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Physicochemical properties and sensory acceptability of sugar-free dark chocolate formulations added with probiotics

Propiedades fisicoquímicas y aceptabilidad sensorial de formulaciones de chocolate negro libres de azúcar y adicionadas con probióticos

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Abstract

The use of natural low-caloric sweeteners as sugar-substitute to formulate foods suitable for people with diabetes has increased in the last years due to the high incidence of the metabolic syndrome. Chocolate is a suitable vehicle of functional ingredients such as probiotics that improve health in people with diabetes. The objective of this study was to evaluate the physicochemical properties (texture, instrumental color, and water activity) and the consumer's acceptability of sugar-free dark chocolate formulation added with *L. plantarum* 299v (L299v) and *L. acidophilus* La 3 (DSMZ 17742). Sweeteners tested were polydextrose (Pol), inulin (Inu), isomalt (Iso), and stevia (Stev) applied in the following combinations: Pol+Inu and Iso+Stev, with and without probiotics (Prob). Probiotics addition in dark chocolate was feasible, maintaining their viability in the final product while not affecting the physicochemical and sensory acceptability of dark chocolate. Sweeteners addition significantly affected the physiochemical and sensory acceptability of the product where chocolates added with Iso+Stev showed the nearest value to the control. Iso+Stev+Prob formulation showed to be a promising sugar-free functional chocolate that could be used with therapeutic and preventive purposes for the diabetic population.

Keywords: metabolic syndrome; sweeteners; probiotics; sugar-free functional food; substitution of sugar by sweeteners; synbiotic chocolate.

Resumen

El uso de edulcorantes naturales bajos en calorías como sustitutos de azúcar para formular alimentos aptos para personas con diabetes se ha incrementado en los últimos años, debido a la alta incidencia del síndrome metabólico. El chocolate es un vehículo idóneo de ingredientes funcionales como los probióticos, que mejoran la salud de las personas con diabetes. El objetivo de este estudio fue evaluar las propiedades fisicoquímicas (textura, color instrumental y actividad de agua) y la aceptabilidad por parte del consumidor de formulaciones de chocolate obscuro sin azúcar adicionados con *L. plantarum* 299v (L299v) y *L. acidophilus* La 3 (DSMZ 17742). Los edulcorantes probados fueron polidextrosa (Pol), inulina (Inu), isomalt (Iso) y estevia (Stev) aplicados en las siguientes combinaciones: Pol+Inu e Iso+Stev, con y sin probióticos (Prob). La adición de probióticos en el chocolate obscuro fue factible, manteniendo su viabilidad en el producto final sin afectar las propiedades fisicoquímicas y aceptabilidad sensorial. Sin embargo, los edulcorantes afectaron significativamente las propiedades fisicoquímicas y aceptabilidad sensorial del producto, en donde la formulación con Iso+Stev mostró el valor más cercano en comparación con el control. La formulación Iso+Stev+Prob demostró ser una formulación prometedora de chocolate funcional sin azúcar que podría usarse con fines preventitos y terapéuticos por la población con diabetes.

Palabras clave: síndrome metabólico; edulcorantes; probióticos; alimento funcional sin azúcar; sustitución del azúcar por edulcorantes; chocolate simbiótico.

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1 Introduction

Type 2 diabetes is affecting millions of people around the world. Therefore, there is an increasing demand of sugar-free food products that can improve health. The production of next-generation functional foods added with bioactive ingredients that can prevent or treat diseases is a feasible approach to obtain food products suitable for people with diabetes (Santana-Gálvez *et al*., 2019; Jacobo-Velázquez *et al*., 2020). Sweeteners are excellent candidates in the food industry to replace sugar from processed products (Carocho *et al*., 2017). These chemical compounds give a sweet taste to food products and low or none calories (Ruiz-Ojeda *et al*., 2019). Chocolate conforms the 55% of the confectionery market in the world and is liked by adults and children due to its sweet taste and pleasurable mouthfeel (Sondhi & Chawla, 2016). Therefore, reformulation of chocolate to produce a sugar-free product that could serve as vehicle of bioactive ingredients could be an interesting approach to develop new food products with preventive and therapeutic properties.

There is a particular interest in the application of natural sweeteners, some of them exert a prebiotic effect. Stevia (Stev) based sweeteners are natural, and highly acceptable in the market. These compounds are extracted from the plant *Stevia rebaudiana*, and the compounds of interest are known as steviol glycosides (Carocho *et al*., 2017; Villamarín-Gallegos *et al*., 2020). Also, Stev is reported to have beneficial effects linked to adjuvant metabolic syndrome diseases such as type 2 diabetes (Philippaert *et al*., 2017). Furthermore, some prebiotics that also contributes with sweet taste includes inulin (Inu) (van der Beek *et al*., 2018; García-Gamboa *et al*., 2020), polydextrose (Pol) (Do Carmo *et al*., 2016), and isomalt (Iso) (Ruiz-Ojeda *et al*., 2019). Prebiotics are defined as non-digestible food ingredients that are metabolized by gut microbiota, improving host health (Gibson & Roberfroid, 1995).

Chocolate has been considered as a suitable matrix to add beneficial compounds such as probiotics (Prob), since its ingredients (cocoa butter, cocoa paste, soy lecithin, and milk) generate low water activity, low oxygen tension, low moisture permeability, and high-fat content, conferring good conditions for probiotics survival during storage (Konar *et al*., 2016; Marcial-Coba *et al*., 2019). The consumption of probiotics (Prob) can be used as a strategy to modulate gut microbiota, highlighting their preventive and therapeutic activity (El Hage *et al*., 2017; González-Figueroa *et al*., 2021). Probiotics are defined as live microorganisms that confer health promoting properties when administrated in adequate amounts to the host (Food and Agriculture Organization of the United Nations / World Health Organization, 2001, 2002).

In addition to the protective characteristics of chocolate, microencapsulation of probiotics comprises the segregation of bacterial cells from the external environment by enclosing them in covalently or ionically crosslinked polymer networks, giving double protection to increase viability (Mirković *et al*., 2018; Marcial-Coba *et al*., 2019).

Spray-drying is the most cost-effective microencapsulation method for probiotic bacteria, and it is suitable for large-scale and industrial applications (Fazilah *et al*., 2019; Macías-Cortés *et al*., 2019). Likewise, spray-drying is a suitable method to preserve the viability of microorganisms (Mirković *et al*., 2018). Some of the most used ingredients for probiotic microencapsulation are sodium alginate and maltodextrin (Krasaekoopt *et al*., 2003). Sodium alginate is a non-toxic linear anionic polymer with a high molecular weight with rigid and flexible regions, giving a structural advantage to protect probiotics (Gutiérrez & Álvarez, 2017). Maltodextrin is a hydrolyzed starch with neutral aroma, low viscosity at higher concentration and, low bulk density giving excellent characteristics to serve as wall material for probiotic microencapsulation (Ray & Chakraborty, 2016; Vázquez-Silva *et al*., 2016).

The combination of sodium alginate and maltodextrin increases the functional properties for the protection of diverse microorganisms (Krasaekoopt *et al*., 2003). According to literature, *Lactobacillus platarum* and *Lactobacillus acidophilus* are adequate probiotic candidates to prevent and ameliorate metabolic syndrome diseases such as type 2 diabetes (Sohag *et al*., 2019; Stevenson *et al*., 2014; Andrade-Velasques *et al*., 2021). Although there are previous reports in literature evaluating the development of chocolates with prebiotic, probiotic and synbiotic characteristics (Konar *et al*., 2016), there is scares information on the design of a chocolate formulations for the diabetic population, which should be sugar-free and also provide prebiotic and probiotic characteristics.

The objective of this study was to evaluate the physicochemical properties (texture, instrumental color, and water activity) and the consumer's

acceptability of sugar-free dark chocolate formulations added with microencapsulated *L. plantarum* 299v (L299v) and *L. acidophilus* La 3 (DSMZ 17742). Sweeteners tested were Pol, Inu, Iso, and Stev applied in the following combinations: Pol+Inu and Iso+Stev, with and without Prob.

2 Materials and methods

2.1 Bacterial strains and chemicals

Probiotic strains *Lactobacillus plantarum* 299v (L299v), and *Lactobacillus acidophilus* La 3 (DSMZ 17742) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and German Collection of Microorganisms and Cell Cultures. MRS agar was obtained from BD Difco™ (NJ, USA), and sodium alginate was purchased from DEIMAN (CDMX, México). Maltodextrin food grade was purchased from Best Ingredients (Monterrey, NL, México). For chocolate bars elaboration, alkalinized cocoa paste, alkalinized cocoa, cocoa butter, whey powder, soy lecithin, polyglycerol polyricinoleate (PGPR), NaCl, vanilla, and sugar, were obtained from Escuela Mexicana de Confitería y Chocolatería (San Luis Potosí, SLP, México). Isomalt LMPF was obtained from Palsgaard Industry de México S de R.L. de C.V. (San Luis Potosí, México). Inulin (Orafti®HSI) from chicory root with an average degree of polymerization (DF) of 10 was purchased from Beneo GmbH (Mannheim, Germnany). Stevia, food grade, was obtained from Grupo Químico Amillán S.A. de C.V (Zapopan, Jal, México). Polydextrose (Polidex Fiber®) was obtained from Ingredion (Guadalajara, Jal, Mexico). Finally, for the microbiological assessment, reconstituted skim milk (Svelty, Nestlé, Lagos de Moreno, JAL, México), Violet Red Bile Agar (VRB agar), potato Dextrose Peptone Agar (DP agar), Xylose Lysine Deoxycholate Agar (XLD agar), *Salmonella Shigella* Agar (SS agar) Tetrathionate Broth Base, Rappaport Vassiliadis Broth and VRBA agar and MRS agar were obtained from BD DifcoTM (NJ, USA).

2.2 Bacterial strains propagation, microencapsulation, and microbial viability assessment

Lactobacillus plantarum 299v and *Lactobacillus acidophilus* La 3 were propagated by inoculating an aliquot (100 μL) from a stock of each strain in 10 mL of MRS broth, which were incubated at 37 ºC (Shel lab 1535, VWR, Randor, PA, USA) during 16 h under aerobic conditions. Then the propagation was scaled-up to a volume of 800 mL under the same incubation conditions. Cells of the two strains were harvested by centrifugation (SL16, Thermo Fisher Scientific Inc., MA, USA) at 10,000 x g at 25 ºC for 15 min. Cell pellets were washed in peptone water (0.1% peptone, 0.85% NaCl, pH 7) and resuspended in a final volume of 30 mL in peptone water.

Suspended cells were added to 750 mL of microencapsulation mix (10% w/v maltodextrin, and w/v 2% food-grade alginate) and spray-dried (ADL 311S, Yamato Scientific Co., Ltd., CA, USA) at 130 ºC inlet, 60 ºC outlet, and 0.13 MPa.

The number of colony-forming units (CFU/g) of probiotics was determined at different points: 1) microencapsulation solution before spray-drying, 2) microencapsulated probiotics by spray-drying, and 3) chocolate samples added with microencapsulated probiotics. To determine CFU/g of probiotics, the microencapsulation solution (1 mL), the powder containing microencapsulated probiotics (0.1 g) or the chocolates added with probiotics (1 g) were homogenized in 90 mL of peptone water preheated at 37 ºC in a stomacher (IUL Instruments, BCN, Spain) for 90 seconds, and a serial dilution was made. Proper dilutions (10^4, 10^6, and 10^8) of each replicate were plated twice on MRS agar and incubated at 37 ºC for 48 h aerobically.

2.3 Chocolate preparation

Chocolate formulations were prepared in a confectionery pilot plant factory (Escuela Mexicana de Confitería y Chocolatería, San Luis Potosí, SLP, México). Six dark chocolate samples were formulated using the same base (alkalized cocoa paste 42%, alkalized cocoa powder 8%, cocoa butter 5%, whey powder 6%, soy lecithin 0.3%, PGPR 0.2%, NaCl 0.08%, and vanilla 0.003% per 100 g of chocolate). Sugar was replaced with sweeteners (Pol, Inu, Iso, and Stev) and added with Prob (*L. plantarum* 299v and *L. acidophilus* La 3) as indicated in Table 1. Sweeteners combinations were determined according to their sweetening power. For instance, Iso is only 40% as sweet as sucrose, therefore it requires a high-potency sweetener such as Stev, which is 300 times sweeter than sucrose (Carocho *et al*., 2017).

Table 1. Sugar-free dark chocolate formulations added with probiotics.

Ingredients	% Percentage in each formulation (w/w)					
	D.C.	D.C.+Prob	Pol+Inu	Pol+Inu+Prob	Iso+Stev	Iso+Stev+Prob
Alkalinized cocoa paste	42	41.96	42	41.96	42	41.96
Alkalinized cocoa	8	7.99	8	7.99	8	7.99
Cocoa butter	5	4.99	5	4.99	5	4.99
Whey powder	6	5.99	6	5.99	6	5.99
Soy lecithin	0.3	0.3	0.3	0.3	0.3	0.3
PGPR	0.2	0.2	0.2	0.2	0.2	0.2
NaCl	0.08	0.08	0.08	0.08	0.08	0.08
Vanilla	0.03	0.03	0.03	0.03	0.03	0.03
Sugar	38.39	38.35	-	-	-	-
Polydextrose	-	-	26.39	26.36	-	-
Inulin	-	-	12	11.99	-	-
Isomalt LMPF	-	-	-	-	38.36	38.32
Stevia	-	-	-	-	0.03	0.03
Probiotic	-	0.1	-	0.1	-	0.1

Abbreviations: D.C. = Dark chocolate control, D.C.+Prob = Dark chocolate control + probiotics, Pol+ Inu = Polydextrose and inulin, Pol+Inu+Prob = Polydextrose and inulin + probiotics, Iso+Stev = Isomalt and stevia, Iso+Stev+Prob = Isomalt and stevia + probiotics

Furthermore, Pol and Inu were combined since Pol provides the bulk and appropriate textural and mouthfeel qualities usually associated with sugar while lacking the sweet taste and caloric value and Inu enhances flavor and sweetness and is used to partially replace sucrose (Aidoo *et al*., 2015).

Each chocolate formulation was produced with the following procedure: 1) melting and heating, 2) coaching, 3) refining, 4) tempering, and 5) molding. The melting process used a water bath at 40 ºC; for the coaching and refining steps, the temperature was 25 ºC, and the duration was for 24 h using a chocolate refiner (Premier, Diamond Custom Machines Corp., NJ, USA). The tempering step had three changes of temperature; the first stage of tempering was maintained at 47 ºC to melt all fat crystals (3-5 min) fully; then, in the second stage, the chocolate was cooled at 29 ºC under manual agitation using a spatula (3-5 min); and then reheated to 31 ºC. Finally, the chocolate formulations were molded at 14 ºC for 1 h and stored at 11 ºC until analysis. Microencapsulated probiotics were added to chocolate after the tempering step (29 ºC) as described by Silva *et al*. (2017) at a ratio of 10^{13} UFC/g, resulting in a product with 10^6 UFC/g per portion of 12 g.

2.4 *Water activity, color and texture determinations*

Water activity (a_w) of chocolate samples was measured shortly after solidification using a water activity meter (Aqualab CX-2, USA) at 25 ºC. The color was determined with a spectrophotometer cm-600d (Konica Minolta, INC, Japan Comintec). Colorimetric parameters obtained were CIE $L*$, $a*$, and $b*$. Chroma ($C*$), hue (h), and white index ($WI*$) were calculated using Eq. 1, 2 and, 3 as follows:

$$C* = \sqrt{(a*)^2 + (b*)^2} \tag{1}$$

$$h = \tan^{-1}(a*/b*) \tag{2}$$

$$WI* = 100 - [(100 - L*)^2 + a*^2 + b*^2]^{1/2} \tag{3}$$

Hardness and work penetration (N) of the samples were analyzed as described by Cikrikci *et al*. (2017) using a Texture analyzer (TVT 6700, Perten Instruments of Australia Pty Limited., NSW, Australia) equipped with a cylinder probe (height 45 mm, diameter 3 mm). The conditions used were sample height: 8 mm; starting distance for sample: 5 mm; compression: 2 mm; initial speed: 0.5 mm/s; test speed: 0.5 mm/s; retract speed: 10 mm/s; trigger force: 5 g; data rate: 500 pps. Texture determinations were performed at room temperature (20 ºC). Five replicates of each treatment were evaluated.

2.5 Evaluation of microbial safety of chocolate

In order to determine the microbial safety of chocolate samples prior to sensory analysis, chocolate formulations were analyzed for total coliforms, yeast, molds and *Salmonella* spp. according to methods previously reported in literature (Feng *et al*., 2018), and the Official Mexican Standard Methods NOM-186-SSA1/SCFI-2013. Briefly, 10 g of each chocolate sample were put into sample bag (Whirl-Pak, Nasco, USA), diluted with sterile peptone water (0.1% peptone, 0.85% NaCl, pH 7) and homogenized for 2 min in a stomacher (IUL Instruments, Spain). Triplicate counts were performed for all dilutions. Total coliforms were determined using violet red bile agar and incubating at 37 °C for 24 h. Fungi and molds were grown in potato dextrose peptone agar and incubated at 25 °C for 5 days. All chocolates presented <10 CFU/g for total coliforms, fungi and molds.

For *Salmonella* spp. analysis, 25 g of chocolate sample were put in 225 mL of reconstituted skim sterilized milk for 60 min at 25 ºC. Then, 1 mL of each sample was put in 10 mL of Vassiliadis-Rappaport and in 10 mL of tetrathionate for 24 h. *Salmonella* spp. counts were performed in XLD agar and SS agar. Chocolate formulations were free of *Salmonella* spp.; thus, all chocolates were safe for human consumption and suitable for sensory evaluations.

2.6 Sensory evaluation

A sensory acceptability test was performed using the 9-point hedonic scale to assess the consumers' acceptability of chocolate formulation. A total of 115 people that consume chocolate at least once a week (59% male and 41% female) were selected with ages ranging between 17 and 21 years.

Each chocolate sample was given with a different random three-digit number. The samples were given in different orders. The temperature of samples was 15 ºC when given to consumers. Participants were asked to eat the chocolate sample one at the time, drink water and eat a cookie with a plain flavor before the evaluation and between samples. For each chocolate, the participants were requested to evaluate the attributes of appearance, flavor, texture, and overall acceptability using a 9-point hedonic scale ranging from 1 to 9 with the following meaning: 1 = "Dislike extremely", 2 = "Dislike very much," 3 = "Dislike moderately," 4 = "Dislike slightly," 5 = "Neither like nor dislike," 6 = "Like slightly," 7 = "Like moderately," 8 = "Like very much," and 9 = "Like extremely".

2.7 Statistical analysis

Results were expressed as mean ± standard error of three independent measurements unless otherwise indicated. Significant differences between mean values were determined by one-way analysis of variance (ANOVA), followed by LSD test ($P < 0.05$). Minitab 19 software (Minitab, Inc., State College, PA, USA) was used.

3 Results and discussion

3.1 Probiotics viability

The present study conducted a spray-drying microencapsulation technique using maltodextrin (10%, w/v) and sodium alginate (2%, w/v) solution as protective ingredients to achieve probiotics' viability. Separate microencapsulated solutions were performed for each bacterium. *Lactobacillus plantarum* 299v microencapsulated solution showed 1.1×10^{12} CFU/mL, and *Lactobacillus acidophilus* La 3 showed 2.0×10^{12} CFU/mL.

Powders with 3×10^{14} CFU/g and 5×10^{10} CFU/g of microencapsulated *L. plantarum* 299v and *L. acidophilus* 3, respectively, were obtained after spray-drying. In agreement with the results obtained herein, previous reports evaluating microencapsulation of probiotics with sodium alginate, demonstrated that it can be used as a heat protector agent of different probiotic strains such as *L. rhamnosus, B. longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei, B. lactis* B1-O4 and *B. lactis* Bi-07 (Ding & Shan, 2007). Other authors also combined sodium alginate with maltodextrin (Krasaekoopt *et al*., 2003), which has been used as a coating agent in microencapsulation of *L. casei* (Hernández-Carranza *et al*., 2014). Also, Boza *et al*. (2004) demonstrated that maltodextrin can protect probiotic strains from heat damage after the spray-drying process with an outlet air temperature of 75 °C.

Microencapsulated bacteria counts in all chocolate formulations showed $>1\times10^{8}$ CFU/g of viable probiotic strains, which is more than the range of minimum count of probiotic bacteria at the recommended intake ($\geq10^{6}$ CFU/g) to have a beneficial effect (Shah, 2007; Mandal *et al*., 2013).

These results are in agreement with the previous report of Silva *et al*., (2017), who evaluated the viability of *L. acidophilus* LA3 and *B. animalis* subsp. lactis BLC1 in semisweet chocolate. The authors found that the low water activity of chocolate kept probiotics in a low metabolic state, increasing their viability in the chocolate matrix. In addition, the high fat content of cocoa paste, decreases the availability of oxygen to the probiotic cell, preventing oxidation, and protecting the cell viability from thermal inactivation (Gaudreau *et al*., 2013; Maukonen & Saarela, 2015). Likewise, Kemsawasd *et al*. (2016) compared the viability of probiotics on different types of chocolates (dark, milk and white) and found that cells remained higher in dark chocolate (50% of cocoa) due to the higher amounts of cocoa and antioxidant compounds (i.e. flavonoids) as compared with milk and white chocolate.

3.2 Physicochemical properties of sugar-free dark chocolate formulations added with probiotics

3.2.1 Instrumental color

Color is one of the critical factors for consumers acceptability. In chocolate, color variation is related to differences in processing parameters during the production and composition of the product (Cikrikci *et al*., 2017). Color values were determined using the CIE $L* a* b*$ system; where $L*$ means luminance ranging from 0 (black) to 100 (white); $a*$ goes from green to red and $b*$ from blue to yellow. Furthermore, chroma ($C*$) and hue (h) values, which are two of the perceptual attributes of color, were also calculated.

The results of the color of dark chocolate formulations are shown in Table 2. In terms of lightness ($L*$) values, dark chocolate control (D.C.) and dark chocolate with probiotics (D.C.+Prob) were the brightest with 15.27 and 16.91 respectively while, Iso+Stev was the darkest. Similar results were reported by Homayouni Rad *et al*. (2019), where the authors tested isomalt and stevia as sucrose substitutes in dark-chocolates. Sucrose crystals have an inter-particle interaction with chocolate, scattering the light in all directions from the matrix, and developing a high level of $L*$ in comparison with other sucrose-free chocolates (Aidoo *et al*., 2015).

Furthermore, sugar-free dark chocolates (Pol+Inu, Iso+Stev, Pol+Inu+Prob, and Iso+Stev+Prob) showed lower values for redness ($a*$) and yellowness ($b*$) as compared to D.C. containing sugar (D.C. and D.C.+Prob). Shourideh *et al*. (2012) and Aidoo *et al*. (2015) obtained similar values in sugar-free chocolates added with inulin and polydextrose. This is explained through absorptivity and scattering factors which depends on the particle size of the matrix. For instance, the combination of inulin and polydextrose changes the crystallinity of the chocolate matrix (Aidoo *et al*., 2015), having the property to absorb moisture, and decrease light scattering and lightness (Shourideh *et al*., 2012). Also, isomalt as a polyol has a different interaction within the fat crystals in chocolate than sugar, impacting in scattering factors decreasing $a*$ and $b*$ values (Homayouni Rad *et al*., 2019).

In terms of brightness, h and $C*$ were evaluated since are of great importance in terms of consumer evaluation and acceptability (Toker *et al*., 2019). $C*$ and h value are quantitative attributes of colorfulness. For instance, high chroma values are perceived by humans as more intense colors and high h values means fewer yellow characters in color samples (Pathare *et al*., 2013). D.C. and D.C.+ Prob result to have higher h and $C*$ values compared with sugar-free chocolates formulations, meaning that D.C. and D.C.+Prob have less colorfulness properties than sugar-free chocolates. Pol+Inu and Iso+Stev+Prob were the lowest h and $C*$ values.

Sample	Color parameters[a]						a_w^a	Hardness[b]	Fracturability
	$L*$	$a*$	$b*$	$C*$	h	$WI*$			
D.C.	15.27 ± 1.5ab	5.15 ± 0.69a	2.32 ± 0.69a	4.83 ± 0.12a	0.41 ± 0.06a	15.07 ± 1.43ab	0.31 ± 0.09b	5426 ± 233.83a	3360.2±174.43b
Pol+Inu	14.68 ± 0.16abc	2.37 ± 0.14b	-0.07 ± 0.11b	2.38 ± 0.14b	-0.04 ± 0.05b	14.65 ± 0.15abc	0.35 ± 0.03a	4937.2± 328.35ab	4341.6±741.43ab
Iso+Stev	12.03 ± 0.68c	2.43 ± 0.56b	0.34 ± 0.72b	2.60 ± 0.68b	0.02 ± 0.22ab	11.99 ± 0.66c	0.441 ± 0.03a	4724±625.06ab	5100.5±772.84a
D.C.+prob	16.91 ± 0.28ab	4.57 ± 0.05a	1.96 ± 0.19a	4.98 ± 0.07a	0.40 ± 0.04a	16.76 ± 0.28a	0.35 ± 0.06b	5236± 226.04ab	3766.5 ±180.40ab
Pol+Inu+Prob	14.94 ± 0.39abc	2.71 ± 0.11b	0.10 ± 0.04b	2.72 ± 0.11b	0.04 ± 0.01ab	14.90 ± 0.39abc	0.35 ± 0.02a	5557 ± 126.22a	3595.8 ± 374.61ab
Iso+Stev+Prob	13.51 ± 1.63bc	1.92 ± 0.32b	-0.05 ± 0.44b	2.02 ± 0.31b	-0.05 ± 0.22b	13.48 ± 1.62bc	0.56 ± 0.03a	3977 ± 926.24b	4843.33±842.15ab

Abbreviations: D.C. = Dark chocolate control, D.C.+Prob = Dark chocolate control + probiotics, Pol+ Inu = Polydextrose and inulin, Pol+Inu+Prob = Polydextrose and inulin + probiotics, Iso+Stev = Isomalt and stevia, Iso+Stev+Prob = Isomalt and stevia + probiotics

Values with different letters within the same column indicate statically significant difference by the LDS test ($P < 0.05$)

aValues represent the mean of 3 replicates with their standard error.

bValues represent the mean of 5 replicates with their standard error.

Once more, scattering and absorptivity factors play an important role in color. According to Homayouni Rad *et al*. (2019) sucrose acts as a nucleating agent and effects crystallization and pre-crystallization within fat crystals impacting light scattering and increasing brightness. For sweeteners such as Iso, it is not common to have this crystallization behavior.

Whiteness index (WI*) is useful to evaluate the occurrence of the fat bloom phenomenon (Silva *et al*., 2017), due to measuring the loss of shine and the white and gray spots generated in the chocolate surface; a higher WI* value indicates increased fat blooming that occurs in the product (Ekantari *et al*., 2019). WI* values showed to be statistically different, where D.C. showed the whitest surface, whereas Iso+Stev presented the darkest meaning that D.C. has more predisposition to fat blooming. According to Bricknell & Hartel (1998), the speed of fat bloom can be determined by observing the WI* in the product, the higher the white index indicates the fatter blooming that occurs in the product.

3.2.2 Sugar-free dark chocolate water activity (a_w) analysis

The water activity (a_w) values of dark chocolate samples are shown in Table 2. Except for Iso+Stev+Prob, all formulations were below the threshold for pathogenic microbial growth in foods (a_w <0.46). Konar (2013) obtained similar values of a_w using inulin as a substitute of sugar in chocolates, and attributed these values to the interaction of inulin and conching process temperature. On the other hand, chocolates Iso+Stev and Iso+Stev+Prob changed the a_w of the dark sugar-free chocolate, increasing the value of a_w, due to the three-dimensional network that is capable of holding water (Franck, 2002). Isomalt possess different number of hydroxyl groups that have hydrophilic potential and are generally involved in intermolecular hydrogen bonds which generate non-polar interaction with chocolate and results in particle agglomeration (Saputro *et al*., 2017). Also, several factors such as raw material used, temperature, and humidity, as well as unit operations of the chocolate making process such as refining and conching, can influence a_w parameter, because in these critical processes amorphous sucrose is capable of absorbing water from the environment (Konar, 2013; Konar *et al*., 2017; Konar *et al*., 2018).

3.2.3 Sugar-free dark chocolate texture analysis: hardness and fracturability

Hardness and fracturability are two texture parameters that have an essential role in the sensory acceptability of chocolate. Results for hardness and fracturability of dark chocolate formulations are shown in Table 2. Hardness describes physical rigidity. Pol+Inu+Prob showed the nearest value to D.C. compared to the other sugar-free dark chocolates evaluated. On the other hand, Iso+Stev+Prob were the less rigid. Aidoo *et al*. (2015), studied Inu and Pol in dark chocolate as bulk agents and they found that Inu increased hardness in sugar-free chocolates (Aidoo *et al*., 2015). In the present study, Inu from chicory root with an average DP of 10 was used as an ingredient. This DP is low compared with other sources of inulin (i.e. agave) (Mueller *et al*., 2016), and allows inulin to emulate the functionality of oil, retaining less water and preventing significant texture changes in the final product. Likewise, the average DP of polydextrose is 12 (Aidoo *et al*., 2014), providing similar physicochemical characteristics as inulin, and thus their combination (Inu+Pol) did not affect hardness value of chocolate as compared with the control.

In terms of fracturability, values ranged between 3,360 N and 5,100 N. Significant differences (P <0.05) can be observed between the control (D.C.) and the treatment Iso+Stev presenting the higher value to reach fracture. This is explained due to the elasticity properties that Stev gives to the chocolate matrix (Palacio-Vasquez *et al*., 2017).

3.3 Consumers sensory acceptability of sugar-free dark chocolate formulations added with probiotics

The replacement of sugar by sweeteners decreased the overall acceptability of the product, whereas probiotics addition alone did not affect the overall acceptability (Table 3). The nearest value to overall acceptability from sugar-free formulations to D.C. were the Iso+Stev and Iso+Stev+Prob. For both treatments the % of consumers that liked the product was > 60%, whereas for Pol+Inu and Pol+Inu+Prob % of acceptability was 54.10% and 44%, respectively. The higher acceptability of chocolates added with Stev as compared with the Poli+Inu mixtures, could be attributed to the contribution of Stev on the perception of bitterness flavor.

Table 3. Sensory acceptability values of sugar-free dark chocolate formulations added with probiotics.

Parameter	D.C.	Pol+Inu	Iso+Stev	D.C.+Prob	Pol+Inu+Prob	Iso+Stev+Prob
Appearance	7.3 ± 0.11a	6.9 ± .16b	6.6 ± .19b	7.6 ± 0.12a	6.6 ± 0.14b	6.9 ± 0.13b
Flavor	7.6 ± 0.12a	4.9 ± 0.18c	6.2 ± 0.20b	7.8 ± 0.10a	4.2 ± 0.20d	6.1 ± 0.21b
Texture	7.8 ± 0.13a	5.8 ± 0.19b	5.7 ± 0.20b	7.8 ± 0.10a	5.7 ± 0.20b	6.0 ± 0.21b
Overall acceptability	7.5 ± 0.14a	5.2 ± 0.19c	5.9 ± 0.20b	7.7 ± 0.13a	4.7 ± 0.18c	6.0 ± 0.21b
% consumers that liked the product	95.3	54.1	64.4	94	44	61.2

Abbreviations: D.C. = Dark chocolate control, D.C.+Prob = Dark chocolate control + probiotics, Pol+ Inu = Polydextrose and inulin, Pol+Inu+Prob = Polydextrose and inulin + probiotics, Iso+Stev = Isomalt and stevia, Iso+Stev+Prob = Isomalt and stevia + probiotics

Values represent the mean obtained from 115 consumers with their standard error. Rows with different letters indicate statistically significant difference by LSD test ($P < 0.05$). The values shown for % of consumers that liked the product represents consumers that graded the product with overall acceptability ≥ 6.

A previous study on the development of sugar-free chocolates added with Stev reports that samples with lower cocoa butter content presented changes in bitterness and melting rate, concluding that the product was suitable for consumers that enjoy bitter chocolate and look for low-calories healthy products (Azevedo *et al*., 2017).

The main parameters that were affected in acceptability due to sugar replacement were the flavor and the texture. These lower values can be attributed to the bitter aftertaste of stevia (Lagast *et al*., 2017). Furthermore, another interesting factor that could explains the lower acceptability values obtained in chocolates added with Inu could be related with the increase in melting rate in the mouth induced by Inu (Shah *et al*., 2010). Moreover, the increase in the number of solid particles due to sweeteners addition in sugar-free chocolate, could be responsible of the lower texture scores (Shah *et al*., 2010).

Conclusions

The results presented herein show that the substitution of sugar by sweeteners modified color, water activity, texture, and consumer acceptability of chocolate. The formulation that showed the nearest value of overall acceptability as compared with the control was the dark chocolate added with Iso+Stev with and without Prob. The chocolate obtained by this formulation represents a feasible product to be introduced in the sugar-free food product market, which is highly increasing in the last years. In addition, probiotics added in the formulation could aid in the prevention and treatment of diabetes. In this context, adding probiotics to chocolate did not affect the physicochemical properties and acceptability of dark chocolate, obtaining a product with $>1\times10^8$ CFU/g being more than the minimum count of probiotic bacteria needed obtain a beneficial health effect.

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Nomenclature

a_w = Water Activity
$C*$ = Chroma
CFU/g = Colony Forming Units per gram
D.C. = Dark chocolate control
D.C.+Prob = Dark chocolate control+probiotics
h = Hue angle
Iso+Stev = Isomalt and stevia
Iso+Stev+Prob = Isomalt and stevia+probiotics
Pol+Inu = Polydextrose and inulin
Pol+Inu+Prob = Polydextrose and inulin+probiotics
WI* = White Index

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