Physico-chemical Content of Carrot and Sweet Potato-Filled Chocolate Bites Incorporated with *Lactobacillus casei* and *Lactobacillus plantarum*

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ABSTRACT---- ***In this study, the physico – chemical content of carrot and sweet potato – filled chocolate bites incorporated with Lactobacillus casei and Lactobacillus plantarumwere determined. Results showed that in sweet potato – filled chocolate bites, there was a significant difference in free fatty acid and peroxide value on both lactic acid bacteria. Meanwhile, the carrot–filled chocolate bites showed significant difference in its values specifically lactic acid, free fatty acid and pH. Notably, L. casei showed significant difference in lactic acid, peroxide value and pH while there was a significant difference in free fatty acid, peroxide value and pH for L. plantarum.***

Keywords – carrots, sweet potato, physico-chemical content, *Lactobacillus casei*, *Lactobacillus plantarum*

1. INTRODUCTION

There is an increasing trend for functional foods that improve the health and well-being of consumers. Foods that are incorporated with probiotics are one of the functional foods that are trending nowadays. Lactic acid bacteria (LAB) are the most communal type of probiotic and these bacteria are generally found in dairy produce, creating lactic acid as one of its main fermentation products. These bacteria mostly grow best in moderate temperature neither too hot nor too cold and may be classified as homo-fermentive or hetero-fermentive. *Lactobacillus casei* is a kind of lactic acid bacteria, a bacteria present particularly in hard cheeses and in cheeses made from raw milk [5]. *Lactobacillus plantarum* on the other hand is a prevalent lactic acid bacteria found in fermented foods as well. Application of *Lactobacillus plantarum* and its probiotic properties partakes superbly over the past several years [12].

Chocolates also often presaged as one of the main reasons in incurring diabetes because of its sugar content but nowadays, studies have shown that chocolates can be a healthy food with the thorough study of the chocolate itself and combining nutrients that can benefit its aficionados. But probiotic cultures can also be incorporated in fruit and vegetable as substrate for it contains nutrients that support probiotics. Vegetable and fruit juices are probably the most popular non-dairy probiotic food applications. It showed that probiotic lactobacilli (*L. acidophilus*, *L. casei* and *L. plantarum*) were able to grow and produce acid in non- supplemented tomato, beetroot and cabbage juice [13]. Carrots comprise of approximately 88% water, 7% sugar, 1% protein, 1% fibre, 1% ash, and 0.2% fat. Sugars are sustainable food for the probiotics thus, carrots can be a potential substrate for probiotic. Free sugars in carrot include sucrose, glucose, xylose and fructose [4]. On the other hand, sweet potato was also used in this study which comprises approximately 54.83% moisture, protein ranging from 0.46% to 2.93%, dietary fiber ranging from 0.49% to 4.71%, lipid ranging from 0.06% to 0.48% and ash ranging from 0.31% to 1.06%. It contains essential mineral nutrients such as Ca, P, Mg, K, S, Fe, Cu, Zn, Mn, Al and B. Sweet potato is also important source of vitamin A, thiamine, riboflavin, niacin, ascorbic acid and many other functional compounds [15]. This indicates that sweet potato can also be a potential substrate for probiotics.

It would be a beneficial impact on human health when lactic acid bacteria are used in food due to its numerous health benefits. Chocolate bars with lactic acid, vegetable and fruit juices incorporated with probiotics are one application that has proven successful. Combination of chocolate, vegetables and fruits might have better results in developing functional foods.

2. MATERIALS AND METHODS

2.1 Microbial Culture and Growth Conditions

Pure cultures of *Lactobacillus casei USTCMS 1050* and *Lactobacillus plantarum* were obtained from the University Of Santo Tomas Collection Of Microorganisms at the Thomas Aquinas Research Complex, Graduate School. The acquired microorganisms served as the mother culture. Three test tubes containing MRS broth for each microorganism were prepared. They served as the sub-culture. The sub-cultures were placed in an incubator for 24-48 hours at 37^{O}C. After the sub-cultures were prepared, disposable petri plates and MRS Agar were prepared. A loop full amount of *Lactobacillus casei* and *Lactobacillus plantarum* that were obtained from the previous sub-cultures were streaked on the MRS Agar blanks prepared in petri plates using multiple streaking. The MRS Agar was then placed inverted in an incubator for 24-48 hours at 37^{O}C [3][16].

2.2 Enumeration of Lactic Acid Bacteria in Carrot and Sweet Potato-Filled Chocolate Bites

For the MRS broth culture containing *L. casei* and *L. plantarum*, conventional plate method was used to determine its initial count. One (1) Mcfarland Barium Sulfate Standard was used to compare the turbidity of the previously washed culture with de-ionized water. First, MRS Broth and MRS Agar were prepared. Then, for the chocolate samples containing the microorganism, 11 grams of chocolate sample was weighed and it was homogenized with a previously prepared and sterilized 99 ml MRS broth using a blender. Serial dilution was conducted up to 10^5 using a micropipette. A 0.1 ml of cell suspension in the 10^5 dilution was obtained and was placed in each triplicate plate for each microorganism on the prepared disposable petri plates using pour plate method. After the agars on the plates were solidified, the plates were placed inverted in an incubator for 24-48 hours at 37^{O}C[3][16]**.**

2.3 Preparation of Carrot and Sweet Potato-filled Chocolate Bites

The milk chocolate and unsweetened chocolate were purchased from All About Baking, Quezon Ave, Quezon City, Metro Manila. The carrots were purchased from a local supermarket. The packaging material (Laminated Foil) was purchased at RM Boxes, Sta. Mesa, Manila.

The carrot fillings were prepared by removing the non-edible portion and it was immersed in an acetic acid-water bath (1:4) solution to remove dirt and extraneous materials from the skin of the carrots. Then it was sliced thinly and was blanched for 4-5 minutes at 100^{o}C. After blanching, the carrots were drained and were submerged into an ice bath. After the carrots were cooled, it was drained and was grinded with a food processor until the sizes of the carrots are fine and even. The sweet potato filling was prepared by removing the non- edible portion as well and was boiled for 15 – 30 minutes to remove the extraneous matters and dirts. It also helps soften the sweet potato. Then after boiling, the boiled sweet potatoes were mashed until good enough for mixing with the melted chocolate. The chocolate coating was prepared by melting and tempering first the milk chocolate at 30-31.1^{o}C. Moulders were filled with 4.0 grams of milk chocolate and the chocolate was evenly distributed within it. The chocolate was cooled immediately with an ice bath. The chocolate filling was prepared by melting and tempering 200 grams of dark chocolate at 32^{o}C. Two hundred grams of the prepared carrots and another 200 grams of prepared sweet potato were mixed with the dark chocolate until all the carrots were evenly distributed. Then 6.5 grams of the chocolate filling was placed inside the prepared chocolate coating. A 100 μL (0.1 ml) culture of *L. casei* or *L. plantarum* was injected at the middle of the chocolate using a micropipette. The chocolate bites were sealed with 2.5 grams of milk chocolate. The chocolate bites were removed from molders and was packaged using a laminated foil.

2.4 pH Analysis

The pH values for the two variations were measured in duplicate weekly using a pH meter **(Jenway, 350 pH meter).**

2.5 Water Activity

The Aw values for two variations were measured in duplicate weekly using a water activity meter from **Novasina.**

2.6 Free Fatty Acid Analysis

The free fatty acid (FFA) was determined using the method described by the American Oil Chemists Society [9] wherein 10 grams of sample was weighed in to an Erlenmeyer flask and 2-3 drops of Phenolphthalein indicator was added into the solution. A 100 mL of a previously neutralized and boiling 95% Ethyl alcohol was added into the solution and it was titrated with 0.01M sodium hydroxide (NaOH) while shaking vigorously until a permanent pale pink color persists or until pH 7 is achieved using a litmus paper.

Free Fatty Acid Content was computed as:

$$\%\ FFA = \frac{mLNaOHxNNaOHxmeqwt.ofoilpresent}{Wtofsample\ (g)} x100$$

2.7 Peroxide Value Analysis

The peroxide value (PV) was determined using the method by the American Oil Chemists Society [10]wherein two (2) grams of sample was weighed into a dried and tarred Erlenmeyer flask and the sample was melted in a water bath. A 2:1 solution of Acetic Acid: Chloroform was added into the melted sample and it was swirled until the sample was dissolved. A 0.5 mL of saturate KI solution was added to the solution and it was allowed to stand for one (1) min. while occasionally stirring it. A 30 mL of distilled water was immediately added into the solution and a 0.5 mL of 1 % starch solution was also added as the indicator. When a blue color appeared, the solution was titrated with 0.01N sodium thiosulfate ($Na_2S_2O_3$) solution until the color disappears.

Peroxide Value was computed as:

$$PV = \frac{(mLsample - mLblank)xNofNa2S2O3\ x\ 1000}{Wtofsample\ (g)}$$

2.8 Determination of Percent Lactic Acid

The titratable acidity (% lactic acid) was determined using the method by Association of Analytical Communities **(AOAC Official Method 947.05)**wherein 20 grams of sample was weighed into an Erlenmeyer flask. A 90 mL of previously boiled distilled water was added into the solution and the flask was swirled. Then 2-3 drops of phenolphthalein indicator was added into the solution and it was titrated with 0.02N sodium hydroxide (NaOH) until faint pink color persists or until the solution reaches pH 7.

Percent lactic acid was computed as:

$$\%\ LacticAcid\ = \frac{mLNaOHxNNaOHx\ 9.0}{Wtofsample\ (g)}$$

2.9 Moisture Analysis

Moisture content was determined by heating the chocolate bites to a constant weight at 105ºC for two (2) hours and measuring the weight lost due to evaporation of water. The **AOAC oven drying method 14.0004** was used [4]**.** The dry matter was calculated as the difference of moisture content from 100**.** Approximately 5 g of sample was weighed in a pre-dried aluminum dish and it was placed in the drying oven at 105^{o}C for 1 hour and 30 minutes. The dish with the dried sample was cooled in a dessicator and was weighed.

2.10Statistical Analysis

Bacterial counts were expressed as mean. Bacterial counts were the average of triplicate experiments. The significant differences between the microbial counts were calculated using the paired - difference t-test. One-way Analysis of Variance was also used to determine the significant difference of microbial counts from week 0 to week 3. Paired-difference t-test was used in determining the significant difference of *L. casei* and L. *plantarum* in its physico-chemical analyses of carrot and sweet potato-filled chocolate bites while one way Analysis of Variance was also used to determine the significant difference of the physico-chemical analysis of carrot and sweet potato - filled chocolate bites from week 0 to week 2.

3. RESULTS AND DISCUSSION

Table 1 shows the changes in the tritratable acidity, free fatty acids, and peroxide value of carrot and sweet potato - filled chocolate bites incorporated with *L. casei* and *L. plantarum* with statistical analysis using three way analysis of variance. The three way analysis of variance was used to compare if there were significant changes that occurred in the values of the physico-chemical content during the course of the two-week analysis.

Table 1. Titratable acidity, free fatty acid and peroxide value of carrot and sweet potato – filled chocolated bites

Substrate	Bacteria	Weeks	Titratable Acidity*	Free Fatty Acid*	Peroxide Value*
Carrots	*Lactobacillus casei*	0	0.05^{b}	0.11^{a}	3.51^{a}
		1	0.13^{a}	0.15^{a}	5.32^{a}
		2	0.14^{a}	0.06^{b}	2.77^{b}
	Lactobacillus plantarum	0	0.07^{a}	0.19^{a}	1.12^{b}
		1	0.06^{a}	0.43^{a}	5.79^{a}
		2	0.11^{a}	0.03^{b}	6.27^{a}
Sweet potato	*Lactobacillus casei*	0	0.07^{a}	0.14^{b}	18.09^{b}
		1	0.09^{a}	0.30^{a}	20.28^{a}
		2	0.05^{a}	0.35^{b}	13.79^{b}
	Lactobacillus plantarum	0	0.08^{a}	0.15^{b}	5.66^{b}
		1	0.08^{a}	0.32^{a}	21.68^{a}
		2	0.05^{a}	0.11^{b}	9.09^{b}

For carrots, *L. casei* showed no significant difference in titratable acidity values from week 1 and week 2 but has a significant difference during week 0. While for free fatty acid and peroxide values also showed no significant difference from week 0 and week 1 but had a significant difference during the second week.Meanwhile, for *L. plantarum*, the titratable acidity values showed no significant difference from week 0 to week 2. Free fatty acids, meanwhile have no significant difference from week 0 and week 1 but had a significant difference during week 2. Peroxide values have no significant difference during week 1 and week 2 but showed significant difference during week 0.

On the other hand, in sweet potato, *L. casei* and *L. plantarum* had no significant difference from week 0 to week 2 for titratable acidity. Meanwhile, there was a recorded significant difference in free fatty acid and peroxide values for both *L. casei* and *L. plantarum* from week 0 to week 2. The *L. casei* and *L. plantarum* variation's free fatty acid and peroxide values had no significant difference in week 0 and week 2 but have significant difference in week 1.

Titratable acidity (TA) is an important parameter in following the progress of a fermentation process. Titratable acidity is either inherent or induced as a result of the conversion of sugars to lactic acid by the lactic acid cultures.Accroding to(Oloo B.; et al, 2014) as cited by (Gardner et al., 2001), of the much titratable acidity, lactic acid is the main organic acid produced by action of *Lactobacilli* . In the analysis, the titratable acidity recorded for carrot-filled chocolate containing *L. casei* and *L. plantarum* were 0.05, 0.13, 0.14 and 0.06, 0.06, 0.11 respectively. On the other hand, the sweet-potato filled chocolate bites obtained a titratable acidity of 0.07, 0.09, 0.05 and 0.08, 0.08, 0.05 correspondingly. As Beckett (2008) stated, the titratable acidity for chocolates in terms of lactic acid is not greater (<) than 0.15, therefore the results obtained for both

carrot and sweet potato filled chocolate bites are in accordance with the standards stated earlier. Hence, the chocolate bites with *L. plantarum* had a higher titratable acidity compared to *L. casei* regardless of the substrate used *(carrots and sweet-potato).*

In addition, fermentable sugars serve as a significant factor in the titratable acidity value of a product. According to Robbins (2013) as cited by www.livestrong.com, the fermentable sugars found in carrots were fructose and glucose. On the other hand, Woolfe (1992) said that the major sugars occurring in raw sweet potato are sucrose, glucose and fructose. Woolfe (1992) also added that there is a trace amount of maltose in the sweet potato and its concentration increases significantly during cooking due to starch hydrolysis which produces dextrin. Relating the literature to the results obtained, it can therefore be noted that the lactic acid bacteria did not utilize the fermentable sugars present in the substrates. High titratable acidity values denote a high lactic acid concentration and since the results obtained were low, the concentrations of the lactic acid in the chocolate bites were also low.

The next parameter measured is free fatty acid. In the current study, the free fatty acid recorded for *L. plantarum* and *L. casei* were relatively low. This may be due to the antioxidant present in the dark chocolate. Dark chocolates contain high amount of polyphenols and flavonoids [9]. Fatty acids undergo a process called auto-oxidation which requires oxygen, hence due to the high amount of antioxidant present, few fatty acids undergone the process and therefore retained its quality for two weeks. The result for carrot-filled chocolate bites containing *L. casei* and *L. plantarum* were as follows: 0.11, 0.15, 0.06 and 0.19, 0.43, 0.03 respectively. Furthermore, the free fatty acid result of sweet potato-filled chocolate bites containing *L. casei* and *L. plantarum* were 0.14 0.30, 0.35, and 0.15, 0.32, 0.10 respectively. In terms of standard values, two literatures were considered. First, according to www.fao.org (2013), the cocoa butter present in the chocolate should not exceed its free fatty acid value for more than 1.75% while according to NPCS Board of Food Technologists (2013), the free fatty acid value of chocolates in general should not exceed at 0.7% therefore, the values obtained for both carrot and sweet potato filled chocolate bite were accepted based on the standards and did not show any significant rancidity.

The peroxide value is used for identifying the onset of oxidative change or rancidity in fats and oils [8]. As seen in table 1, the values for peroxide of carrot-filled chocolate bites containing *L. casei* and *L. plantarum* from week one to three were 3.51, 5.32, 2.77 and 1.12, 5.79, 6.27 correspondingly while the sweet potato-filled chocolate bites containing *L. casei* and *L. plantarum* obtained 18.09, 20.28, 13.79 and 5.66, 21.68, 9.09. In terms of standards, according to Minifie (2012), the peroxide value of a chocolate product must not exceed at 50%. The onset of rancidity in chocolates can be detected through taste. Relating the standard to the results, the peroxide values obtained were therefore accepted and have not undergone significant oxidative rancidity.

Table 2 shows the changes in the moisture, water activity, and pH of carrot and sweet potato -filled chocolate bites incorporated with *L. casei* and *L. plantarum* with statistical analysis using three - way analysis of variance. For carrots, *L. casei's* pH values showed no significant difference from week 0 and week 1 but had a significant difference during the second week. On the other hand, moisture content and water activity showed no significant difference from week 0 to week 2. Meanwhile, the pH values of *L. plantarum* variations are significantly different from week 0 to week 2. Lastly, there was no recorded significant difference for both moisture content and water activity for *L. plantarum* from week 0 to week 2. While for sweet potato, *L. casei* had no significant difference from week 0 to week 2 for pH, moisture content, and water activity. On the other hand, *L. plantarum* showed no significant difference in pH and water activity from week 0 to week 2 while there was a significant difference in moisture content from week 2.

The data obtained shows evidence of a minimal decrease in pH from week 0 and week 1 in carrot and sweet potato-filled chocolate bites containing *L. plantarum* and *L. casei*. In accordance to(Oloo B.; et al, 2014), the decrease in pH was attributable to the increasing acidity due to the action of the lactic acid fermenters. To further justify, *L. plantarum* and *L. casei* breaks down sugars and produce organic acids which contributes to acidity. Minimal decrease in pH values under low temperature storage indicates active metabolism of microorganism. In contrary, the results showed an increase in pH in week 2. The fluctuation in pH throughout the analysis however was not expected. A possible explanation may be due to the varying temperature in the refrigerator. Noticeably, the chocolate bites containing *L. casei* and *L. plantarum* did not taste sour for the reason that the optimum pH was not met. In this study, it shows that the lactic acid bacteria were not able to utilize the sugar present due to the high amount of fat present in the chocolate [1]. In terms of chocolate standards, the standard pH values in chocolate products is in the range of 6.25 – 6.7, slightly acidic to neutral, therefore the pH values obtained for the carrot and sweet potato–filled chocolate bites were accepted based on the standards.

Table 2. Moisture, water activity and pH of carrot and sweet potato – filled chocolated bites

Substrate	Bacteria	Weeks	Moisture[ns]	Water activity (Aw)[ns]	pH*
Carrots	*Lactobacillus casei*	0	18.65[a]	0.78	7.07[a]
		1	20.36[a]	0.78	6.44[a]
		2	20.99[a]	0.78	6.48[b]
	Lactobacillus plantarum	0	23.40[a]	0.81	6.99[a]
		1	15.79[a]	0.81	6.43[b]
		2	21.34[a]	0.81	6.46[c]
Sweet potato	*Lactobacillus casei*	0	18.99[a]	0.73	6.45[a]
		1	17.48[a]	0.73	6.47[a]
		2	10.26[a]	0.73	6.76[a]
	Lactobacillus plantarum	0	15.54[a]	0.72	6.53[a]
		1	20.30[a]	0.72	6.41[a]
		2	9.62[b]	0.72	6.50[a]

Based on the standard value, the moisture content of the chocolate is 0.21% [19], another literature from Tablot (2009) states that the standard value for filled chocolate is not greater than 1% and this statement is also supported by Canovas et al. (2008) that filled chocolates have 1.2% of moisture but this is contrary to the results obtained. As seen in the results, the carrot-filled and sweet potato-filled chocolate bites recorded higher moisture content than that of the standard. This may be related to the filling because carrots and sweet potato are relatively high in moisture. As said by(Lahtinen et al, 2012), vegetable like carrots, comprise of approximately 88% water. On the other hand, sweet potato comprises approximately 54.83% moisture [16]. Another contributing factor in high moisture content of the chocolate bites is that the bacterial suspension is contained in de-ionized water which was inoculated in the filling. Evidently, carrot – filled chocolate bites had a higher moisture content than that of sweet potato-filled chocolate bites. In the analysis, the moisture content of carrot-filled chocolate bites containing *L. casei* and *L. plantarum* were 18.65% and 23.40% respectively. For sweet potato filled chocolate bites the moisture content were 18.99% and 15.54% respectively.

The results of carrot filled chocolate bites containing *L. casei* showed a stable water activity from week 0 to week 2. The data obtained was 0.78 accordingly. The same trend was seen for carrot filled chocolate bites containing *L. plantarum.* From week 0 to week 2, the value obtained was 0.81. Meanwhile, sweet potato filled chocolate bites containg *L. casei* had a stable water activity of 0.73 from week 0 to week 2. As for the *sweet* potato filled chocolate bites containg *L. plantarum*, the water activity had a very minimal decrease. As seen in the table, the water activity was 0.72 respectively. Water activity is a comparison of vapour pressure of water in a food sample with vapour pressure of pure water. High salted foods and those with very high sugar content have values of between 0.80 and 0.90 [7]. Hence, small water activity and high concentration of sugars in the carrot and sweet potato - filled chocolate practically eliminate the growth of bacteria.

4. CONCLUSION

The study conducted dwelt on the physico-chemical analysis of the developed product. The results in physico–chemical analysis of sweet potato-filled chocolate bites showed that there were significant differences in the percent free fatty acid and peroxide values of both the samples. For carrot–filled chocolate bites it showed that there were significant differences in percent lactic acid, percent free fatty acid and pH for the chocolate bites with *L. casei* while there were significant differences in the percent free fatty acid, peroxide value and pH for the chocolate bites with *L. plantarum*.

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