Physicochemical, and microbiological characteristics of probiotic dark chocolate bar sweetened with palm sugar and coconut sugar

*Nurhayati, R., Zulfa, N., Herawati, E.R.N. and Laila, U.

1Research Division for Natural Product Technology, Indonesian Institute of Sciences. Jl Jogja-Wonosari km 31.5 ds Gading, Playen, Gunungkidul, Yogyakarta, Indonesia

2Department of Food Science and Technology, Faculty of Agricultural, Universitas Sebelas Maret, Surakarta, Indonesia

Abstract

Dark chocolate is considered a nutritious food due to its high polyphenol and antioxidant activity. The improvement of chocolate functionality can be conducted by enriching it with probiotics. Replacement of sucrose in probiotics-enriched chocolate with palm or coconut sugar serves as a probiotic protective agent and increases its functional value. The addition of probiotics and sugar replacement will change the physical, chemical and microbiological properties of dark chocolate products. This research aimed to investigate the physical, chemical and microbiological characteristics of probiotic dark chocolate with sucrose replacement by coconut sugar and palm sugar. The viscosity, colour, melting profile and surface microstructure were investigated for physical properties of probiotic dark chocolate. Total phenolic content was measured by Folin-Ciocalteu method, and the antioxidant activity was quantified by the DPPH radical method. Total lactic acid bacteria were evaluated for microbiological properties of chocolates. As a reference, probiotics enriched dark chocolate with sucrose were also studied. The results showed that the viscosity of all the samples was significantly different, and chocolate sweetened with coconut sugar was the most viscous. Based on the DSC profile, chocolate sweetened with sucrose had a lower melting temperature than chocolate sweetened with coconut and palm sugar. The antioxidant activity of chocolate sweetened with palm sugar was significantly higher than that of chocolate sweetened with coconut sugar and reference. Probiotic dark chocolate sweetened with coconut sugar and palm sugar had significantly higher total polyphenols than reference. Sugar replacement did not significantly influence the calorie value, but palm sugar was proven in reducing the calorie of the probiotic dark chocolate. Dark chocolate provided suitable protection in fulfilled adequate viability for being claimed as a probiotic food, i.e., 6.88, 6.94, 7.16 log CFU/g respectively. Microscopy visualization showed that sugar agglomeration happened in both probiotic dark chocolates sweetened with coconut sugar and palm sugar. In general, the replacement of sucrose with palm and coconut sugar slightly increases the total phenolic content and antioxidant activity of probiotics-enriched dark chocolate.

1. Introduction

Cocoa as nutritious food is rich in polyphenols and antioxidants (Muhammad et al., 2017). One of the cocoa-derived products is chocolate. Vinson et al. (1999) reported that chocolate contains the highest polyphenols compared to 23 vegetables and some fruits. Epicatechin, catechin, procyanidin, and minerals (K, Mg, Cu, Fe) are found in chocolate. There are three types of chocolate, namely dark chocolate, milk chocolate, and white chocolate. The difference is based on the composition of a chocolate bar, which dark chocolate contains the highest amount of chocolate paste. Nutritionally, dark chocolate is believed to provide more health benefits. The high content of antioxidants in dark chocolate can contribute an important regulatory role in maintaining the immune system, lowering blood pressure and strengthening blood flow (Albrecht et al., 2010; Latif, 2013). In Europe, there is a specific trend related to the consumption of dark chocolate products with high cocoa content without the addition of milk (Alberts and Cidell, 2006).
Several studies have changed the formula of dark chocolate to improve product functionality, such as by the addition of phytosterols, plant extracts, synbiotics, and probiotics (Botelho et al., 2014; Erdem O et al., 2014; Dwijatmoko et al., 2016; Muhammad et al., 2019). As it relates to chocolate, current market surveys of functional food show that digestive health is an important thing desired by consumers (Council, 2018). Probiotics are defined as live microorganisms in which a suitable amount can confer health benefits by increasing the balance of the gastrointestinal tract. The number of viable cells of probiotics in the food product is not less than 10^6 CFU/mL during the shelf life of the product (Nebesny et al., 2007; Kharat and Deshpande, 2017). Among some microorganisms, Lactic Acid Bacteria (LAB) is dominantly applied as a probiotic in the food industry. Probiotics not only play a role in balancing the intestinal system, but they also give favourable advantages by producing metabolites (vitamins, short-chain fatty acids, and enzymes), improving the immune system, and playing a role in the prevention of some diseases, such as diarrhoea, hepatic disease, inflammation, arthritis, and some allergies (Parvez et al., 2006). Moreover, some studies have reported beneficial effects of lactic acid bacteria in overcoming diabetes and lowering blood glucose. Some lactic acid bacteria produce α-glucosidase inhibitor activity which is capable to inhibit carbohydrate metabolism and absorption, thereby lowering blood glucose levels (Chen et al., 2014). Our previous study found Pediococcus lolii L2 that is LAB isolated from Lingzhi (Ganoderma lucidum) has α-glucosidase inhibitor activity (Nurhayati et al., 2016). The addition of LAB in dark chocolate can increase the functional value of the product.

The improvement of dark chocolate functionality can also be conducted by replacing sucrose as a sweetener. Sucrose is known as the most common sweetener usually used up to 50% in making dark chocolate. Therefore, it is necessary to substitute sucrose with other sweeteners, such as sugar alcohol, low-calorie polysaccharides, coconut sugar and palm sugar. Coconut sugar (Cocos nucifera) and palm sugar (Arenga pinnata Merr) are natural sweeteners made from sap/nectar, claimed to be healthier sweeteners than sucrose in the presence of minerals and vitamins, proteins, phenols, and low glycemic index. These two natural sweeteners will provide sweetness, enrich the taste, and improve the colour and aroma of the product (Apriyantono et al., 2002; Purnomo, 2007; Saputro et al., 2019). Palm and coconut sugar developed and supported the growth of probiotics (Akin et al., 2007; Pin Low et al., 2015) due to its inulin content which functions as a cryoprotective agent (Saxelby, 2014). Coconut and palm sugar have a low glycemic index which plays an important role in the dietary management of obesity, diabetes, hypertension and heart disease (Trinidad et al., 2010; Srikaeo and Thongta, 2015). Coconut sugar and palm sugar can be developed as a sweetener in food products based on the advantages of their functional properties.

The addition of probiotics and sugar replacement improve the functional properties of dark chocolates, however, it will influence the physical, chemical and microbiological properties of the product. This research aimed to evaluate the physical, chemical, and microbiological characteristics of probiotic dark chocolate bars sweetened with palm and coconut sugar.

2. Materials and methods
2.1 Materials
Chocolate paste and cocoa butter were obtained from Nglanggeran, Gunungkidul district. Coconut sugar and palm sugar were obtained from a farmer in Kulon Progo district as local product Probiotic microcapsules (spray-dried Pediococcus lolii L2 with skim milk powder as a carrier) were obtained from Research Division for Natural Product Technology Indonesian Institute of Sciences (Nurhayati et al., 2020).

2.2 Probiotic dark chocolate bar processing
The processing of probiotic dark chocolate bar used the method of Nurhayati et al. (2019) and Patent number P00201910409. Cocoa liquor, cocoa butter, sucrose/coconut sugar/palm sugar, lecithin, and probiotic microcapsules were used to formulate a 65% dark chocolate bar. Cocoa liquor and cocoa butter were melted using the bain-marie method. Melted cocoa liquor -cocoa butter, sugar and soy lecithin were mixed into the ball mill. The refining and conching processes were conducted for 18 hrs. Ball mill temperature then was lowered and the probiotic microcapsules were added for mixing. The chocolate was further moulded and cooled. There were 3 samples of probiotics dark chocolate, namely chocolate sweetened with sucrose, chocolate sweetened with palm sugar and chocolate sweetened with coconut sugar. The probiotic dark chocolate bar was then packaged using aluminium foil packaging and stored at 4°C before being analyzed. The three types of probiotics in dark chocolate were analyzed for physical, chemical and microbiological properties in three replications for each analysis.

2.3 Preparation of chocolate extract
The chocolate extract was prepared according to the method of Nurhayati et al. (2019) with modification. Ground probiotic dark chocolate bar (30 g) was extracted in 150 mL petroleum benzene for 5 hrs using the
soxhlation method. The defatted chocolate dried at 50°C for 12 hrs. Approximately 5 g of the dried defatted chocolate was extracted in 25 mL of 70% ethanol for 2 hrs at 50°C and homogenized by a magnetic stirrer. The mixture was filtered through filter paper. The filtrate was considered as chocolate extract and used for the antioxidant activity and total phenolic content assay. The filtrate was stored in a dark room at 4°C.

2.4 Chemical characteristics

2.4.1 Proximate analysis

The standard Official Method of Analysis (AOAC, 2000) was used to determine the macronutrient contents, which included moisture content (thermogravimetric method), protein content (Kjeldahl method), fat content ( Soxhlet method), ash content (combustion method), and carbohydrate by difference.

2.4.2 Antioxidant activity

The antioxidant activity was estimated according to Nurhayati et al. (2019). The chocolate extracts (200 µL) were mixed with 3 mL (500 µM) of 2,2-diphenyl-2-picrylhydrazyl (DPPH). In the absence of light, the mixture was homogenized and incubated for 20 mins at room temperature. Absorbance was measured at λ 517 nm. The DPPH solution alone was measured before the addition of the samples (A₀) and 70% ethanol was used as a blank. Butylated hydroxytoluene (BHT) and Butylated hydroxyanisole (BHA) were used as antioxidant references (1 mg/mL). The antioxidant activity represented as% of radical scavenging activity (RSA) remaining after each time according to the equation below:

\[
% \text{RSA} = \frac{A_0 - A_t}{A_0} \times 100\%
\]

Where \(A_0\) means the absorbance of the DPPH solution alone at zero time, and \(A_t\) means the absorbance of each at 20 mins following the addition of the DPPH solution.

2.4.3 Total phenolic content

Total phenolic content was assayed using the Folin-ciocalteu method of Lee et al. (2003) with modifications. The chocolate extracts (200 µL) were mixed with the Folin-ciocalteu reagent (200 µL) and homogenized. After 5 mins, 2 mL of 7% Na₂CO₃ solution was added and diluted to a volume of 5 mL with aquadest and homogenized. After incubation for 90 mins at 23°C, the absorbance was measured at λ 750 nm using a UV-Vis Spectrophotometer. The amount of total phenolic content was calculated using a standard curve of gallic acid. The total phenolic content of the defatted chocolate was expressed as mg gallic acid equivalent per g chocolate (mg GAE/g chocolate).

2.4.4 Calorie

The determination of the total calorie value in the probiotic dark chocolate bars was measured using a bomb calorimeter (Cohen and Schilke, 1994). The sample (1 g) was placed in a stainless cup inside the bomb. A filament wire and burner string were weighed, and the filament wire was installed passing close to the sample. Aquadest (1 mL) was placed into the bomb and the bomb was sealed and filled with 20–30 atm of oxygen. The bomb was placed in the bucket containing 2.1 L of water and the bucket was placed inside the jacket. The stirrer was run, and the thermometer temperature was observed. When proper temperature relationships were achieved, recorded the temperature as the initial temperature. Burning is initiated by passing a high current through the filament for 5 s. The rising temperature of the water in the bucket was monitored until equilibrium was reached and recorded as the final temperature (rising temperature = final temperature - initial temperature). Benzoic acid was used as standard. The combustion heat of the sample was expressed in calories per g of the sample.

2.5 Viability of lactic acid bacteria

The viability of lactic acid bacteria in a probiotic dark chocolate bar was determined using the total plate count method. Initially, 25 g of chocolate sample was blended with 225 mL of 0.85% NaCl solution (10⁻¹), followed by preparation of serial dilution. Approximately 1 mL of each last three dilution series was poured into a sterile petri dish and MRS agar was poured onto the sample. After 48 hrs of incubation at 37°C, the colonies were counted and the results were expressed as colonies forming units per g of product (CFU/g).

2.6 Physical characteristics

2.6.1 Viscosity

The viscosity was measured using DV-E Brookfield Viscometer coupled with a disc spindle (no. 64) at 20 rpm following the Saidin et al. (2014) method. The melted chocolate was incubated at 38°C for 1 hr prior to the analysis. The viscosity measurement was obtained every 30 s for 10 mins.

2.6.2 Colour

The colour of the probiotic dark chocolate bar was analysed using Konica Minolta Chromameter (CR-20) following the Saidin et al. (2014) method. The colour parameters shown by the CIELAB system were expressed in L* (luminance), a* (green-red), and b*
(blue-yellow). The total colour difference (ΔE) is calculated to determine the effect of the colour difference of the treatment sample on the control sample (Briones et al., 2006) according to the equation below:

$$\Delta E = \sqrt{(L_f - L_i)^2 + (a_f - a_i)^2 + (b_f - b_i)^2}$$

Where f (final) is the value of the treatment sample and i (initial) is the value of the control sample. Chocolate sweetened with sucrose was used as a control sample.

2.6.3 Melting profile

The melting profile of chocolate was measured using a DSC-60 Plus Shimadzu (Differential Scanning Calorimeter). Approximately 4.4 mg of chocolate were sealed in hermetically sealed cups. During thermal profiling, the samples were equilibrated at 30°C and then followed by a heating step to 300°C at a rate of 10°C/min.

2.6.4 Surface microstructure of chocolate bar

Surface microstructure analysis was performed following the James and Smith (2009) method. The chocolate bar sample was immobilized by freezing. The sample was sputtered with gold for 10 mins, 10 mA. The sample was observed and photographed using 3.00 kV accelerating potential. Scanning Electron Microscopy (SEM) was carried out on Hitachi SU 3500.

2.7 Data analysis

Data analysis was performed by SPSS 17.0 software by applying analysis of variance (one-way ANOVA). DMRT (Duncan’s Multiple Range Test) was performed to analyze the differences in means. The differences were considered significant at a 0.95 confidence level.

3. Results and discussion

3.1 Chemical characteristics of probiotic dark chocolate bar

3.1.1 Macronutrient content

Macronutrients of food products are fat content, protein content, ash content, moisture content and carbohydrate content. The proximate analysis gives information about the quality of the food product. According to Figure 1, the protein and ash content of chocolate with coconut sugar and chocolate with palm sugar is higher than chocolate with sucrose. The increased protein and ash content is due to the high protein and mineral content in coconut and palm sugar. The protein content of sucrose, palm sugar, and coconut sugar used in this research were 0%, 0.91%, and 0.72%, respectively. The ash content of sucrose, palm sugar, and coconut sugar was used in this research at 0%, 2.60%, and 2.10%, respectively. Coconut and palm sugars contain sucrose, amino acids, vitamins, and minerals (Purnomo, 2007; Saputro et al., 2019). Ho et al. (2007) reported that 15 amino acids were found in palm sugar with asparagine, arginine and glutamine for the major content. The value of moisture, fat and carbohydrate content of probiotic dark chocolate had not significant. This macronutrient content of probiotic chocolate is similar to the research of (Mehta, 2017) and (Al-Marazeeq, 2018) who evaluate the proximate analysis of some branded chocolate and dark chocolate fortified with a white germ. Encapsulated probiotic bacteria did not affect the nutrition composition of dark chocolate due to a little concentration added (Mirkovic et al., 2018).

![Figure 1. The macronutrient content of probiotic dark chocolate with sugar substitute](image)

3.1.2 Calorie value

All food consumed will be burned to generate energy, referred to as calories. A kilocalorie is the amount of energy required to raise the temperature of one kilogram of water by one degree on a centigrade (Celsius) thermometer located at sea level. The calorie values of chocolate sweetened with sucrose, chocolate sweetened with coconut sugar and chocolate sweetened with palm sugar respectively 6.44 kcal/g, 6.48 kcal/g and 6.35 kcal/g (Table 1). The calorie values of chocolate sweetened with palm sugar were lower than chocolate sweetened with coconut sugar, but not significantly different from chocolate sweetened with sucrose. In comparison to chocolate sweetened with sucrose or coconut sugar, chocolate sweetened with palm sugar has lower carbohydrate and fat content. This could explain why chocolate sweetened with palm sugar has fewer calories. The human body's primary energy sources are fat, glucose, and protein. Additionally, organic acids and alcohol are energy sources, although in trace levels in the diet. The probiotic dark chocolate in this research provided a slight calorie value compared to Mirkovic et al. (2018), Al-Marazeeq (2018) and Mehta (2017). The higher fat content explained the high calorie of chocolate in this study.
Table 1. Chemical properties of probiotic dark chocolate bar with sugar substitute

| Chocolate          | % Antioxidant activity | Total phenolic content (mg GAE/g sample) | Calorie (cal/g) |
|--------------------|------------------------|----------------------------------------|-----------------|
| Choc sucrose       | 81.08±2.170            | 2.938±0.171                            | 6,445.31±37.65  |
| Choc coconut sugar | 81.47±0.794            | 3.657±0.210                            | 6,482.86±86.85  |
| Choc palm sugar    | 83.84±0.041            | 3.830±0.357                            | 6,352.06±25.11  |
| BHA                | 85.99±0.950            | -                                      | -               |
| BHT                | 86.33±0.239            | -                                      | -               |

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different (p < 0.05) among samples.

3.1.3 Antioxidant activity

Radical scavenging activity showed antioxidant activity. The antioxidant can control autoxidation by inhibiting the formation of free radicals, interrupting the propagation of free radicals, and subsequently reducing oxidative stress. This action will improve immune function, increase healthy longevity and become a therapeutic agent in the management of diabetes mellitus (Ayepola et al., 2014). Table 1 shows that the radical scavenging activity of chocolate sweetened with palm sugar is significantly higher than chocolate sweetened with sucrose and chocolate sweetened with coconut sugar. The radical scavenging activity of chocolate sweetened with palm sugar was significantly lower than BHA and BHT as antioxidant references. Palm sugar and coconut sugar are known to contain antioxidant compounds, such as 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-piran-4-one (DDMP). DDMP formed by the Maillard reaction between reducing sugars and proteins shows antioxidant activity (Ho et al., 2006). Ban et al. (2007) reported that DDMP could inhibit the growth of colon cancer cells by inducing apoptotic cell death through the inhibition of NF-KB. Probiotic bacteria also contribute to antioxidant activity, in accordance with Afify et al (2012) who obtained antioxidant activity from the probiotic cell-free extract.

The antioxidant activity correlated with the total phenolic content of probiotics in dark chocolate. Chocolate sweetened with palm sugar and coconut sugar had higher total phenolic content than chocolate sweetened with sucrose. This result showed that the addition of 30% coconut sugar and palm sugar had an impact on the total phenolic content and antioxidant activity of probiotic dark chocolate. Palm sugar and coconut sugar contain vitamins, minerals, amino acids and higher phenolic content (Saputro et al., 2018). The antioxidant activity can be influenced by the presence of other bioactive components besides polyphenols, such as methylxanthines, theobromine and Maillard’s reactions to the product (Oracz and Nebesny, 2016), vitamin C and peptides (Asghar et al., 2020). Table 1. exhibits that the antioxidant activity of chocolate sweetened with sucrose and chocolate sweetened with coconut sugar showed no significant difference. In addition, the antioxidant activity can also be influenced by molecular weight, structure, and concentration of compounds. The configuration and number of hydroxyl groups (-OH) in flavonoid molecules can also affect the efficiency of radical inhibition.

3.1.4 Total phenolic content

Table 1 shows that the total phenolic content of chocolate sweetened with coconut sugar and chocolate sweetened with palm sugar were significantly higher than chocolate sweetened with sucrose. The total phenolic content of chocolate sweetened with palm sugar, chocolate sweetened with coconut sugar and chocolate sweetened with sucrose were 3.83 mg GAE/g chocolate, 3.66 mg GAE/g chocolate and 2.94 mg GAE/g chocolate respectively. The sugar substitution with palm sugar and coconut sugar increases the total phenolic content in probiotic dark chocolate. This can be explained by the high content of polyphenols in palm sugar and coconut sugar. The total phenolic contents of sucrose, coconut sugar, and palm sugar used for this research were 0.03 mg GAE/g, 1.48 mg GAE/g, and 1.70 mg GAE/g, respectively. The total phenolic content generally shows a positive correlation with antioxidant activity, where the higher the total phenolic content, the higher the antioxidant activity as in Table 1. Coconut sugar and palm sugar contain high content of polyphenols, such as gallic acid, protocatechuic acid, caffeic acid, p-coumaric acid, and galangin (Asghar et al., 2020)

Polyphenols possess antioxidant activity in-vitro, exhibit anticarcinogenic, anti-inflammatory, anti-allergic, and antiviral properties (Ayepola et al., 2014). Cocoa and cocoa products are rich in polyphenols such as catechin, epicatechin, cyanidin, and procyanidin which contributed to health benefits. Dark chocolate intervention for 3 weeks in 45 healthy volunteers significantly increased the high-density lipoprotein cholesterol (HDL-c) dan decreased the low-density lipoprotein (LDL) diene conjugates, a marker of lipid peroxidation in-vivo (Mursu et al., 2004). Tokode et al. (2011) also reported that dark chocolate or cocoa product intervention significantly reduced serum LDL and total cholesterol levels in human trials. This health benefit of cocoa consumption is due to the high content of

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3.2 Viability of lactic acid bacteria

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO, 2002). The addition of probiotics is expected to improve the functional properties of chocolate products. Consumption of live probiotics that are able to colonize the intestines is expected to increase the population of good bacteria in the gastrointestinal tract. Table 2. shows that the total amount of LAB in chocolate samples was not significantly different with values ranging from 6.88-7.16 log CFU/g chocolate. These values have fulfilled the minimum therapeutic level of viable probiotic microorganisms which should be at least 6 log CFU/g of viable cells in a food product (Terpou et al., 2019).

Research by Foong et al. (2013) showed that dark chocolate with the fortification of L. plantarum probiotics during 3 months of storage had 6-8 log CFU/g total amount of LAB in chocolate. Chocolate is an excellent vehicle for probiotic bacteria. According to Possemiers et al. (2010), chocolate was an effective vehicle for the oral delivery of a probiotic mixture consisting of L. helveticus and B. longum. In the gastrointestinal tract simulation, the higher probiotics combination was seen when embedded in chocolate matrices compared to milk matrices. Previous studies established that chocolate is an excellent carrier for L. helveticus and B. longum probiotics when consumed orally. The lipid portion of cocoa butter provided good protection against the hostile environment for probiotic bacteria (Possemiers et al., 2010). Palm sugar and coconut sugar also work as a protection for probiotic microorganisms in chocolate. Magnesium, manganese, and amino acids all occur naturally in palm and coconut sugar and serve as growth stimulants for bacteria (Low et al., 2015). Additionally, because chocolate is a low-moisture food, it may work as an effective carrier for bacteria. A successful oral probiotic carrier should be acceptable for human consumption, maintain viability throughout the food manufacturing, distribution, and storage process, preserve the probiotics during passage through the upper intestine, and transfer the probiotics to the colon.

3.3 Physical characteristics

3.3.1 Colour

The visual attribute is a critical factor in determining the product’s quality. The processing methods, composition, and roughness of the chocolate may all have an effect on the colour of the chocolate. Dark chocolate’s look is determined by its shine, shape, surface, and smoothness or roughness. (Briones et al., 2006)

Table 2. shows that the L*, a* and b* values of chocolate sweetened with coconut sugar and chocolate sweetened with palm sugar were significantly lower than chocolate sweetened with sucrose. The L* value indicated the product’s lightness. In this study, we discovered that chocolate sweetened with palm and coconut sugar had a darker colour than chocolate sweetened with sucrose. This could be a result of the Maillard reaction and caramelization processes involved in the production of palm and coconut sugar. Positive a* values indicated the presence of red, while positive b* values showed the presence of yellow. Sucrose-sweetened chocolate was more red and yellow in colour than chocolate sweetened with palm sugar or coconut sugar. According to (Saputro, Van de Walle, Aidoo et al., 2017), coconut sugar and palm sugar have lower particle densities than sucrose, occupy a greater fraction of the particle volume, and hence enhance particle contact. Palm sugar and coconut sugar both contain reducing sugars (glucose and fructose) and proteins that have the potential to cause Maillard reactions. It may intensify the blackness of chocolate made with coconut sugar or chocolate made with palm sugar.

The total colour difference (ΔE*) was evaluated to determine the effect of sugar replacement on the dark chocolate probiotics. The data showed that chocolate sweetened with palm sugar had ΔE* of 3.5672±0.5434 and chocolate sweetened with coconut sugar had ΔE* of 2.8695±0.2499. According to Bodart et al. (2008), the acquired values of ΔE* were equivalent to what is visible or not to the human eye:

ΔE* < 1 means the colour distinctions are imperceptible to the human eye

1 < ΔE* < 3 means minor colour distinctions are perceivable by the human eye

| Chocolate      | Total LAB (log CFU/g) | Viscosity (cP) | L*     | a*     | b*     | ΔE     |
|----------------|-----------------------|----------------|--------|--------|--------|--------|
| Choc sucrose   | 7.16a                 | 7,364b         | 27.53±0.609b | 6.05±0.176b | 5.23±0.398c | -      |
| Choc coconut sugar | 6.94a      | 8,172c         | 25.75±0.217a | 4.60±0.089a | 3.46±0.320b | 2.8695±0.2499 |
| Choc palm sugar | 6.88a                 | 5,968a         | 25.80±0.494a | 4.40±0.996a | 2.78±0.508a | 3.5672±0.5434 |

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different (p < 0.05) among samples.
Viscosity is also affected by the particle density and moisture of the chocolate. The higher moisture and the smaller density of the chocolate, the higher the viscosity. This is caused by the increase in particle-particle interaction when the density of the particles gets smaller. The presence of a high moisture content in chocolate sweetened with coconut and palm sugar created an agglomeration of the particles (Saputro, Van de Walle, Aidoo et al., 2017).

The study by Saputro, Van de Walle, Aidoo et al. (2017) showed the highest to the lowest particle density is occupied by sucrose (1.570 g/cm³), palm sugar (1.560 g/cm³), and coconut sugar (1.525 g/cm³) respectively. It was assumed that the higher the particle density, the lower the viscosity. Table 2 expresses the viscosity of all samples, which were significantly different. The highest viscosity was owned by chocolate sweetened with coconut sugar, then followed by chocolate sweetened with sucrose, and the lowest viscosity was owned by chocolate sweetened with palm sugar. This disparate result may be influenced by the particle density factor, the chocolate composition, or the chocolate’s moisture content. It appears as though the increased viscosity was caused by the increased moisture and carbohydrate content of chocolate sweetened with coconut.

3.3.3 Melting profile

The melting profile of chocolate illustrates the melting behaviour of chocolate in the mouth. In this study, differential scanning calorimetry (DSC) analysis records some melting properties of the chocolate including $T_{\text{onset}}$ (the temperature at which a specific crystal form starts to melt), $T_{\text{max}}$ (the temperature at which melting rate is highest), and enthalpy (the amount of latent heat absorbed during melting) (Afoakwa et al., 2008). The DSC profile of chocolates at 30–200°C showed two endothermic peaks (Figure 2). The first and second peaks are attributed to the melting of cocoa butter and sugar, respectively. This is not directly related to the sensory properties, but the presence of melting sugar in chocolate can be used to know the presence of amorphous sugar and/or moisture content (Saputro, Van de Walle, Aidoo et al., 2017).

Table 3 shows that the $T_{\text{onset}}$ and $T_{\text{max}}$ of the fat melting parameter of chocolates were significantly different and chocolate sweetened with sucrose had a lower melting temperature than chocolate sweetened with coconut and palm sugar. This might be correlated to particle size, moisture content, and sugar composition that affect the splitting and melting temperature. The enthalpy values were also significantly different for all the samples, and it is related to the different energy needed to complete the melting of fat. Coconut sugar and palm sugar have a higher viscosity and moisture content, which may have contributed to the increased energy required for fat melting. The $T_{\text{onset}}$ and $T_{\text{max}}$ values of probiotics dark chocolate were found to be greater in this study than in Afoakwa et al. (2008) and Saputro, Van de Walle, Kadivar et al. (2017). This could be because of the difference in fat content and the addition of probiotic bacteria encapsulated in milk matrices. Probiotics dark chocolates used in this study contain 46–48% fat, whereas Afoakwa’s dark chocolate contains 25–35% fat. Variations in fat content resulted in variations in crystallinity and melting characteristics.

Figure 2. The melting profile of probiotic dark chocolate with sugar substitute

Table 3 shows that the $T_{\text{onset}}$ and $T_{\text{max}}$ of the sugar melting parameter of chocolates were significantly different and chocolate sweetened with sucrose had higher sugar melting temperature than chocolate sweetened with coconut and palm sugar. This result was according to Saputro, Van de Walle, Aidoo et al. (2017), chocolate sweetened with palm sugar and coconut sugar had lower $T_{\text{onset}}$ and $T_{\text{max}}$ than chocolate sweetened with sucrose. Coconut sugar and palm sugar contain higher moisture content than sucrose which might cause the formation of amorphous material during chocolate production.

$\Delta E^* > 3$ means distinct colour contrasts are readily apparent to the human eye.
processing. Moisture content is related to $T_{\text{max}}$ and enthalpy. The presence of impurities (protein, minerals, and reducing sugars) in coconut sugar and palm sugar could contribute to the decrease of $T_{\text{onset}}$ and $T_{\text{max}}$ of coconut sugar and palm sugar. Thus, the decreasing of the melting point of a material can be related to the presence of impurities.

The existence of agglomeration in chocolate is known by measuring the melting point and peak area in the sugar phase of chocolate. Chocolate that contains high moisture content has a lower melting point and a narrower peak area than chocolate with low moisture content. Moisture content which acts as a plasticizer contributes to a decrease in the melting temperature of the sugar phase in chocolate. In Saputro, Van de Walle, Kadivar et al. (2017), chocolate sweetened with sucrose had a greater enthalpy value than chocolate sweetened with palm sugar and coconut sugar.

3.3.4 Surface microstructure

Fat (cocoa butter), cocoa powder, sugar and emulsifiers affected the microstructure of chocolate (Delbaere et al., 2016). The microstructure of probiotic dark chocolate (Figure 3) showed that the particle was closely packed. Cocoa particles had a rounded shape and sugar particles had sharp-edge and irregular shapes. Microscopy visualization showed that sugar agglomeration happened in both probiotic dark chocolates sweetened with coconut sugar and palm sugar, respectively. Meanwhile, the probiotic dark chocolate-sweetened sucrose had less agglomeration. It occurred because coconut sugar and palm sugar have high hygroscopicity. The moisture content on the surface of coconut sugar and palm sugar might induce the agglomeration of the sugar particle.

![Figure 2. The melting profile of probiotic dark chocolate with sugar substitute](A) (B) (C)

4. Conclusion

There were specific effects of probiotic dark chocolates comprising physical, chemical, and microbiological characteristics by replacing sucrose with palm sugar and coconut sugar. Visually, replacing sweeteners with palm sugar and coconut sugar made the darker colour, increase the fat melting profile, and increase the total phenolic content and antioxidant activity of probiotic dark chocolate compared to the reference. The antioxidant activity, total phenolic content and the amount of probiotic content of chocolate sweetened with palm sugar and coconut sugar showed the functional properties of dark chocolate. According to the total phenolic content, antioxidant activity, calorie value and viability of probiotics, palm sugar replacement was suggested for the next research and product development. This probiotic dark chocolate has the potential to be developed into functional foods, especially for blood glucose control, however, in-vivo testing and clinical trials are needed in future research to determine the effectiveness of the products and their effects on diabetes mellitus.

Conflict of interest

The authors declare no conflict of interest.

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Table 3. Fat and sugar melting parameter of probiotic dark chocolate bar

| Chocolate            | Fat melting parameter | Sugar Melting Parameter |
|----------------------|-----------------------|-------------------------|
|                      | $T_{\text{onset}}$ ($^\circ$C) | $T_{\text{max}}$ ($^\circ$C) | Enthalpy (J/g) | $T_{\text{onset}}$ ($^\circ$C) | $T_{\text{max}}$ ($^\circ$C) | Enthalpy (J/g) |
| Choc sucrose         | 32.10$^a$             | 35.54$^a$              | 23.52$^b$     | 188.73$^c$          | 190.16$^c$          | 34.30$^c$       |
| Choc coconut sugar   | 32.21$^b$             | 36.43$^b$              | 20.78$^a$     | 148.38$^b$          | 161.28$^b$          | 19.69$^b$       |
| Choc palm sugar      | 34.72$^c$             | 37.63$^c$              | 34.58$^b$     | 145.43$^c$          | 158.17$^c$          | 11.33$^a$       |

Values with different superscript within the same row are significantly different (p < 0.05) among samples.
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