Food Research 6 (5) : 97 - 107 (October 2022)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

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

[1,*]Nurhayati, R., [2]Zulfa, N., [1]Herawati, E.R.N. and [1]Laila, U.

[1]*Research Division for Natural Product Technology, Indonesian Institute of Sciences. Jl Jogja-Wonosari km 31,5 ds Gading, Playen, Gunungkidul, Yogyakarta, Indonesia*
[2]*Department of Food Science and Technology, Faculty of Agricultural, Universitas Sebelas Maret, Surakarta, Indonesia*

Article history:
Received: 3 September 2021
Received in revised form: 4 October 2021
Accepted: 26 January 2022
Available Online: 12 September 2022

Keywords:
Dark chocolate,
Probiotic,
Palm sugar,
Coconut sugar,
Antioxidant activity,
Total phenolic content

DOI:
https://doi.org/10.26656/fr.2017.6(5).591

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).

*Corresponding author.
Email: *rifa.lipi@gmail.com*

eISSN: 2550-2166
Published by Rynnye Lyan Resources

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 (A_0) 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 :

$$\% \, RSA = \frac{Ao - At}{Ao} \times 100\%$$

Where A_o 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% Na_2CO_3 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 Viscocity

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:

$$\Delta E = \sqrt{(Lf - Li)^2 + (af - ai)^2 + (bf - bi)^2}$$

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).

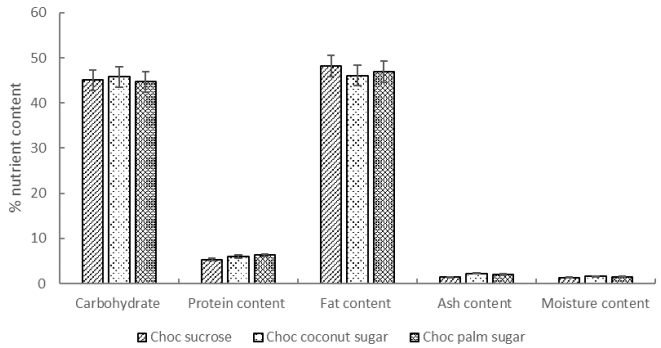

Figure 1. The macronutrient content of probiotic dark chocolate with sugar substitute

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	81.088±2.170[a]	2.938±0.171[a]	6,445.31±37.65[ab]
Choc coconut sugar	81.474±0.794[a]	3.657±0.210[b]	6,482.86±86.85[b]
Choc palm sugar	83.843±0.041[b]	3.830±0.357[b]	6,352.06±25.11[a]
BHA	85.991±0.950[c]	-	-
BHT	86.336±0.239[c]	-	-

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). Tokede *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

polyphenols in chocolate.

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 perceptible by the human eye

Table 2. Physical properties of probiotic dark chocolate bar with sugar substitution

Chocolate	Total LAB (log CFU/g)	Viscosity (cP)	Colour			
			L*	a*	b*	ΔE
Choc sucrose	7.16[a]	7,364[b]	27.533±0.609[b]	6.050±0.176[b]	5.233±0.398[c]	-
Choc coconut sugar	6.94[a]	8,172[c]	25.750±0.217[a]	4.600±0.089[a]	3.467±0.320[b]	2.8695±0.2499
Choc palm sugar	6.88[a]	5,968[a]	25.800±0.494[a]	4.400±0.996[a]	2.783±0.508[a]	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.

ΔE* > 3 means distinct colour contrasts are readily apparent to the human eye.

This result demonstrated that palm sugar replacement provided a colour contrast on the probiotics dark chocolate, whereas coconut sugar replacement produced a minor colour contrast with chocolate sweetened with sucrose.

3.3.2 Viscosity

Viscosity plays an important role in the chocolate manufacturing industry, such as regard to the mouthfeel, consumer acceptance, and handling properties during application. Solid materials, process parameters, and fat content can influence the viscosity of chocolate (Chetana *et al*., 2013). 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/cm^3), palm sugar (1.560 g/cm^3), and coconut sugar (1.525 g/cm^3) 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_{onset} (the temperature at which a specific crystal form starts to melt), T_{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).

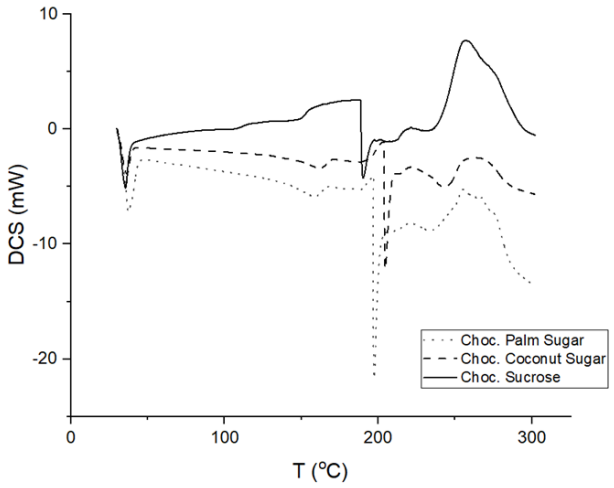

Figure 2. The melting profile of probiotic dark chocolate with sugar substitute

Table 3 shows that the T_{onset} and T_{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_{onset} and T_{max} values of probiotics dark chocolate were found to be greater in this study than in Afaokwa *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 Afaokwa's dark chocolate contains 25-35% fat. Variations in fat content resulted in variations in crystallinity and melting characteristics.

Table 3 shows that the T_{onset} and T_{max} of the sugar melting parameters 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_{onset} and T_{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

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

Chocolate	Fat melting parameter			Sugar Melting Parameter		
	T_{onset} (°C)	T_{max} (°C)	Enthalpy (J/g)	T_{onset} (°C)	T_{max} (°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[c]	145.43[a]	158.17[a]	11.33[a]

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

processing. Moisture content is related to T_{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_{onset} and T_{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.

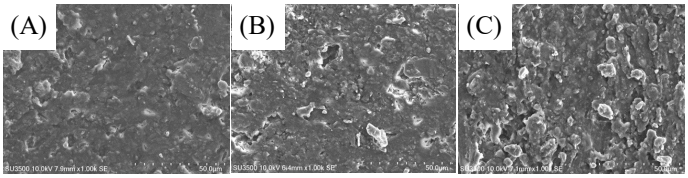

Figure 2. The melting profile of probiotic dark chocolate with sugar substitute

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.

Acknowledgements

We would like to thank Insentif Riset Nasional Grant (2018-2019) and PRN Grant (2020) by the Ministry of Research and Technology, Republic of Indonesia for funding this research.

References

Afify, A.E.M.R., Romeilah, R.M., Sultan, S.I.M. and Hussein, M.M. (2012). Antioxidant activity and biological evaluations of probiotic bacteria strains. *International Journal of Academic Research Part A*, 4(6), 131-139. https://doi.org/10.7813/2075-4124.2012/4-6/A.19

Afoakwa, E.O., Paterson, A., Fowler, M. and Vieira, J. (2008). Characterization of melting properties in dark chocolates from varying particle size distribution and composition using differential scanning calorimetry. *Food Research International*, 41(7), 751–757. https://doi.org/10.1016/j.foodres.2008.05.009

Al-Marazeeq, K.M. (2018). Evaluation of Proximate Composition and Sensory Attributes of Dark

Chocolate Fortified with Wheat Germ. *Advance Journal of Food Science and Technology*, 14(3), 103 –107. https://doi.org/10.19026/ajfst.14.5843

Alberts, H.C. and Cidell, J.I. (2006). Chocolate consumption, manufacturing and quality in western Europe and the United States. *Geography*, 91(3), 218 –226. https://doi.org/10.1080/00167487.2006.12094169

Albrecht, J.A, Schwarz, C.J. and Schnepf, M. (2010). Chocolate — A Functional Food? University of Nebraska-Lincoln Extension. Retrieved on April 16, 2021 from https://extensionpublications.unl.edu/assets/pdf/hef599.pdf

Apriyantono, A., Aristyani, A., Nurhayati, Lidya, Y., Budiyanto, S. and Soekarto, S.T. (2002). Rate of browning reaction during preparation of coconut and palm sugar. *International Congress Series*, 1245(C), 275–278. https://doi.org/10.1016/S0531-5131(02)00882-8

Asghar, M.T., Yusof, Y.A., Mochtar, M.N., Ya'acob, M.E., Ghazali, H.M., Chang, L.S. and Manaf, Y.N. (2020). Coconut (*Cocos nucifera* L.) sap as a potential source of sugar: Antioxidant and nutritional properties. *Food Science and Nutrition*, 8(4), 1777–1787. https://doi.org/10.1002/fsn3.1191

AOAC. (Association Official Analytical Chemists). (2000). Official Methods of Analysis of AOAC International. 17th ed. Method 920.181. USA: AOAC.

Ayepola, O.R., Brooks, N.L. and Oguntibeju, O.O. (2014). Oxidative Stress and Diabetic Complications: The Role of Antioxidant Vitamins and Flavonoids. In Oguntibeju, O. (Ed.) Antioxidant-Antidiabetic Agents and Human Health, InTech Open E-Book. https://doi.org/10.5772/57282

Ban, J.O., Hwang, I.G., Kim, T.M., Hwang, B.Y., Lee, U.S., Jeong, H.S., Yoon, Y.W., Kimz, D.J. and Hong, J.T. (2007). Anti-proliferate and pro-apoptotic effects of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyranone through inactivation of NF-kappaB in human colon cancer cells. *Archives of Pharmacal Research*, 30(11), 1455–1463. https://doi.org/10.1007/BF02977371

Bodart, M., de Peñarada, R., Deneyer, A. and Flamant, G. (2008). Photometry and colourimetry characterization of materials in daylighting evaluation tools. *Building and Environment*, 43(12), 2046–2058. https://doi.org/10.1016/j.buildenv.2007.12.006

Botelho, P.B., Galasso, M., Dias, V., Mandrioli, M., Lobato, L.P., Rodriguez-Estrada, M.T. and Castro, I.A. (2014). Oxidative stability of functional phytosterol-enriched dark chocolate. *LWT-Food Science and Technology,* 55(2), 444-451. https://doi.org/10.1016/j.lwt.2013.09.002

Briones, V., Aguilera, J.M. and Brown, C. (2006). Effect of surface topography on colour and gloss of chocolate samples. *Journal of Food Engineering*, 77 (4), 776–783. https://doi.org/10.1016/j.jfoodeng.2005.08.004

Chen, P., Zhang, Q., Dang, H., Liu, X., Tian, F., Zhao, J., Chen, Y., Zhang, H. and Chen, W. (2014). Screening for potential new probiotic based on probiotic properties and α-glucosidase inhibitory activity. *Food Control*, 35(1), 65–72. https://doi.org/10.1016/j.foodcont.2013.06.027

Chetana, R., Reddy, S.R.Y. and Negi, P.S. (2013). Preparation and Properties of Probiotic Chocolates Using Yoghurt Powder. *Food and Nutrition Sciences*, 4(3), 276–281. https://doi.org/10.4236/fns.2013.43037

Cohen, B.L. and C.A. And Schilken. (1994). A Laboratory Experiment Introducing Measuring by Calorimetry. *Journal of Chemical Education,* 71(4), 342-345. https://doi.org/10.1021/ed071p342

Council, I.F.I. (2018). (2018). Food and Health Survey Report. In *European University Institute*. Retrieved on April 16, 2021 from Food Insight Website: https://foodinsight.org/wp-content/uploads/2018/05/2018-FHS-Report.pdf

Delbaere, C., Van de Walle, D., Depypere, F., Gellynck, X. and Dewettinck, K. (2016). Relationship between chocolate microstructure, oil migration, and fat bloom in filled chocolates. *European Journal of Lipid Science and Technology*, 118(12), 1800–1826. https://doi.org/10.1002/ejlt.201600164

Dwijatmoko, M.I., Praseptiangga, D. and Muhammad, D.R.A. (2016). Effect of cinnamon essential oils addition in the sensory attributes of dark chocolate. *Nusantara Bioscience*, 8(2), 301–305. https://doi.org/10.13057/nusbiosci/n080227

Erdem, O., Gültekin-Özgüven, M., Berktaş, I., Ersan, S., Tuna, H.E., Karadag, A., Özçelik, B., Günes, G. and Cutting, S.M. (2014). Development of a novel synbiotic dark chocolate enrched with *Bacillus indicus* HU36, maltodextrin and lemon fiber: Optimization by response surface mehodology. *LWT - Food Science Technology,* 56(1), 187-193. https://doi.org/10.1016/j.lwt.2013.10.020

FAO. (2002). Guidelines for the Evaluation of Probiotics in Food: Report of a Join FAO/WHO. Retrieved on April 17, 2021 from FAO website: https://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf

Foong, Y.J., Lee, S.T., Ramli, N., Tan, Y.N. and Ayob, M.K. (2013). Incorporation of potential probiotic *Lactobacillus plantarum* isolated from fermented cocoa beans into dark chocolate: Bacterial viability and physicochemical properties analysis. *Journal of Food Quality*, 36(3), 164–171. https://doi.org/10.1111/jfq.12028

Ho, C.W., Aida, W.M.W., Maskat, M.Y. and Osman, H. (2007). Changes in volatile compounds of palm sap (Arenga pinnata) during the heating process for production of palm sugar. *Food Chemistry*, 102(4), 1156–1162. https://doi.org/10.1016/j.foodchem.2006.07.004

Ho, C.W., Wan Aida, W.M., Maskat, M.Y. and Osman, H. (2006). Optimization of headspace solid phase microextraction (HS-SPME) for gas chromatography mass spectrometry (GC-MS) analysis of aroma compound in palm sugar (*Arenga pinnata*). *Journal of Food Composition and Analysis*, 19(8), 822–830. https://doi.org/10.1016/j.jfca.2006.05.003

James, B.J. and Smith, B.G. (2009). Surface structure and composition of fresh and bloomed chocolate analysed using X-ray photoelectron spectroscopy, cryo-scanning electron microscopy and environmental scanning electron microscopy. *LWT - Food Science and Technology*, 42(5), 929–937. https://doi.org/10.1016/j.lwt.2008.12.003

Kharat, V.T. and Deshpande, H.W. (2017). Studies on proximate analysis and microbial analysis of probiotic chocolate. *Journal of Pharmacognosy and Phytochemistry*, 6(5), 407–411.

Latif, R. (2013). Chocolate/cocoa and human health: A review. *Netherlands Journal of Medicine*, 71(2), 63–68.

Lee, K.W., Kim, Y.J., Lee, H.J. and Lee, C.Y. (2003). Cocoa Has More Phenolic Phytochemicals and a Higher Antioxidant Capacity than Teas and Red Wine. *Journal of Agricultural and Food Chemistry*, 51(25), 7292–7295. https://doi.org/10.1021/jf0344385

Saidin, M.S., Ramli, N., Nee, Y.T. and K. Ayob, M. (2015). The Effect of Milk Replacer Incorporation on the Physicochemical Properties of Milk Chocolate. *The Open Food Science Journal*, 8(1), 9–17. https://doi.org/10.2174/1874256401408010009

Mehta, M. (2017). Proximate Analysis of Branded Chocolate. *Global Journal for Research Analysis*, 6 (7), 352–354.

Mirkovic, M., Seratlic, S., Kilcawley, K., Mannion, D., Mirkovic, N. and Radulovic, Z. (2018). The Sensory Quality and Volatile Profile of Dark Chocolate Enriched with Encapsulated Probiotic *Lactobacillus plantarum* Bacteria. *Sensors*, 18(8), 2570. https://doi.org/10.3390/s18082570

Muhammad, D.R.A., Praseptiangga, D., Van de Walle, D. and Dewettinck, K. (2017). Interaction between natural antioxidants derived from cinnamon and cocoa in binary and complex mixtures. *Food Chemistry*, 231, 356–364. https://doi.org/10.1016/j.foodchem.2017.03.128

Muhammad, D.R.A., Lemarcq, V., Alderweireldt, E., Vanoverberghe, P., Praseptiangga, D., Juvinal, J.G. and Dewettinck, K. (2019). Antioxidant activity and quality attributes of white chocolate incorporated with Cinnamomum burmannii Blume essential oil. *Journal of Food Science and Technology*, 57(5), 1731–1739. https://doi.org/10.1007/s13197-019-04206-6

Mursu, J., Voutilainen, S., Nurmi, T., Rissanen, T.H., Virtanen, J.K., Kaikkonen, J., Nyyssönen, K. and Salonen, J.T. (2004). Dark chocolate consumption increases HDL cholesterol concentration and chocolate fatty acids may inhibit lipid peroxidation in healthy humans. *Free Radical Biology and Medicine*, 37(9), 1351–1359. https://doi.org/10.1016/j.freeradbiomed.2004.06.002

Nebesny, E., Żyżelewicz, D., Motyl, I. and Libudzisz, Z. (2007). Dark chocolates supplemented with Lactobacillus strains. *European Food Research and Technology*, 225(1), 33–42. https://doi.org/10.1007/s00217-006-0379-9

Nurhayati, R., Frediansyah, A., Rahmawati, F., Retnaningrum, E. and Sembiring, L. (2016). Screening of α-glucosidase Inhibitor-Producing Lactic Acid Bacteria from Ganoderma lucidum as Functional Food Candidate for Diabetic. *International Conference on Appropriate Technology Development (ICATDev)*, 1(2003), 76–81.

Nurhayati, R., Herawati, E.R.N., Oktaviani, B. and Rachmawati, I.D. (2019). The effect of refining time to the antioxidant capacity, phenolic content, sensory and physical properties of dark chocolate couverture. *IOP Conference Series: Earth and Environmental Science*, 251(1). https://doi.org/10.1088/1755-1315/251/1/012047

Nurhayati, R., Miftakhussolikhah, Oktavianingrum, D.A. and Pratiwi, R. (2020). The effect of encapsulated *Pediococcus lolii* L2 on its cell viability and alfa glukosidase inhibition activity. *IOP Conference Series : Earth and Environmental Science*, 462, 012024. https://doi.org/10.1088/1755-1315/462/1/012024

Oracz, J. and Nebesny, E. (2016). Antioxidant Properties of Cocoa Beans (*Theobroma cacao* L.): Influence of Cultivar and Roasting Conditions. *International*

Journal of Food Properties, 19(6), 1242–1258. https://doi.org/10.1080/10942912.2015.1071840

Parvez, S., Malik, K.A., Ah Kang, S. and Kim, H.Y. (2006). Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology*, 100(6), 1171–1185. https://doi.org/10.1111/j.1365-2672.2006.02963.x

Pin Low, R.H., Baba, A.S. and Aboulfazli, H. (2015). Effect of different level of refined cane sugar and unrefined coconut palm sugar on the survivability of *Lactobacillus acidophilus* in probiotic ice cream and its sensory and antioxidant properties. *Food Science and Technology Research*, 21(6), 857-862. https://doi.org/10.3136/fstr.21.857

Possemiers, S., Marzorati, M., Verstraete, W. and Van de Wiele, T. (2010). Bacteria and chocolate: A successful combination for probiotic delivery. *International Journal of Food Microbiology*, 141(1–2), 97–103. https://doi.org/10.1016/j.ijfoodmicro.2010.03.008

Purnomo, H. (2007). Volatile components of coconut fresh sap, sap syrup and coconut sugar. *ASEAN Food Journal*, 14(1), 45–49.

Saputro, A.D., Van de Walle, D., Aidoo, R.P., Mensah, M.A., Delbaere, C., De Clercq, N., Van Durme, J. and Dewettinck, K. (2017). Quality attributes of dark chocolates formulated with palm sap-based sugar as nutritious and natural alternative sweetener. *European Food Research and Technology*, 243(2), 177–191. https://doi.org/10.1007/s00217-016-2734-9

Saputro, A.D., Van de Walle, D., Kadivar, S., Bin Sintang, M.D., Van der Meeren, P. and Dewettinck, K. (2017). Investigating the rheological, microstructural and textural properties of chocolates sweetened with palm sap-based sugar by partial replacement. *European Food Research and Technology*, 243(10), 1729–1738. https://doi.org/10.1007/s00217-017-2877-3

Saputro, A.D., de Walle, D.V., Hinneh, M., Durme, J.V., and Dewettinck, K. (2018). Aroma profile and appearance of dark chocolate formulated with palm sugar-sucrose blends. *European Food Research and Technology*, 244, 1281-1292. https://doi.org/10.1007/s00217-018-3043-2

Saputro, A.D., Van de Walle, D. and Dewettinck, K. (2019). Palm Sap Sugar: A Review. *Sugar Tech*, 21 (6), 862–867. https://doi.org/10.1007/s12355-019-00743-8

Srikaeo, K. and Thongta, R. (2015). Effects of sugarcane, palm sugar, coconut sugar and sorbitol on starch digestibility and physicochemical properties of wheat based foods. *International Food Research Journal*, 22(3), 923–929.

Terpou, A., Papadaki, A., Lappa, I.K., Kachrimanidou, V., Bosnea, L.A. and Kopsahelis, N. (2019). Probiotics in Food Systems: Significance and Emerging Strategies Towards Improved Viability and Delivery of Enhanced Beneficial Value-Review. *Nutrients*, 11(7), 1591. https://doi.org/10.3390/nu11071591

Tokede, O.A., Gaziano, J.M. and Djoussé, L. (2011). Effects of cocoa products/dark chocolate on serum lipids: A meta-analysis. *European Journal of Clinical Nutrition*, 65(8), 879–886. https://doi.org/10.1038/ejcn.2011.64

Trinidad, T.P., Mallillin, A.C., Sagum, R.S. and Encabo, R.R. (2010). Glycemic index of commonly consumed carbohydrate foods in the Philippines. *Journal of Functional Foods*, 2(4), 271–274. https://doi.org/10.1016/j.jff.2010.10.002

Vinson, J.A., Proch, J. and Zubik, L. (1999). Phenol antioxidant quantity and quality in foods: Cocoa, dark chocolate, and milk chocolate. *Journal of Agricultural and Food Chemistry*, 47(12), 4821–4824. https://doi.org/10.1021/jf990312p

eISSN: 2550-2166