Fermentation and drying of cocoa beans

The fermentation of the cocoa beans was conducted based on the method of Djaafar et al. (2019) and Marwati et al. (2019). Fresh cocoa pods were broken to obtain the wet beans. Around 40 kg of wet cocoa beans were used for each treatment. Only mature and healthy cocoa beans were used in this study. The wet beans were fermented with *L. plantarum* HL-15 (S1) and without *L. plantarum* HL-15 (S2) in duplicate. In the fermentation with the addition of starter culture, the cocoa beans were inoculated with 20 mL of culture *L. plantarum* HL-15 or an amount of 10^{10} CFU/kg of wet cocoa beans. The fermented dry beans were packed in sealed plastic containers and stored at room temperature until analysis and their use for chocolate bar processing.

2.4 Probiotic chocolate bar processing

The production of probiotic chocolate was as follows. Around 4.5 kg of fermented cocoa beans were steamed for 20 min before roasting. Roasting was done at a temperature of 100–110°C for 15–19 min until the cocoa beans turned brown and easily crushed. The nibs were then separated from their husk using a desheller machine equipped with a winnower. The nibs were then crushed into a paste (cocoa mass/liquor) by using a cocoa grinder machine. The cocoa liquor was then refined and mixed with cocoa butter, full-cream milk powder, and sugar using a ball mill machine. Refining was carried out at 50°C for 10–11 h, by turning the liquor two times in the second and fourth hours. The addition of lecithin, baking soda, and vanilla was done during the conching process. The conching process was carried out at 60–70°C for 2 h. In the final 10 min of the conching, the temperature was lowered to 50°C. The *L. plantarum* Dad-13 probiotic with a concentration of 10^8–10^9 CFU/g was then added and stirred for 10 min (Cielecka-Piontek et al., 2020). The chocolate paste was then tempered. The temperature was set as follows: the initial temperature was 40°C and then lowered to 30°C before increasing to 40°C. The temperature was then lowered to 34 °C, and the molding for the chocolate bar was done. After molding, the chocolate was put into the fridge (10–15°C) to accelerate the solidification of the chocolate. Packaging of chocolate was carried out at 26°C. The packaged chocolate was stored at 4 and 26°C for 10 weeks until analysis.

2.5 Analysis

2.5.1 Quality of fermented cocoa beans

Analysis of bean quality was carried out after the beans were dried and before they were used to make probiotic chocolate. The moisture content was determined using the

thermo-gravimetric method based on AOAC (2005). The quality of beans was determined by the cut-test method based on the Indonesian National Standards (The National Standardization Agency of Indonesia, 2008).

2.5.2 Fungi analysis of fermented cocoa beans

The analysis of fungal contamination on cocoa beans was carried out using direct platting and dilution plating methods according to Black (2020). DG-18 media were prepared by dissolving 15.75 g of DG-18 Agar Base media in 500 mL aquadest. Heating was carried out until all media were dissolved. Around 110 g of glycerol and 1 vial of chloramphenicol were then added. Sterilization was done by autoclaving at 121°C for 15 min. The direct plating media were prepared by pouring 10–15 mL of sterile DG-18 media into a sterile petri dish aseptically. The mixture was then allowed to solidify. A total of 5 fermented cocoa beans were placed on the media and then incubated for 5 days at room temperature. The growth of fungi was observed every 24 h. The analysis was done in duplicate.

The dilution plating method was carried out according to Black (2020), Suhartatik et al. (2013) and Utami et al. (2020). A series of sample dilutions were performed at several dilution levels (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). A total of 5 g of the sample was added with 45 mL of peptone water (0.1%) (a dilution concentration of 10^{-1}) and was crushed with a stomacher for 2 min. As much as 1 mL of the sample was then dissolved in 9 mL of peptone water (0.1%) to obtain a dilution level of 10^{-2}. The steps were then repeated until the dilution level of 10^{-4}. A volume of 1 mL of the solution of 10^{-2}, 10^{-3}, and 10^{-4} dilution levels was then put into a sterile disposable petri dish. Around 10–15 mL of sterile DG-18 media was added and mixed evenly. After the media were thickened, sterile DG-18 media were poured as an overlay. The incubation was then carried out for 5 days at room temperature. Fungal growth in the form of colonies was then calculated and expressed as the average number of fungi per gram of cocoa beans sample.

2.5.3 Physical and chemical quality

Water activity (Aw) analysis was carried out using an Aw meter (Decagon Pawkit) (NE Hopkins Ct, Pullman, WA 99163, United States). The pH and fat content of chocolate were measured according to AOAC (2005).

The peroxide value (PV) was determined using the titration method according to the AOAC (2005) with modifications. A total of ± 1 g of fat extracted from the chocolate sample was put into an Erlenmeyer flask and added with 25 mL of chloroform: acetic acid (2:3) solution. Notably, 1 mL of potassium iodide solution was added, and the solution was shaken for 1 min. Distilled water (35 mL) was added, and starch was used as an indicator. The titration was done with 0.02 N sodium thiosulfate solution and was stopped when the blue color disappeared. PV was expressed as mEq O_2/kg of the chocolate sample.

2.5.4 Viability of Lactiplantibacillus plantarum Dad-13

The viability of *L. plantarum* Dad-13 in probiotic chocolate bars was analyzed using the total plate count method. The evaluation was done during 2 months of storage at 1-week intervals. Notably, 25 g of the probiotic chocolate bar was added with 225 mL of 0.1% peptone water buffer and then crushed using a stomacher. The mixture was then diluted at 10^{-4}–10^{-8}. A total of 0.1 mL of the suspension was spread on sterile MRS-$CaCO_3$ media and then incubated at 37°C for 24–48 h. Colonies were counted and expressed as log CFU/g (Utami et al., 2020).

2.5.5 Sensory analysis

Sensory evaluation was assessed by the affective testing method using a five-level hedonic scale based on the method developed by Sharif et al. (2017). The analysis involved 60 untrained panelists. Consumers' rate of liking and product acceptance was measured on a hedonic scale. Sensory attributes analyzed include color, appearance, aroma, taste, texture, and aftertaste. An analysis of commercial chocolate (without probiotics) purchased in the local market was carried out as a reference.

2.6 Research design and statistical analysis

This research was conducted using a factorial completely randomized design with two factors in triplicate. During cocoa bean fermentation, the first factor was *S1* = fermentation with *L. plantarum* HL-15 starter and *S2* = fermentation without *L. plantarum* HL-15 starter. The storage of probiotic chocolate was used as a second factor, namely, P1 = storage temperature of 26°C and *P2* = storage temperature of 4°C. The data obtained were averaged and analyzed using two-way ANOVA ($p < 0.05$). Statistical analysis was carried out using Microsoft Excel (Microsoft, USA) and the SPSS 22.0 program.

3 RESULTS AND DISCUSSION

3.1 Quality of fermented cocoa beans

The moisture content of cocoa beans fermented without *L. plantarum* HL-15 was lower than that of fermented with *L. plantarum* HL-15. Beans obtained from the fermentation with the starter *L. plantarum* HL-15 had better quality (Table 1).

Table 1. The quality of fermented cocoa beans.

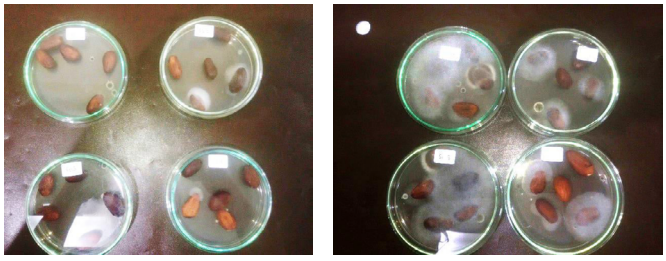
Treatment	Bean count/100 g	Moldy bean (%)	Slatey bean (%)	Insect-infested bean (%)	Foreign matter (%)	Clustered bean (%)	Germinated bean (%)	Moisture content (%)	Quality group
S1	101 ± 3.55[a]	0	3.47[b]	0	0	0	0	6.76 ± 0.00[a]	II-B
S2	100 ± 4.24[a]	0	6.49[a]	0	0	5.65	0	4.88 ± 0.00[b]	II-B

Values ($n = 3$) with different letters in the same column were significantly different ($p < 0.05$). S1: fermentation with *L. plantarum* HL-15; S2: fermentation without *L. plantarum* HL-15.

The beans were mostly well-fermented. Fermentation with *L. plantarum* HL-15 produced significantly ($p < 0.05$) a lower number of slatey beans (3.47%) than that of fermented without *L. plantarum* HL-15 (6.49%). However, both were included in the same quality group (II-B) based on the Indonesian National Standards.

Direct visual observation on fermented cocoa beans showed no fungal growth in both treatments (Table 1). However, analysis using direct plating and dilution platting methods showed that cocoa beans from fermentation with *L. plantarum* HL-15 had less fungal contamination (Figure 1 and Table 2, respectively). There were three groups of observable fungi based on their color variations: white and gray (identified as *Rhizopus* sp.), dark green (identified as *Cladosporium* sp.), and brown (identified as *Aspergillus* sp.). There was more fungal contamination in the beans from fermentation without *L. plantarum* HL-15 (Figure 1). The fungi were identified as xerophilic fungi. A previous study by Rahayu et al. (2021c) showed that beans obtained from fermentation without *L. plantarum* HL-15 were found to produce mycotoxins-producing fungi, namely, *Aspergillus* sp. This was similar to the result found in this study. Beans obtained from fermentation without *L. plantarum* HL-15 had more contamination of *Aspergillus* sp.

The LAB is one of the essential microorganisms in cocoa bean fermentation, which contributed to the formation of the aroma compounds (Viesser et al., 2020, 2021). The use of *L. plantarum* HL-15 in bean fermentation showed that the LAB, apart from functioning as an antifungal, could also maintain the quality of beans during storage (Marwati et al., 2020). This was in agreement with the previous results of Marwati et al. (2019) and Romanens et al. (2019). Cocoa beans fermented with *L. plantarum* HL-15 have low fungi contamination. This indicated that using *L. plantarum* HL-15 in cocoa bean fermentation could suppress fungi growth. Therefore, cocoa beans fermented with *L. plantarum* HL-15 are safe to use as a raw material for probiotic chocolate bar processing.



(A) (B)

Figure 1. The results of the fungal contamination test using the direct plating method on fermented cocoa beans. (A) Fermentation with *L. plantarum* HL-15. (B) Fermentation without *L. plantarum* HL-15.

Table 2. Total fungi of fermented cocoa beans by the dilution plating method.

Sample code	Number of cells (CFU/mL)
S1	4.2×10^2
S2	4.5×10^2

Values were the average of three replications. S1: fermentation with *L. plantarum* HL-15; S2: fermentation without *L. plantarum* HL-15.

3.2 Physicochemical properties of probiotic chocolate bar

The Aw values of probiotic chocolate ranged from 0.61 to 0.71 (Table 3). The Aw value increased during the storage at both 26 and 4°C for all the treatments. The Aw of probiotic chocolate made from beans of *L. plantarum* HL-15 fermentation and storage at 26°C was significantly higher ($p < 0.05$) than the other treatments. Longer storage duration significantly increased the Aw ($p < 0.05$). This may be due to the absorption of water vapor into the chocolate from the environment. The chocolate bar in this study contained sugar, wherein sugar is known as a hygroscopic component that can absorb water from the environment.

The Aw in food indicates the water availability for metabolic activity and the growth of microorganisms. Hence, it is commonly used to determine the microbial stability of food products (England, 2020). In probiotic chocolate products, the water activity highly relates to the viability of probiotic cells. Probiotic bacteria cannot grow in low Aw conditions (< 0.6) (Silva et al., 2017; Vesterlund et al., 2012). In this study, the Aw value of chocolate was in a safe condition for the growth of probiotic *L. plantarum* Dad-13. The Aw in the chocolate product was affected by various factors, including the type and amount of chocolate material and process conditions, especially in the refining and conching process of chocolate (Razavizadeh & Tabrizi, 2021).

The pH values of all treatments were in a narrow range of 6.22–6.80 (Table 4). Longer duration of storage resulted in a significant decrease in the pH values ($p < 0.05$) for all treatments but not significantly different unless the probiotic chocolate S2P1 treatment had significantly higher pH values than the other treatments. This could be due to the activity of *L. plantarum* Dad-13 contained in the chocolate. The LAB produce lactic acid in the presence of sugar, which causes a decrease in pH value.

The fat content in probiotic chocolate decreased during 10 weeks of storage (Table 5). The decrease was smaller in

Table 3. Water activity (Aw) of probiotic chocolate during 10 weeks of storage at 26 and 4°C.

Sample code	Storage time (weeks)					
	0	2	4	6	8	10
S1P1	0.61 ± 0.00^{aA}	0.62 ± 0.01^{bB}	0.66 ± 0.01^{cC}	0.68 ± 0.01^{dC}	0.71 ± 0.00^{eC}	0.71 ± 0.00^{eB}
S1P2	0.61 ± 0.00^{aA}	0.61 ± 0.01^{aA}	0.63 ± 0.00^{bB}	0.67 ± 0.01^{cB}	0.68 ± 0.01^{dA}	0.69 ± 0.01^{eA}
S2P1	0.61 ± 0.00^{aA}	0.61 ± 0.00^{aA}	0.62 ± 0.01^{bA}	0.66 ± 0.00^{cA}	0.69 ± 0.00^{dB}	0.69 ± 0.00^{dA}
S2P2	0.61 ± 0.00aA	0.62 ± 0.01^{bB}	0.62 ± 0.01^{bA}	0.66 ± 0.00^{cA}	0.69 ± 0.01^{dB}	0.69 ± 0.01^{dA}

Values ($n = 3$) with different lowercase letters in the same row were significantly different ($p < 0.05$). Values ($n = 3$) with different uppercase letters in the same column were significantly different ($p < 0.05$). S1P1: probiotic chocolate from fermented beans by *L. plantarum* HL-15 at 26°C; S1P2: probiotic chocolate from fermented beans by *L. plantarum* HL-15 at 4°C; S2P1: probiotic chocolate from fermented beans without *L. plantarum* HL-15 at 26°C; S2P2: probiotic chocolate from fermented beans without *L. plantarum* HL-15 at 4°C.

Table 4. The pH values of probiotic chocolate during 10 weeks of storage at 26 and 4°C.

Sample code	Storage time (weeks)					
	0	2	4	6	8	10
S1P1	6.80 ± 0.01^{aC}	6.80 ± 0.01^{aC}	6.68 ± 0.02^{aC}	6.56 ± 0.04^{aB}	6.32 ± 0.01^{aB}	6.31 ± 0.01^{aA}
S1P2	6.80 ± 0.01^{aC}	6.80 ± 0.01^{aC}	6.60 ± 0.01^{aC}	6.56 ± 0.01^{aB}	6.25 ± 0.05^{aB}	6.22 ± 0.03^{aA}
S2P1	6.80 ± 0.01^{aC}	6.81 ± 0.02^{bC}	6.75 ± 0.03^{bC}	6.68 ± 0.02^{bB}	6.39 ± 0.03^{bB}	6.38 ± 0.02^{bA}
S2P2	6.80 ± 0.01^{aC}	6.64 ± 0.01^{aC}	6.61 ± 0.01^{aC}	6.63 ± 0.01^{aB}	6.33 ± 0.02^{aB}	6.32 ± 0.01^{aA}

Values ($n = 3$) with different lowercase letters in the same row were significantly different ($p < 0.05$). Values ($n = 3$) with different uppercase letters in the same column were significantly different ($p < 0.05$). S1P1: probiotic chocolate from fermented beans by *L. plantarum* HL-15 at 26°C; S1P2: probiotic chocolate from fermented beans by *L. plantarum* HL-15 at 4°C; S2P1: probiotic chocolate from fermented beans without *L. plantarum* HL-15 at 26°C; S2P2: probiotic chocolate from fermented beans without *L. plantarum* HL-15 at 4°C.

Table 5. Fat content of probiotic chocolate during 10 weeks of storage at 26 and 4°C.

Sample code	Storage time (weeks)					
	0	2	4	6	8	10
S1P1	48.08 ± 0.01^{bD}	44.42 ± 0.69^{aD}	$43.70. \pm 0.15^{aD}$	42.03 ± 0.85^{aC}	40.42 ± 0.30^{aB}	39.92 ± 0.60^{aA}
S1P2	48.08 ± 0.01^{bD}	48.98 ± 0.27^{cD}	49.51 ± 0.16^{cD}	47.50 ± 0.54^{cC}	46.35 ± 0.42^{cB}	45.53 ± 0.12^{cA}
S2P1	48.08 ± 0.01^{bD}	43.84 ± 0.78^{aD}	43.46 ± 0.29^{aD}	43.33 ± 0.04^{aC}	42.47 ± 0.22^{aB}	40.48 ± 0.44^{aA}
S2P2	48.08 ± 0.01^{bD}	$48.12. \pm 1.38^{bD}$	47.28 ± 0.65^{bD}	45.95 ± 0.02^{bC}	45.57 ± 0.41^{bB}	43.14 ± 1.10^{bA}

Values ($n = 3$) with different lowercase letters in the same row were significantly different ($p < 0.05$). Values ($n = 3$) with different uppercase letters in the same column were significantly different ($p < 0.05$). S1P1: probiotic chocolate from fermented beans by *L. plantarum* HL-15 at 26°C; S1P2: probiotic chocolate from fermented beans by *L. plantarum* HL-15 at 4°C; S2P1: probiotic chocolate from fermented beans without *L. plantarum* HL-15 at 26°C; S2P2: probiotic chocolate from fermented beans without *L. plantarum* HL-15 at 4°C.

probiotic chocolate stored at 4°C than that stored at 26°C. Fat content plays an important role in the characteristics of chocolate. The lipid phase in chocolate is responsible for thermal stability, mouthfeel, the release of flavor, and overall consumer satisfaction (Ostrowska-Ligęza et al., 2018). In probiotic chocolate, fat protects the probiotic strain during processing and storage (Konar et al., 2018; Mirkovic et al., 2018).

The PV of probiotic chocolate increased during 10 weeks of storage. There was a sharp increase in the PV from 0 to 6 weeks of storage for the chocolate stored at 26°C. Likewise, the PV of chocolate stored at 4°C was significantly lower than that stored at 26°C (Table 6). These results were in line with research conducted by Azarpazhooh et al. (2021), who found that the PV of milk chocolate at 4°C for 7 days was very low. Storage of chocolate at 26°C resulted in higher liquid fat content in chocolate. This was in agreement with the result of Zhao and James (2019) that the liquid ratio of chocolate fat is higher at high temperatures. Fat had a high surface area in liquid form, allowing a higher contact rate with free radicals. This resulted in a high rate of oxidation. The increase in PV was due to the primary oxidation of unsaturated lipids (mainly oleic acid) that starts with hydroperoxide formation (Rasti et al., 2017). On the contrary, there was no significant difference in the treatment of probiotic LAB addition on the fat content and PV of the chocolate. This indicated that the addition of LAB probiotics did not contribute to fat oxidation in the chocolate.

Table 6. Peroxide value (mEq O_2/kg) of probiotic chocolate during 10 weeks of storage at 26 and 4°C.

Sample code	Storage time (weeks)			
	0	6	8	10
S1P1	0.05 ± 0.00^{aC}	0.28 ± 0.009^{bA}	0.32 ± 0.013^{bA}	0.37 ± 0.039^{bB}
S1P2	0.05 ± 0.00^{aA}	0.02 ± 0.002^{aA}	0.05 ± 0.001^{aA}	0.07 ± 0.003^{aB}
S2P1	0.05 ± 0.00^{aC}	0.29 ± 0.003^{bA}	0.32 ± 0.011^{bA}	0.39 ± 0.012^{bB}
S2P2	0.05 ± 0.00^{aA}	0.04 ± 0.004^{aA}	0.05 ± 0.001^{aA}	0.07 ± 0.002^{aB}

Values ($n = 3$) with different lowercase letters in the same column were significantly different ($p < 0.05$). Values ($n = 3$) with different uppercase letters in the same row were significantly different ($p < 0.05$). S1P1: probiotic chocolate from fermented beans by *L. plantarum* HL-15 at 26°C; S1P2: probiotic chocolate from fermented beans by *L. plantarum* HL-15 at 4°C; S2P1: probiotic chocolate from fermented beans without *L. plantarum* HL-15 at 26°C; S2P2: probiotic chocolate from fermented beans without *L. plantarum* HL-15 at 4°C.

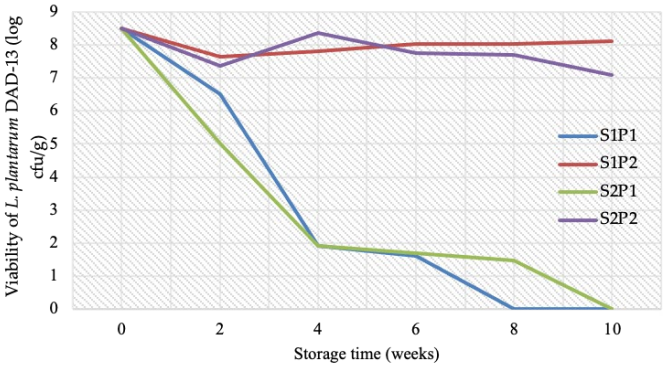
3.3 Viability of probiotic L. plantarum Dad-13

The viability of probiotic cells upon consumption is a critical quality attribute for probiotic products. The viability of *L. plantarum* Dad-13 during chocolate storage at 4°C was at 7–8 log cycles for both cocoa bean fermentation treatments. However, the viability of the probiotics greatly decreased when it was stored at 26°C. No viable cells were evaluated in the tenth week of storage (Figure 2). This was in agreement with the results of Montel Mendoza et al. (2013), who stated that dry cultures of *Lactococcus lactis* CRL 1584, *L. lactis* CRL 1827, *Lactococcus garvieae* CRL 1828, and *L. plantarum* CRL 1606 had higher viability when they were stored at cold temperatures (4°C) than those stored at room temperature (25°C) for 18 months. Meanwhile, Cielecka-Piontek et al. (2020) found that probiotic strains of BLr – *Lactobacillus rhamnosus* and BBb – *B. breve* DSM 16604 still survived and were in stable number (10^8 CFU/g) in dark chocolate samples after stored for 6 months at both 20 and 4°C. Similarly, Cozentino et al. (2022) found that the viability of the probiotic *Enterococcus faecium* CRL 183 in the chocolate spread was quite stable at 20°C for 180 days, without suffering any significant reduction in their viability ($p > 0.05$). Hence, the viability of the probiotic in chocolate is highly dependent on the species of the microorganisms used as the probiotics. In this case, it was found that probiotic chocolate enriched with *L. plantarum* Dad-13

must be stored at a cold temperature (4°C) to maintain the viability of the probiotic.

3.4 Sensory properties of probiotic chocolate bar

Sensory analysis showed that the panelists' preference for color, appearance, aroma, taste, texture, and aftertaste on the probiotic chocolate stored at 26°C was similar to that of the reference ($p < 0.05$). On the contrary, the texture and aftertaste of chocolate stored at 4°C were significantly different ($p < 0.05$) compared with the reference. The treatment of cocoa bean fermentation with and without *L. plantarum* HL-15 did not show significant differences ($p < 0.05$) on all tested attributes, namely, color, appearance, aroma, taste, texture, and aftertaste (Table 7). However, the appearance of the reference chocolate was glossier than the probiotic chocolate bar *L. plantarum* Dad-13. These results showed that the production of probiotic chocolate using *L. plantarum* Dad-13 is feasible. The consumer can easily accept it due to its similarity with readily available chocolate products.



S1P1: probiotic chocolate bar from fermented beans by *L. plantarum* HL-15 and storage at 26°C; S1P2: probiotic chocolate bar from fermented beans by *L. plantarum* HL-15 and storage at 4°C; S2P1: probiotic chocolate bar from fermented beans without *L. plantarum* HL-15 and storage at 26°C; S2P2: probiotic chocolate bar from fermented beans without *L. plantarum* HL-15 and storage at 4°C.

Figure 2. The *L. plantarum* Dad-13 viability during probiotic chocolate bar storage at 26 and 4°C. The *L. plantarum* Dad-13 viability can be maintained during storage at 4°C for 10 weeks.

Table 7. The sensory attribute of probiotic chocolate during storage at 26 and 4°C.

Sensory attribute	Sample code				Reference sample
	S1P1	S1P2	S2P1	S2P2	
Color	3.90[a]	4.38[a]	3.08[a]	3.65[a]	4.00[a]
Appearance	3.00[a]	3.05[a]	2.73[b]	3.18[a]	3.72[a]
Aroma	2.57[a]	2.67[a]	2.50[a]	2.78[a]	2.47[a]
Taste	3.93[a]	3.82[a]	3.23[a]	3.88[a]	3.82[a]
Texture	2.90[a]	4.00[b]	4.32[b]	3.97[b]	1.87[a]
Aftertaste	2.45[a]	3.03[b]	2.52[a]	2.75[b]	3.13[b]

Values ($n = 60$) with different lowercase letters in the same row were significantly different ($p < 0.05$). S1P1: probiotic chocolate bar from fermented beans by *L. plantarum* HL-15 and storage at 26°C; S1P2: probiotic chocolate bar from fermented beans by *L. plantarum* HL-15 and storage at 4°C; S2P1: probiotic chocolate bar from fermented beans without *L. plantarum* HL-15 and storage at 26°C; S2P2: probiotic chocolate bar from fermented beans without *L. plantarum* HL-15 and storage at 4°C. Reference was a chocolate product available in the market.

4 CONCLUSION

In this research, a new chocolate product enriched with probiotic bacteria *L. plantarum* Dad-13 that was produced from fermented cocoa beans using *L. plantarum* HL-15 was successfully developed. The chocolate products could support the growth of probiotic *L. plantarum* Dad-13. However, it should be stored at 4°C to suppress changes in cell viability, water activity, fat, and PV. The probiotic chocolate bar enriched by *L. plantarum* Dad-13 had a viability of 10^7–10^8 CFU/g. This product was generally accepted by the consumer due to its similarity to readily available chocolate products.

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