PHYSICO-CHEMICAL PROPERTIES OF CHOCOLATE OF *Lactobacillus Plantarum* FROM FERMENTED COCOA BEANS

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Abstract
The existence and isolation of Lactobacillus plantarum during cocoa bean fermentation was examined in this study. The results showed significant growth at the 24 and 48 h time points compared to the 0, 72 and 96 hour time points. L. plantarum 1, which represented rod-shaped bacteria, catalase negative and homofermentative with identification (ID) of 99.9% through a phenotypic API 50 HCL system (Biomerieux) was isolated. The effect of isolated L. plantarum on dark chocolate over a three month storage period was studied. The growth level of L. plantarum in dark chocolate and the mousse decreased until there were 81.25 ± 0% (6.5 ± 0 log cfu/g) and 76.88 ± 0.88% (6.2 ± 0.07 log cfu/g) in the samples, respectively. The physico-chemical properties of dark chocolate with L. plantarum were almost similar to control dark chocolate based on measurements of viscosity, texture hardness, pH, colour and water activity. However, the physico-chemical properties of dark chocolate mousse containing L. plantarum were significantly different compared to control and dark chocolate with L. plantarum over three months of storage at 4ºC. Overall, L. plantarum was able to grow in dark chocolate without changing the physico-chemical properties of the dark chocolate.

Keywords: dark chocolate, cocoa fermentation, *Lactobacillus plantarum*, mousse chocolate, physico-chemical properties

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1. INTRODUCTION

There are three stages involved in cocoa processing: primer, secondary and production. The fermentation process is grouped in the primer stage (Ardhana and Fleet, 2003). Fermentation involves releasing energy through oxidation and the reduction of a substrate product by a microorganism. This process, which is performed by yeast, lactic acid bacteria (LAB) and acetic acid bacteria, occurs on the inside and outside of cocoa beans, which can cause turbulence in internal hydrolytic processes and enzyme activity (Camu et al., 2007). The fermentation process is crucial for ensuring chocolate quality and flavour (Beckett, 2008). Cocoa pulp is rich in carbohydrates such as glucose, fructose and sucrose. The low pH of cocoa pulp, which is around pH 3.0-3.5, corresponds to the high citric acid content of the pulp. The acidic conditions promote the growth of yeast in the first 24 h of fermentation. When the growth of yeast declines, LAB replace the yeast and cause degradation of the pulp, which flows out of the cocoa bean as pulp juice on the second day of fermentation. Oxygen enters the cocoa bean due to the decreasing amount of pulp, leading to acetic acid bacteria growth. The increase in acetic acid levels and temperature cause the cocoa bean cotyledon to die, thereby finishing the fermentation process. *L. plantarum* is the dominant LAB species that is found during the cocoa fermentation process in Malaysia (Rose, 1998).

L. plantarum is a gram positive, rod-shaped, mesophilic and facultative anaerobic bacteria. It can survive in the pH range of 4.0-4.5 and temperatures around 4°C-45°C. The bacteria are also able to adapt to different circumstances, especially sugar sources inside vegetables and human beings. Previous research studies have successfully demonstrated the probiotic nature of *L. plantarum* and its benefits in food production (Vries et al., 2006). There are two strains of *L. plantarum* that have being identified as being probiotic, 299V and LP01 (Kleerebezem, 2002; Nagendra, 2001). Isolauri (2004) also reported that both *Lactobacillus and Bifidobacterium*

promotes beneficial and good impact to our body. Probiotics bacteria such as lactobacilli and bifidobacteria are natural and viable that is beneficial to human gut (Homayouni et al. 2008; FAO/WHO, 2001). They help our body by protecting our body from dangerous microorganisms which colonizes and infected our digestive tracts (Sanders, 2003; Ouwehand et al., 2002).

Currently, probiotic foods are being marketed as sources of bacteria that help maintain a healthy body. Generally, probiotics are always being assimilated into dairy products and also fruit juices (Possemiers et al., 2010). Therefore, chocolate is believed to have a higher lipid contents from cocoa butter (Lahtinen et al., 2007) which could protect and preserve the probiotics. In order to increase the efficacy of probiotics and consumer acceptance of food product, the suggested level of probiotic bacteria consumption is at 10^6 cfu/g (Boylston et al., 2004; Aragon-Alegro et al., 2007).

The production of dark chocolate with *L. plantarum* is viewed as a functional and bioactive food due to the polyphenol, isomalt and living bacteria content of the chocolate. The antioxidant activity in chocolate is considered to be a good source for reducing the oxidation of low density lipoprotein (LDL), which can cause artery damage (Beckett, 2008). Also, the high content of saturated fat in dark chocolate may explain its ability to reduce the level of lipid oxidation products *ex vivo* (Mursu et al., 2004). There are certain companies that have developed probiotic chocolate such as the Attune Foods Company (America) and Barryl Callebaut Sdn. Bhd (Malaysia). This study was carried out to determine the physiochemical effects of *L. plantarum* that were isolated from fermented cocoa beans in dark and mousse chocolate as well as to determine the survival of *L. plantarum* in dark chocolate. MRS agar was used in this experiment as a selective medium for *Lactobacillus* growth while the API 50 HCL test was conducted to confirm the identification of the *L. plantarum* species

2. MATERIALS AND METHOD

Fermented Cocoa Bean and Chocolate

Cocoa beans and the fermenting pulp were obtained from Pahang (Malaysia). Sampling techniques were performed according to Ardhana and Fleet (2003). The dark chocolate was supplied by Barry Callebault (Malaysia). The asetonitril solution was purchased from Sigma-Aldrich (St. Louis, MO, USA). The potassium dehydrogenate phosphate (KH_2PO_4) glucose, fructose, sucrose, acid lactic, acid acetic and acid citric were purchased from Merck (Steinheim, Germany).

Bacterial Isolation, Chocolate Sample and Microbiology Analysis

A 10^{-1} of dilution was prepared by mixing 50 g of cocoa beans and pulp into 450 mL of 0.1% peptone water. A 10^{-8} serial dilution was performed. As much as 1 mL of each dilution was mixed with 15 mL of MRS agar and incubated overnight at 37°C. These methods were repeated in duplicate for samples from Days 1, 2, 3 and 4 of the fermentation process. The colonies that formed were counted using the plate counting method (TPC). Next, 0.1 mL from dilutions 10^{-5}, 10^{-6}, and 10^{-7} for Day 1 and 2 of the fermentation process was spread onto MRS agar. The colonies for Days 1 and 2 were selected randomly. Up to 20 different strains from each day were selected for streaking. The morphology of the strains was analyzed and gram staining was performed on 40 different strains that were selected for lactic acid bacteria confirmation. The API 50 HCL test was carried out on colonies with positive gram stain results in order to identify the bacterial species. A catalase and a Durham test were performed to determine the characteristics of the isolated Lactobacillus species. Three types of chocolate samples were used including S1, S2 and S3. S2 and S3 were prepared by adding of 10^8 cfu/g of *L. plantarum* to melted dark and mousse chocolates, respectively. Both chocolates were cooled at 34°C and 14ºC. Then 25 g of the chocolate samples were mixed with 225 g of peptone water to obtain a 10^{-1} dilution. A 10^{-8} serial dilution was carried out. On

millilitre of each dilution was mixed with 20 mL of MRS agar. The colony log and the percentage of living bacteria were calculated for each sample.

Chocolate Physico-chemical Analysis

The viscosities before and during the storage of S1 and S2 samples were measured using a Brookfield DV-I + Programmable viscometer and the rigidity of the chocolates were determined using Shidmazu Twin Column Texture Analysis. The pH of the samples was measured using a pH meter (Eutech – Instrument pH 510). The Commission International Enlarge (CIE) colour system, which is also known as the L*, a*, b* system, was used to analyze the samples colour. This system measured the intensity of the red, blue, yellow and green indices. The water activity (a_w) of the samples was determined by analyzing 5 g of melted chocolate using Novasina Lab Partner.

4. RESULTS

Microbiology Analysis

The total plate counting (cfu/g) for *Lactobacillus* growth on selective MRS agar isolated from fermented cocoa beans for 0-96 h was determined for each sample (Figure 1). The highest percentage of *Lactobacillus* growth was obtained 24-48 h in the fermentation process. A significant change ($p<0.05$) in total plate count was found during the first 0-24 h of fermentation. The morphologies of the colonies that were observed after the spread plate method are big, spherical and cream coloured, which matches the colony morphology of *Lactobacillus* described by Johansson et al., (1998). Microscopic observation and biochemical tests showed that all forty colonies that were selected were rod-shaped, gram positive and catalase negative. The genera of *Lactobacillus* and *Carnobacterium* are the only genera in the taxonomy of LAB that have a rod shape (Hutkins, 2006). Nine colonies that were Gram positive, rod-shaped, catalase negative and grew well on the MRS agar were chosen to be identified using API 50 CHL. From the nine colonies, Colony no. 5 contained the species *L. plantarum* with an ID of 99.9% (Table 1). Colony no. 5 had a round and convex-shaped morphology with a smooth and creamy structure on the MRS agar. The bacteria appeared to be rod-shaped and catalase negative based on microscope observation. The isolated *L. plantarum* 1 exhibited homofermentative features in a Durham test. No bubble was produced, indicating that no carbon dioxide gas was released. The colony log (cfu/g) and living percentage of *L. plantarum* within the chocolate samples was plotted (Figure 2). A significant difference between dark chocolate and mousse samples only occurred on Days 14 and 84 of storage. Both samples had similar conditions for allowing *L. plantarum* growth. The living percentage of *L. plantarum* in dark chocolate was 81.25%, which was low compared to other species of *Lactobacillus*.

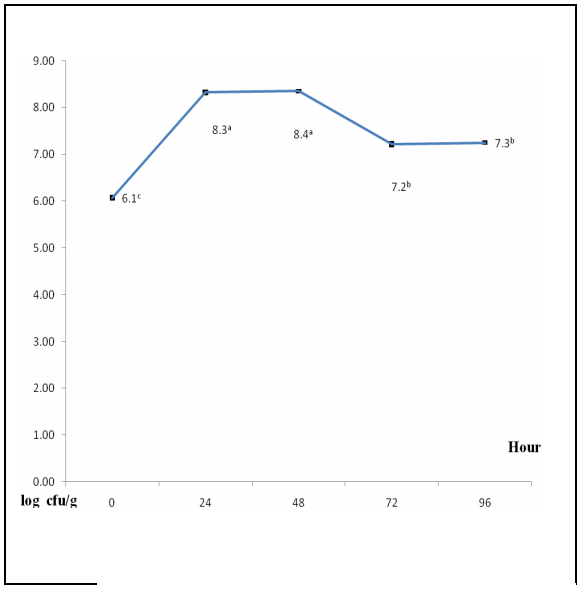

Figure 1Total Plate Count (log cfu/g) of *Lactobacillus* during cocoa bean fermentation.

a - c Different alphabet indicated there are significant different at $p \leq 0.05$.

Table 1: Result of phenotype identification for nine colonies of *L. plantarum* using API CHL50

	Colony								
Carbohydrate	1	2	3	4	5	6	7	8	9
a-Methyl-D-mannosid	+	-	-	+	+	+	+	-	+
a-Methyl-D-glucoside	-	-	-	-	-	-	-	-	-
N-acetyl glucosamine	+	+	+	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+	+	-	+
Arbutin	+	+	+	+	+	+	+	-	+
Esculin D-ribose	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+
Malyose	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+
Melibose	+	+	+	+	+	+	+	-	-
Sucrose	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	+	-	-
Melezitose	+	+	-	+	+	+	+	-	-
D-raffinose	-	-	+	-	+	+	+	-	-
Starch	-	+	-	+	-	-	+	+	-
glycogen	-	+	-	-	-	-	+	-	-
Xylitol	-	-	-	-	-	-	-	-	-
Gentiobiose	+	+	+	+	+	+	+	-	+
Contol	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-
Erythithitol	-	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	+	+	+	+	+	+	+
D-ribose	+	+	-	-	+	+	+	-	+
D-xylose	-	-	-	-	-	-	-	-	-
L-xylose	-	-	-	-	-	-	-	-	-
b-Methyl-xyloside	-	-	-	-	-	-	-	-	-
Galactose	-	-	-	-	-	-	-	-	-
D-Glucose	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+
L-Sorbose	+	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-
Inositol	-	-	+	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	+	+	+	+
D-Turanose	-	+	+	-	+	+	+	+	-
D-Lyxose	+	+	+	+	+	+	+	+	+
D-Tagatose	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-	-
-2-Ketoglutarate	-	-	-	-	-	-	-	-	-
-5-Ketoglutarate	-	-	-	-	-	-	-	-	-
Taxon Identification using API CHL 50	93.1% *plantarum1* *Lactobacillus*	99.3% *plantarum1* *Lactobacillus*	74.5% *plantarum1* *Lactobacillus*	99.9% *plantarum1* *Lactobacillus*	**99.9% *plantarum1* *Lactobacillus***	99.7 % *plantarum1* *Lactobacillus*	99.9% *plantarum1* *Lactobacillus*	50.9 % *Lactobacillus*	98.8% *Lactobacillus*

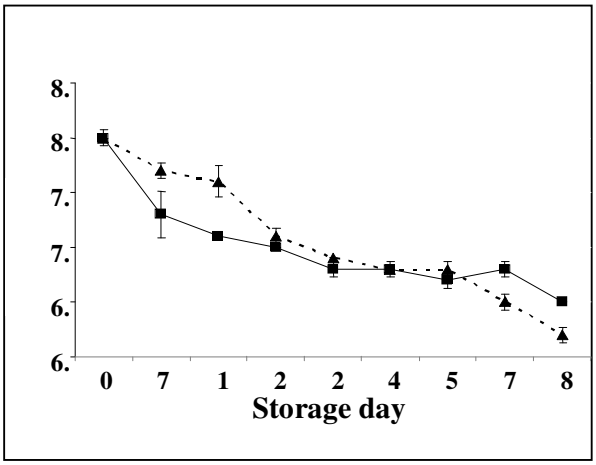

Figure 2Living colony of *L. plantarum* log (cfu/g) found in chocolate during three months of storage.

S2 – Dark chocolate with *L. plantarum;* S3–Dark mousse chocolate *L. plantarum.*

Physico-Chemical Analysis

A significant difference ($p<0.05$) in viscosity was observed between the S1 and S2 samples (Figure 3). The S2 chocolate sample was more viscous compared to the S1 (control) sample due to the presence of *L. plantarum.* No differences in chocolate texture were observed on day 0 of storage for any of the three samples that were prepared. Significant difference was observed on day 56 between the control and chocolate with *L. plantarum* samples and on Day 7 between the S3 sample and the two other samples. The average hardness of texture for both chocolate S1 and S2 was 1500-2200 texture hardness (gf) (Figure 4) while the S3 was the most breakable samples. The lowest pH value was obtained from S3 while S1 and S2 had similar values. The average pH value was in the 6.2-6.6 range (Figure 5). Colour analysis results for S1 and S2 revealed that these samples were more intense and reddish (L*, a*) compared to S3. However, the negative value of b* for the S3 sample was higher than S1 and S2 (Figure 6). The measured water activity of the samples showed that none of the samples were suitable for *L. plantarum* growth, which requires a water activity of at least 0.91 (Nebesny et al., 2007).

Significant differences ($p<0.05$) were found between S3 and the two other samples (S1, S2), which had a range of 0.45-0.70 (Fig. 7).

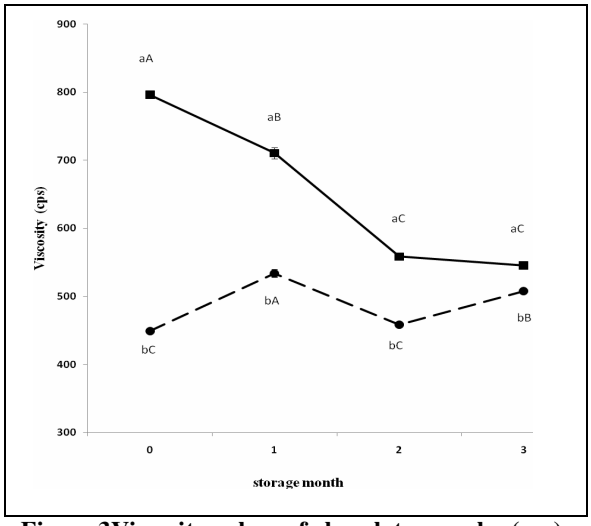

Figure 3Viscosity values of chocolate samples (cps) during three months of storage

: S1 – Control dark chocolate sample; : S2 – Dark chocolate sample with *L. plantarum*); [a ,b] –No significant difference between samples; [A, B, C] – No significant difference between months.

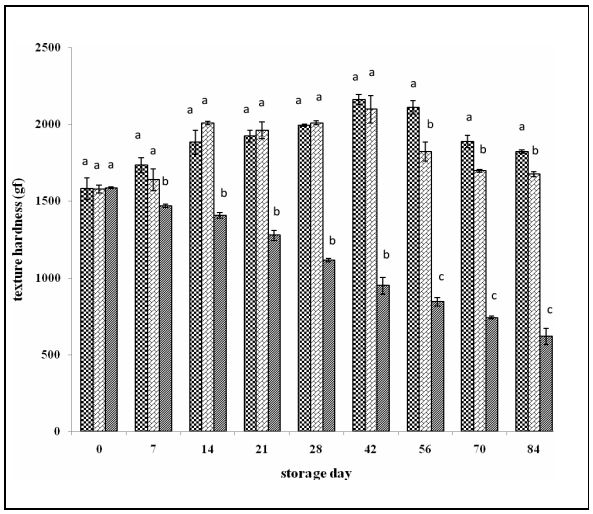

Figure 4The values of texture hardness (gf) for chocolate samples during three months of storage

S1 – Control chocolate sample; S2 – Dark chocolate with *L. plantarum;* S3 – Dark mousse chocolate with *L. plantarum*; [a, b, c] – No significant difference between samples.

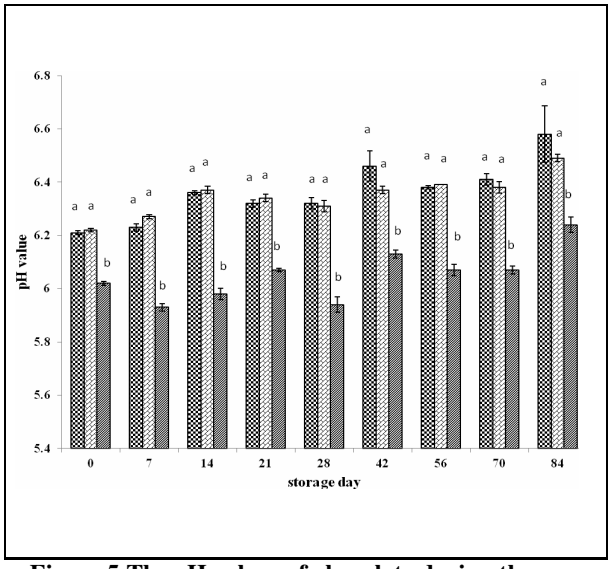

Figure 5 The pH values of chocolate during three months of storage

S1 – Control; S2 – Dark chocolate with *L. plantarum;* S3 – Dark mousse chocolate with *L. plantarum*) [a, b, c] – No significant difference between samples.

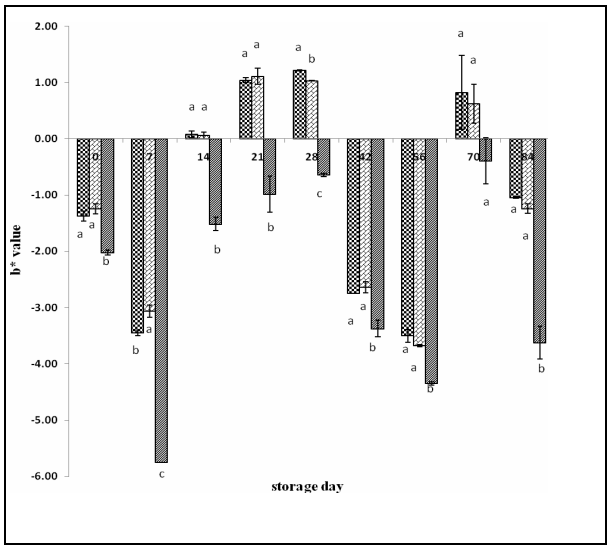

Figure 6The b* values of chocolate samples during three months of storage

S1 – Control; S2 – Dark chocolate with *L. plantarum;* S3 –Dark mousse chocolate with *Lactobacillus plantarum*).
[a,b,c] – No significant difference between samples.

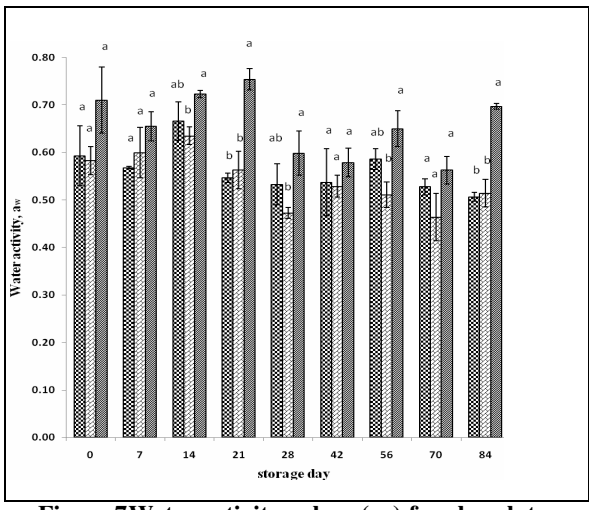

Figure 7Water activity values (a_w) for chocolate samples during three months of storage

S1 – Control; S2 – Dark chocolate *L. plantarum;* S3 – Dark mousse chocolate with *L. plantarum*) ^{a,b} – No significant difference between samples.

5. DISCUSSIONS

Microbiology Analysis

The pH of the medium used for bacteria growth was adjusted to 5.84, which is suitable for *Lactobacillus* growth but represses the growth of yeast and mould. The highest percentage of *Lactobacillus* growth was obtained 24-48 h into the fermentation process due to the rich amounts of carbohydrates and organic acids in cocoa pulp provide for sequential metabolism during the fermentation process. A previous study also showed a similar result where the maximum growth of *Lactobacillus* occurred at 36 h into the fermentation process (Ardhana, & Fleet, 2003). The first microorganism to begin fermentation is yeast followed by lactic acid bacteria and finally, acetic acid bacteria (Camu et al., 2007). Depectinization by the yeast reduces the viscosity of the pulp and changes sugars into ethanol, carbon dioxide and water. Reducing the pulp levels along with the constant production of ethanol indirectly promotes the growth of lactic acid and acetic acid bacteria (Ardhana, & Fleet, 2003; Camu et al., 2007). The number of *L. plantarum* colonies that formed within the chocolate and mousse samples reached the minimum limit for probiotic colonies in food, which is 10^6 cfu/g (Boylston et al., 2004). Both samples had similar conditions for allowing *L. plantarum* growth. The living percentage of *L. plantarum* in dark chocolate was low compared to other species of *Lactobacillus* such as *L. casei* and *L. paracasei*, which was 5.8 x 10^7 cfu/g, was reported to be much higher in another study (Nebesny et al., 2007). *L. plantarum* survived best in the mousse samples during the early storage stage but the amount of bacteria declined due to exhausted milk nutrients in the mousse.

Physico-Chemical Analysis

In a previous study, the best pH for *L. plantarum* in antimicrobial activity towards *Eschericeae coli, Staphylococcus aureus* and *Staphylococcus enteric* was 4.0 (Saarela et al., 2003), indicating that the *L. plantarum* colonies in S2 and S3 were not active enough to respond to other microorganisms. Colour analysis results revealed that S1 and S2 were more intense and reddish (L*, a*) compared to S3, the S3 sample was more blue, but less yellow, than the two other samples. The values obtained in this study for water activity were higher than values that were measured in previous studies, which were between 0.1-0.3. This discrepancy may be due to the decreased in the temperature of the environment during the sample melting process. The highest water activity was recorded for the S3 sample for every test that was conducted during the storage period. The presence of fresh milk and cream, which have high water activities, within the S3 samples might explain why the water activity for S3 was so high.

6. CONCLUSION

Based on the research conducted, we can conclude that none of the effects being studied showed a significant change compare to the normal dark chocolate. These results proved the ability of *L. plantarum* growth in dark chocolate and without causing any physicochemical changes in dark chocolate.

Also, the research result show the *L. plantarum* was successfully isolated from the fermented cocoa bean with highest percentage obtained 24-48 h in the process.

7. ACKNOWLEDGEMENT

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