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Development of a potentially functional chocolate spread containing probiotics and structured triglycerides

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ABSTRACT

New functional hazelnut and chocolate spreads, containing the probiotic strain *Enterococcus faecium* CRL 183 and different concentrations of medium-long-medium (MLM)-type structured triacylglycerols (TAGs) were developed. TAGs did not alter the viability of the probiotic strain, during 180 days of storage at 20 °C. The formulations presented similar contents of proteins, lipids, and carbohydrates, water activity below 0.5, thermal stability and were microbiologically stable. The decrease of saturated fat had a negative effect on the rheological properties of the spreads, with reduction on viscosity, Casson yield value and texture parameters. However, only the spread with 75% substitution of vegetable fat by structured TAG showed severe reduction in rheological properties that affected the product spreadability. The use of probiotic strain and replacement of fat (50%) by MLM-type TAG resulted in a new functional chocolate spread with significant increment in medium-chain and unsaturated fatty acids.

1. Introduction

The consumer awareness and interest in healthier eating has driven the development of functional foods that promote health benefits and disease risk reduction (Prasanth Kumar, Jeyarani, & Gopala Krishna, 2016; Yüksel-Bilsel & Şahin-Yeşilçubuk, 2019).

Probiotics are known as: "live microorganisms that, when administered in appropriate doses, confer a benefit to the health of the host" (FAO/WHO, 2001; Hill et al., 2014). In order to exert their systemic effects, they need to survive the different processing steps, storage conditions and during the passage through the gastrointestinal tract, and colonize the intestine, even temporarily (Markowiak & Ślizewska, 2017; Monteagudo-Mera, Rastall, Gibson, Charalampopoulos, & Chatzifragkou, 2019).

The probiotic strain *Enterococcus faecium* CRL 183 presents beneficial health effects such as modulation of the lipid profile and intestinal microbiota (Bedani et al., 2011; Cavallini et al., 2011), reduced risk of developing colon (Sivieri et al., 2008) and breast cancer (Kinouchi et al., 2012) and improve of the symptoms of inflammatory bowel diseases (Celiberto, Bedani, Rossi, & Cavallini, 2015; Celiberto et al., 2017).

Chocolate is the generic name for the homogenous products obtained from a mixture of cocoa derivatives (*Theobroma cacao* L.), milk products, sugars and/or sweeteners and aditives. Cocoa solids content varies according with type of chocolate (at least 25% for milk chocolate and 35% for dark chocolate). Other ingredients, except flour, starch and other animal fats, can be added to form different chocolate products. Chocolate spreads are widely consumed in breakfast meals or by confectionery professionals, being prepared by mixing cocoa with solid fats (hydrogenated fats or with high levels of saturated fatty acids), nut paste, macadamia, cashew or hazelnut (Codex Alimentarius, 2003; Jeyarani, Banerjee, Ravi, & Krishna, 2015). The property of spreadable at room temperature is reached by an amount usually greater than 40% of fats added to the dry ingredients (Manzocco, Calligaris, Camerin, Pizzale, & Nicoli, 2014; Patel et al., 2014), which unfortunately gives the product

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undesirable nutritional characteristics.

Chocolate and chocolate-based products are suitable vehicles for probiotics, preserving the viability of different bacteria strains (Coman et al., 2012; Possemiers, Marzorati, Verstraete, & Van de Wiele, 2010), besides having beneficial health properties associated to natural antioxidants and sensory characteristics that attract the consumer (El-kalyoubi, Khallaf, Abdelrashid, & Mostafa, 2011). Such products have also been reformulated, reducing sugar and fat content to, attend the consumer's search for healthier products. (Konar, Toker, Oba, & Sagdic, 2016).

To attend the demanding of consumer market, the development of products with a more balanced lipid profile is required (Bouaziz et al., 2017; Manzocco et al., 2014). However, considering the importance of lipid concentration and source, the great technological challenge is to develop spreads with low contents of saturated fats and similar flavor, texture, and spreadability characteristics as the original products (Bouaziz et al., 2017; El-Hadad, Youssef, Abd El-Aal, & Abou-Gharbia, 2011; Jeyarani et al., 2015; Manzocco et al., 2014; Patel et al., 2014).

Enzymatic modification of vegetable oils aiming the synthesis of specific structured TAGs, allows the substitution of fatty acids in the TAG molecule (Abed et al., 2018; Kadivar, De Clercq, Mokbul, & Dewettinck, 2016; Xie & Hu, 2016; Yüksel-Bilsel & Şahin-Yeşilçubuk, 2019; Zhao et al., 2015), constituting an effective means of supplying fatty acids for therapeutic or nutritional purposes (Abed et al., 2018; Kadivar et al., 2016).

The structured TAG has new functional and nutritional properties that depend on the composition of fatty acids and their distribution in the glycerol molecule (Kadivar et al., 2016; Kavadia, Yadav, Odaneth, & Lali, 2018; Wang, Xia, Xu, Xie, & Duan, 2012) and the position of the fatty acids in TAG molecules (*sn*-1, *sn*-2, and *sn*-3) may have a significant impact on human metabolism (Nunes, Pires-Cabral, & Ferreira-Dias, 2011). The TAG with medium-chain fatty acids (6–12 carbon atoms) in the *sn*-1 and *sn*-3 positions, and long-chain in the internal position (MLM), offers benefits during digestion, since the medium-chain fatty acids (caproic, caprylic, capric and lauric acids) are hydrolyzed more quickly by the action of the regioselective pancreatic *sn*-1.3 lipase, and are transported directly to the liver, through the portal circulation (Kim & Akoh, 2015; Lee, Tang, & Lai, 2012; Willett, Martini, & Akoh, 2019). Furthermore, the consume of MLM-type structured TAG may also be associated with the modulation of the immune system (Kono, Fujii, Ishii, Hosomura, & Ogiku, 2010) and of the lipid profile (Sengupta & Ghosh, 2011) and reduced risk of developing atherosclerosis (Korrapati et al., 2018).

Plant seeds are recognized as a good source of vegetable oils with nutritional, technological, and pharmaceutical importance. Grape seeds are agricultural residues rich in antioxidants, such as vitamin E, polyphenols, and flavonoids and their use to produce TAGs could add economic value to the main residue of the wine industry (Hashemi et al., 2017; Maszewska et al., 2018; Sabir, Unver, & Kara, 2012). In addition, grape seed oil is rich in polyunsaturated fatty acids (PUFA), mainly linoleic acid (58–78%) (Chambre, Tociu, Stanescu, & Popescu, 2019; Codex Alimentarius, 2017; Da Porto & Natolino, 2017; Davidov-Pardo & McClements, 2015; Malićanin et al., 2014) being considered a promising raw material for producing structured TAGs by enzymatic acidolysis catalyzed by a specific enzyme 1,3, once it have linoleic acid in the *sn*-2 position of their molecule (Costa et al., 2018).

Seeking at diversifying the functional food market, this study aimed to develop and characterize chocolate hazelnut spreads with potential functional properties. For this, we chose to use the probiotic strain *E. faecium* CRL183, whose health benefits have been proven in different studies, and to partially replace the fat with MLM-type structured TAGs from grape seed oil, which has better nutritional properties and contributes to the utilization of food industry waste.

2. Materials and methods

2.1. Materials

Grape seed (*Vitis vinifera* L.) oil was provided by Distriol (São Paulo, Brazil), capric acid was obtained from Sigma Aldrich (São Paulo, Brazil) and commercially immobilized lipase Lipozyme RM IM® (*Rhizomucor miehei*) was provided by Novozymes A/S (Bagsvaerd, Denmark).

2.2. Methods

2.2.1. Probiotic inoculum

The probiotic inoculum was prepared by transferring 10% of the stock culture of *Enterococcus faecium* CRL 183 (Reference Center for Lactobacilos - CERELA - Argentina) to M17 medium (Himedia®, India), followed by incubation at 37 °C for 14–16 h. The inoculum was lyophilized and stored at room temperature.

2.2.2. MLM-type structured TAGs

Enzymatic acidolysis of MLM-type TAG was carried out in a packed bed reactor, operating in continuous mode, with grapeseed oil and capric acid (C10:0), using lipase, ratio 1:3 (grape oil: capric acid). The optimal conditions for this synthesis (molar ratio and temperature) were established in a previous work, using batch reactors (Bassan et al., 2019) and evaluated in associated packed bed reactors at 45 °C for 120h. Reaction progress was monitored by analyzing TAG and the maximum incorporation degree (ID) was 43.00 ± 5.87% at 54h (Cozentino et al., 2020).

2.2.3. Chocolate hazelnut spreads

The spreads were prepared at the pilot plant of Cereal Chocotec (Institute of Food Technology, Campinas, Brazil) using as ingredients: 35.42% crystal sugar (União, Brazil), 11.94% cocoa powder (Barry Callebaut, Brazil), 14.93% hazelnut paste (Carino, Brazil), 18.84% grape seed oil, 9.91% vegetable fat (Chocofill™ TC 50, Aarhus Karlshamn, Sweden - non-hydrogenated and non-trans fats, with triglyceride structure similar to that of cocoa butter), 8.46% skimmed milk powder (Itambé, Brazil), 0.30% soy lecithin (Solae, Brazil) and 0.20% polyglycerol polyricinoleate (PGPR, Danisco, Brazil). The spreads were processed in a ball mill, capacity 5kg (Caotech model CAO-B5, Netherlands) for 1 h at 50 °C. Fat, soy lecithin, and PGPR were added 10 min before completing this step. The lyophilized probiotics were incorporated to the spreads at the end of this stage, in sufficient quantity to reach 8 log CFU/g (colony forming units per gram). During the process, the granulometry monitoring was performed to assess the maximum particle size (mm) (Beckett, 2008).

Five spreads formulations were developed, two of which without (MLM)-type TAGs: SC – without structured TAGs and probiotics and SP - without structured TAGs and with probiotics. The other spreads formulations were prepared with partial replacement of conventional vegetable fat (9.91%) by structured TAGs and addition of the probiotic strain: SPT25 – 25% (2.48g/100g) of structured TAGs; SPT50 - 50% (4.96g/100g) of structured TAGs; SPT75 – 75% (7.43g/100g) of structured TAGs. The products were stored in airtight glass bottles at a temperature of 20 °C for 180 days.

2.3. Characterization of probiotic chocolates hazelnut spreads and evaluation of their stability

Proximate composition, water activity (Aw), fatty acid composition and rheological properties were evaluated in triplicate and color in quintuplicate. Microbiological parameters were monitored every 30 days during the 180 days of storage period.

2.3.1. Proximate composition and Aw

Moisture, ash, protein and lipid contents were analyzed according to

Association of Official Analytical Chemists, 2005. Total carbohydrate content was determined by difference: total carbohydrates (%) = 100% - (moisture %+ ash % + proteins % + lipids%). The caloric value was calculated using the equation: TCV (total caloric value - Kcal/100 g) = [proteins (g) x 4)] + [carbohydrates (g) x 4] + [lipids (g) x 9] (Association of Official Analytical Chemists, 2005). A_w was determined in each chocolate hazelnut spread using the Aqualab device (A_w43® – ETEC, São Paulo, Brazil).

2.3.2. Color analysis

Determined on the HunterLab® colorimeter (Konica Minolta CR-410, Osaka, Japan), using a D65 illuminant and 10° visual angle. Color images were converted into the CIELAB system and expressed as L*, a* and b* values. The angle hue (h°) = arctan (b*/a*) and chroma (C*) = $[(a^*)^2 + (b^*)^2]^{1/2}$ were calculated. The average values of five repeated measurements and standard deviations were calculated.

2.3.3. Rheological properties and texture

2.3.3.1. Plastic casson viscosity (ηca) and casson flow limit (τca). The spread samples were melted at 45 °C to determine the rheological properties using a programmable rheometer, (Brookfield RVDVIII, Stoughton, USA), with an adapter for small samples and a thermostatic bath at 40 ± 0.1 °C with cylindrical spindle (S15) (Chevalley, 1975). After the data collection, curves were constructed correlating the experimental results of strain rate and shear stress and the parameters were calculated: Casson's plastic viscosity and Casson's flow limit. Casson's parameters were determined by regression.

2.3.3.2. Texture analysis. Texture parameters of hardness, consistency, and adhesiveness were evaluated using a TA-XT plus Texture Analyzer (Stable Micro Systems, Surrey, England). A total of 12.50 g of spread samples were analyzed at a room temperature using an analytical conical probe. The samples were compressed at a speed of 0.5 mm/s and a depth of 10 mm. Five seconds after the first entry of the probe into the sample, a second compression was performed under the same conditions. The experimental data were recorded and processed with Exponent Lite® Software.

2.3.4. Fatty acid composition

The fatty acid composition was determined by gas chromatography, according to the Ce 2–66 method of the American Oil Chemist's Society (American Oil Chemists' Society, 2017), using a mix of fatty acid ester standards (FAMES mix, C4 to C25). The qualitative composition was determined by comparison of retention times with those of fatty acid standards.

2.3.5. Differential scanning calorimetry (DSC)

The crystallization and melting profiles were obtained on a DSC 1 equipment (Mettler Toledo, Gieben, Switzerland). The instrument was calibrated with an Indian standard (99.99% purity). The analysis was performed under a nitrogen atmosphere (flow of 50 mL min^{-1}). Aluminum crucibles (40 μL) were used as a sample holder and as a reference. Approximately 5.0 mg of the sample was used for analysis. The curves obtained by DSC were recorded at the rate of cooling and subsequent heating at 10 °C min^{-1} of the environment to −80 °C and subsequently from −80 to 150 °C.

2.3.6. Microbiological analysis

Samples of 25g of chocolate hazelnut spreads were homogenized with 225 mL of peptone water and serial micro dilutions were prepared to determine the viability of the probiotic strain and product microbiological safety.

E. faecium CRL 183 were enumerated on M17 agar (Himedia®, India) under aerobic incubation at 37 °C for 48 h. The colonies were counted and the results expressed as log CFU/g (Rossi et al., 2008).

For the determination of microbiological safety, concentration of Coliforms at 45 °C and *Staphylococcus aureus* were determinate by Petrifilm™ (3M Microbiology, USA); The detection of *Salmonella* spp. was done using HE (Hektoen Enteric Agar, Accumedia®, USA) and XLD (Xylose Lysine Desoxycholate Agar, Accumedia®, USA) and all samples were incubated at 37 °C for 48 h (Downes & Ito, 2001). Yeast and mold was analyzed on PDA (Potato Dextrose Agar, Himedia®, India) and incubated at 30 °C/120 h (Kornacki, Gurtler, & Stawick, 2015).

2.4. Statistical analysis

The data were submitted to analysis of variance (ANOVA) and Tukey's post-test. All the analyses were performed using the Minitab Statistical Analysis Software (Statistic Analysis Software, Pennsylvania, USA), with a level of significance of 5% ($p < 0.05$). The color results were analyzed by Welch's ANOVA and Games-Howell post-test.

3. Results and discussion

3.1. Nutritional composition and Aw

The spread formulations showed caloric values between 605.18 and 612.54 kcal/100g and protein, lipid, and carbohydrate contents close to the commercials and those reported in the literature for conventional and modified products, without differing from each other ($p > 0.05$) (Table 1) (Altan, Lavenson, McCarthy, & McCarthy, 2011; Amevor, Laryea, & Barimah, 2018; Food Standards Agency, 2014; TACO, 2011).

The chocolate spreads presented water activity values that ranged from 0.37 to 0.40 and moisture contents from 0.13 to 0.23%, without statistically differing from each other ($p > 0.05$) (Table 1). Almeida, Lannes (2017) found higher A_w values (0.65–0.89) and concluded that the partial substitution of fat for gelatin in chocolate spreads, led to an increase in the water phase, and, as a consequence, a greater water activity in the products.

The literature reports that water activity values < 0.70 are considered low (Gurtler, Doyle, & Kornacki, 2014) and in general, for chocolate products, the low water activity (usually between 0.40 and 0.50) is due to its composition (Konar, Özhan, Artık, & Poyrazoglu, 2014; Rossini, Noreña, & Brandelli, 2011). Besides, A_w is one of the main factors that contribute to the conservation of the product, and the viability of lyophilized microorganisms throughout the storage period, and a high A_w may reduce the viability of the probiotic culture (Vesterlund,

Table 1
Proximal composition of chocolate spreads.

Treatments					
	SC	SP	SPT25	SPT50	SPT75
Ashes (%)	2.08 ± 0.03 [a]	2.01 ± 0.02 [a]	2.02 ± 0.02 [a]	2.07 ± 0.06 [a]	2.01 ± 0.03 [a]
Proteins (%)	7.74 ± 0.25 [a]	7.28 ± 0.24 [a]	7.22 ± 0.55 [a]	7.80 ± 0.33 [a]	7.45 ± 0.06 [a]
Lipids (%)	43.91 ± 1.79 [a]	43.70 ± 1.48 [a]	42.83 ± 1.01 [a]	42.81 ± 1.03 [a]	44.25 ± 0.75 [a]
Moisture (%)	0.18 ± 0.09 [a]	0.13 ± 0.03 [a]	0.23 ± 0.07 [a]	0.13 ± 0.03 [a]	0.16 ± 0.09 [a]
Carbohydrates (%)	46.09 ± 2.11 [a]	46.88 ± 1.66 [a]	47.70 ± 1.49 [a]	47.19 ± 1.31 [a]	46.13 ± 0.74 [a]
kcal (kcal/100g)	610.54 ± 8.65[a]	609.95 ± 7.48[a]	605.18 ± 5.32[a]	605.24 ± 5.18[a]	612.54 ± 4.02 [a]
Water activity (A_w)	0.40 ± 0.02 [a]	0.40 ± 0.01 [a]	0.40 ± 0.01 [a]	0.37 ± 0.02 [a]	0.38 ± 0.02 [a]

Averages (±standard deviation) followed by different lower case letters on the same line differ statistically ($p \leq 0.05$). SC - without the addition of structured TAGs and probiotics; SP - without the addition of structured TAGs and with probiotic; SPT25 – 25% of structured TAGs and probiotics; SPT50 – 50% of structured TAGs and probiotics; SPT75 – 75% of structured TAGs and probiotics.

Salminen, & Salminen, 2012). The substitution of fat for structured TAG did not interfere in this parameter and all formulations can be classified as products with low water activity.

3.2. Color analysis

Table 2 shows low L* values (lightness) and positive values a* and b* reflecting brown color of the samples, characteristic of products made with chocolate and its derivatives (Afoakwa, Paterson, Fowler, & Vieira, 2008).

The control formulation (SC) was considered slightly clearer because it had the highest values of L*, differing statistically from each other's ($p < 0.05$). This result is indicative of fat or sugar bloom. Fat bloom results from the formation of fat crystals on the surface of chocolates, giving a whitish appearance with color changes and the development of non-uniform color patterns (Afoakwa, Paterson, Fowler, & Vieira, 2009; Briones & Aguilera, 2005). Sugar bloom can be caused either by the phase separation of TAGs within the cocoa butter matrix or by the deposition of water (air moisture) onto the chocolate, dissolving the sugar on the surface. So, when the water diffuses back into the air, the sugar recrystallizes on the surface of the chocolate, leaving a mottled appearance. Considering the spreads formulation, higher values of L* observed should be related to sugar bloom (Acan et al., 2021; Ghosh, Ziegler, & Anantheswaran, 2002).

The control formulation (SC) and the probiotic formulation performed with the highest level of substitution of the modified oil (SPT75) presented the highest a* values (1.59 ± 0.46 and 0.72 ± 0.08, respectively) without statistically differing from each other ($p > 0.05$), which indicates that these samples present a tendency to red color. The highest values of b*, indicating a more intense yellow color, were also observed in the SC and SPT75 samples (1.54 ± 0.26 and 0.68 ± 0.08, respectively) and could be related to the fat bloom or sugar bloom phenomenon. Son et al. (2018) also observed an increase in the value of a* and b* in all formulations as the sugar bloom proceeded in chocolates containing maltitol and tagatose.

The chroma C* values were influenced by the presence of probiotic and modified oil in the samples, causing a reduction in color intensity, except for the SPT75 sample, which presented similar behavior to the control (SC) ($p > 0.05$).

Hue angle values h° ranged from 54.35 ± 6.46 to 41.40 ± 1.48 being within the red to yellow quadrant, and the formulations SC, SP, and SPT75 presented the lowest h° values, without statistically differing from each other.

In general, as a complex matrix, the chocolates differ in structural and particle size distribution arrangements, influencing in the light scattering coefficients and appearance (Afoakwa et al., 2008; Almeida & Lannes, 2017; Glicerina et al., 2014; Hadnađev et al., 2014). The samples with replacement of 25% and 50% of structured TAG exhibited better behavior, indicating the absence of fat or sugar bloom in these treatments. In a densely compacted medium, the crystalline network of cocoa fat disperses light, reducing the luminance and saturation indexes in products with a higher fat content (Afoakwa et al., 2008).

3.3. Rheological properties and texture

The monitoring of the rheological parameters of chocolate is important, as they are affected by its processing (shelling time, refinement, tempering and temperature) and composition (fat and fatty acid content, type of emulsifier, and particle size distribution). Furthermore, the addition of probiotic cultures also influences the microstructure of the product, as a new processing stage is introduced (Laličić-Petronijević et al., 2015).

Table 3 shows how the addition of probiotics and structured TAGs influenced the rheological parameters of chocolate spreads. The SPT75, with the highest level of structured TAGs, showed an almost fluid behavior making instrumental analysis of its rheological and texture parameters impossible. Spreadable foods are elastoplastic materials that have the ability to spread easily and therefore flow when deformed, but they should not flow like liquid (Di Monaco, Giancone, Cavella, & Masi, 2008). An alternative for the samples that did not show ideal behavior for a spread would be to use them in confectionery fillings.

All formulations presented a Casson's plastic viscosity significantly lower than the control spread (SC) ($p < 0.05$). The addition of the probiotic strain resulted in a product with similar viscosity to that of spreads produced with structured TAG and the proportion of modified oil added had no significant effect on this parameter ($p > 0.05$).

It can be noted that the values of the Casson flow limit parameter, which represents the minimum strength required for the chocolate to start flowing (Aidoo, Afoakwa, & Dewettinck, 2015), decreased progressively with increasing structured TAG content. The Casson's flow limit was significantly higher for the SC (5.35 ± 0.14 Pa), and the other spreads recorded averages between 4.09 ± 0.23 Pa and 1.31 ± 0.12 Pa ($p < 0.05$).

According to Żyżelewicz, Nebesny, Motyl, and Libudzisz (2010) viscosity of dark chocolate masses for the manufacture of chocolate bars should be around 1.0–2.0 Pa s (Agibert & Lannes, 2018; Kadivar et al., 2016). However, topping type chocolates assume lower values of Casson's viscosity and Casson's flow limit compared to other types of

Table 2
Color parameters of the different spread treatments.

Color parameters					
	L*	a*	b*	C*	h°
SC	1.83 ± 0.35 [a]	1.59 ± 0.46 [a]	1.54 ± 0.26 [a]	2.09 ± 0.73 [a]	41.40 ± 1.48[b]
SP	0.17 ± 0.02 [c]	0.20 ± 0.05 [b]	0.22 ± 0.02 [c]	0.30 ± 0.04 [b]	47.51 ± 7.76 [a,b]
SPT25	0.37 ± 0.33 [b,c]	0.18 ± 0.04 [b]	0.19 ± 0.01 [c]	0.25 ± 0.02 [b]	49.66 ± 2.40 [a]
SPT50	0.19 ± 0.05 [c]	0.17 ± 0.04 [b]	0.24 ± 0.04 [c]	0.29 ± 0.05 [b]	54.35 ± 6.46 [a]
SPT75	0.97 ± 0.14 [b]	0.72 ± 0.08 [a]	0.68 ± 0.08 [b]	0.99 ± 0.11 [a]	43.40 ± 1.65 [a,b]

L*: light; a*: red/green; b*: yellow/green; C*: chroma; h°: hue angle.
Averages (±standard deviation) followed by different lower case letters in the same column differ statistically ($p < 0.05$) ($n = 5$).
SC - without the addition of structured TAGs and probiotics; SP - without the addition of structured TAGs and with probiotic; SPT25 – 25% of structured TAGs and probiotics; SPT50 – 50% of structured TAGs and probiotics; SPT75 – 75% of structured TAGs and probiotics.

Table 3
Physico-chemical characteristics of chocolate spreads.

Parameters	SC	SP	SPT25	SPT50	SPT75
Rheology					
Viscosity η_{Ca} (Pa.s)[a]	1.45 ± 0.04[a]	0.89 ± 0.05[b,c]	0.85 ± 0.02[c]	0.94 ± 0.01[b]	nd
Flow limit τ_{Ca} (Pa)[a]	5.35 ± 0.14[a]	4.09 ± 0.23[b]	2.54 ± 0.08[c]	1.31 ± 0.12[d]	nd
R^2	1.00	0.99	1.00	1.00	nd
Texture					
Hardness (g)[a]	154.70 ± 40.13 [a]	128.20 ± 27.04 [a]	55.61 ± 11.83 [b]	42.17 ± 16.04 [b]	nd
Consistency (g.sec)[a]	2484.42 ± 792.87 [a]	2058.61 ± 223.51 [a,b]	913.00 ± 309.57[b,c]	502.94 ± 125.05 [c]	nd
Adhesion (g)[a]	31.37 ± 3.25 [a]	25.04 ± 6.02 [a]	3.90 ± 1.16 [b]	3.95 ± 2.48 [b]	nd

[a] Average (±standard deviation) followed by different lower case letters on the same line differ statistically ($p < 0.05$). SC - without the addition of structured TAGs and probiotics; SP - without the addition of structured TAGs and with probiotic; SPT25 – 25% of structured TAGs and probiotics; SPT50 – 50% of structured TAGs and probiotics; SPT75 – 75% of structured TAGs and probiotics. Nd = not determined.

chocolates (Beckett, 2008; Chevalley, 1975).

Hazelnut and chocolate spreads were produced in ball mills, and the processing time and rotation speed used may have affected the maximum particle size and morphological properties, altering their flow behavior (Acan et al., 2021). Konar et al. (2018) reported that the addition of inulin (DP < 10) and *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*) to white chocolates (sucrose made) also reduced its rheological attributes, such as plastic viscosity and yield value. This effect was associated with the presence of smaller particles in the product, with more surface area, which will be covered with fat, facilitating the flow, and reducing the viscosity of the chocolate.

The determination coefficients (R^2) obtained ranged from 0.99 to 1.00, indicating a good fit of the model to the experimental data and confirming the adequate rheological behavior in the chocolate spreads.

According to the results presented in Table 3, the spread without structured TAG and probiotic (SC) had significantly higher values for all texture parameters (hardness, consistency, and adhesion) ($p < 0.05$) with similar behavior as the probiotic spread (SP). These results are in agreement with those obtained from the rheological analysis, and confirm that these samples are firmer and more viscous. As the proportion of structured TAGs increased, the spreads became more spreadable and with lower levels of hardness and adhesion compared to SC and SP, without significantly differing from each other ($p < 0.05$). It is noteworthy that the sample with probiotic (SP) had a consistency and viscosity behavior like that of the sample with 25% (SPT25) of structured TAGs. Similar results were reported by Mayfield et al. (2015) who concluded that chocolate spreads rich in conjugated linoleic acid had significantly less hardness than control chocolate samples (with higher solid fat content).

Some vegetable fats, with triglyceride composition similar to cocoa butter, can be added in any amount to chocolate without causing a significant effect on texture (Afoakwa, Paterson, & Fowler, 2007). However, when liquid oil is used in the production of chocolate spreads, their physical stability can decrease significantly, leading to oil release and a tendency for phase separation during storage (Doan et al., 2016; Fayaz et al., 2017; Manzocco et al., 2014). Thus, the commercial fat (CHOCOFILL™ TC) and the structured TAG composition may explain the reduction in the rheological properties and texture of the spreads SPT25, SPT50 and SPT75. CHOCOFILL™ TC has a triglyceride composition like cocoa butter (most of which are saturated fat acids - palmitic and stearic acids), while structured TAG is composed by a mixture of capric acids in the *sn*-1 and *sn*-3 positions, and long-chain unsaturated fatty acid in the internal position (MLM). The molecular structure makes the saturated fat solid and unsaturated oil liquid at the room temperature, explained the lower viscosity, flow limit and texture parameters of spreads with substitution of CHOCOFILL™ TC by MLM-type structured TAGs. Furthermore, the compatibility (ability of the TAG of two distinct fats to crystallize together) between the lipids used in the formulations is also essential for good crystallization. Partial replacement of commercial fat with structured TAGs may have resulted in a mixture with lower chemical compatibility influencing the crystallization process (Fayaz et al., 2017).

3.4. Fatty acid composition

The fatty acid profiles of the chocolate spreads are presented in Table 4. The spreads presented abundant levels of mono and polyunsaturated fatty acids, with a greater average amount of oleic (C18:1; 38.11%) and linoleic (C18:2; 37.00%) acids, followed by saturated fatty acids, palmitic (C16:0), which ranged from 11.76% to 14.58% and stearic (C18:0; 5.47%) acids. As expected, the addition of MLM-type TAG resulted in an increase in the content of medium-chain fatty acid, specifically capric acid, as a result of the partial replacement of fat in spreads.

The data also showed that the replacement of vegetable fat by 50% (SPT50) and 75% (SPT75) of MLM-type TAG resulted in higher capric acid levels ($p < 0.05$) and an increase in polyunsaturated fatty acids (PUFA) content compared to SC and SP formulations.

Table 4
Fatty acid composition (%) of chocolate spreads.

Fatty acids	Treatments				
	SC	SP	SPT25	SPT50	SPT75
(C10:0) capric	nd	nd	1.08 ± 0.02^{c}	1.61 ± 0.11^{b}	1.91 ± 0.01^{a}
(C16:0) palmitic	14.58 ± 1.75 ^{a}	12.92 ± 0.45 ^{a}	12.67 ± 0.02 ^{a}	12.24 ± 0.15 ^{a}	11.76 ± 0.40 ^{a}
(C18:0) stearic	5.58 ± 0.27 ^{b}	5.39 ± 0.03 ^{b,c}	6.21 ± 0.08 ^{a}	5.25 ± 0.06 ^{b,c}	4.92 ± 0.01 ^{c}
(C18:1) oleic	38.87 ± 1.08 ^{a}	38.51 ± 0.53 ^{a,b}	39.60 ± 0.45 ^{a}	37.29 ± 0.33 ^{a,b}	36.28 ± 0.08 ^{b}
(C18:2) linoleic	35.48 ± 0.96 ^{b,c}	37.30 ± 0.40 ^{a,b}	34.95 ± 0.47 ^{c}	38.17 ± 0.40 ^{a}	39.36 ± 0.20 ^{a}
(C18:3) linolenic	2.85 ± 0.03 ^{c}	3.09 ± 0.03 ^{b}	2.88 ± 0.04^{c}	3.13 ± 0.02^{b}	3.50 ± 0.01^{a}
(C20:0) arachidic	1.14 ± 0.77 ^{a}	0.84 ± 0.24 ^{a}	0.84 ± 0.42 ^{a}	0.75 ± 0.36 ^{a}	0.75 ± 0.28 ^{a}
(C20:1) gadoleic	0.50 ± 0.26 ^{a}	0.60 ± 0.04 ^{a}	0.50 ± 0.25 ^{a}	0.49 ± 0.24 ^{a}	0.57 ± 0.15 ^{a}
(C22:0) Behenic	1.01 ± 0.45 ^{a}	1.34 ± 0.11 ^{a}	1.28 ± 0.26 ^{a}	1.07 ± 0.21 ^{a}	0.94 ± 0.23 ^{a}
Σ saturated fatty acids	22.31	20.49	22.08	20.92	20.28
Σ unsaturated fatty acids	77.70	79.50	77.93	79.08	79.71
MUFA	39.37	39.11	40.10	37.78	36.85
PUFA	38.33	40.39	37.83	41.30	42.86

Averages (±standard deviation) followed by different lower case letters on the same line differ statistically ($p \leq 0.05$).
SC - without the addition of structured TAGs and probiotics; SP - without the addition of structured TAGs and with probiotic; SPT25 – 25% of structured TAGs and probiotics; SPT50 – 50% of structured TAGs and probiotics; SPT75 – 75% of structured TAGs and probiotics. nd not detected.
Monounsaturated fatty acids (MUFA).
Polyunsaturated fatty acids (PUFA).

3.5. Thermal behavior of chocolate spreads and grape oil

The melting profiles obtained by the grape oil DSC before and after the enzymatic acidolysis reaction and the spread formulations are shown in Figs. 1 and 2, respectively.

The melting profile of grape oil showed three events, two endotherms referring to the melting, is possible to note the presence of the main event from −27.41 °C (Fig. 1). As expected, after the acidolysis

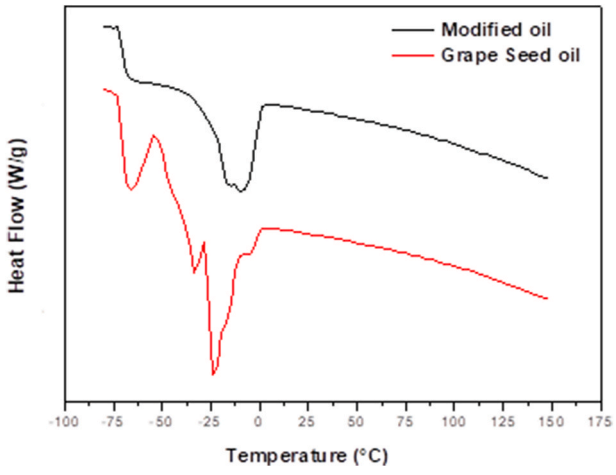

Fig. 1. DSC curves: (a) grape oil melting profiles before and after the enzymatic acidolysis reaction in a packed bed reactor (oil/capric: 1:3, p/p, 45 °C).

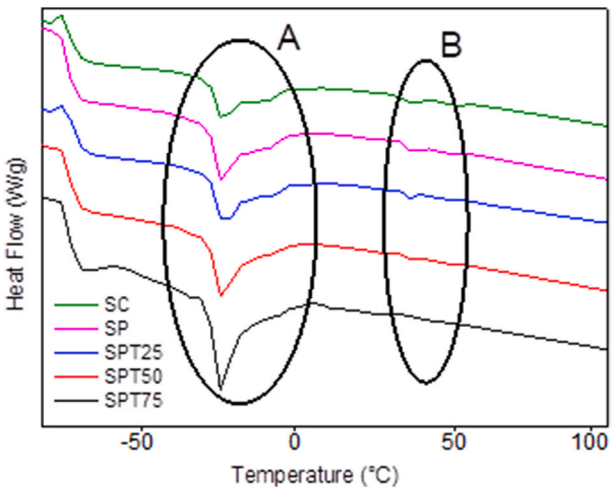

Fig. 2. DSC curves: (a) melting profiles of chocolate spread with the addition of structured TAG in different proportions: SC - without the addition of structured TAGs and probiotics; SP - without the addition of structured TAGs and with probiotics; SPT25 – 25% of structured TAGs and probiotics; SPT50 – 50% of structured TAGs and probiotics; SPT75 – 75% of structured TAGs and probiotics.

reaction, there was a variation in the melting behaviors compared to the starting product due mainly to the change in the composition of the fatty acids and their positional distribution in the skeleton of the glycerol molecule (Abed et al., 2018).

The structured TAG showed only one melting peak at a lower temperature −40.37 °C that may be attributed to the new formation of triglyceride, consisting of medium-chain fatty acids (capric acid) that present a lower melting temperature range due to the lower number of carbon atoms compared to the triglycerides containing only long-chain fatty acids (Abed et al., 2018; Kavadia et al., 2018; Álvarez & Akoh, 2016). Besides, the first melting events were not observed in the curve obtained by DSC of the structured TAG, confirming that significant changes in the physical-chemical properties of grape oil were observed due to the altered composition of triglycerides and fatty acids (Fig. 1).

The fusion phenomenon depends on the disruption of the non-covalent bonds of the crystalline structure, mainly of hydrophobic bonds, indicating a new three-dimensional arrangement after the modification (Himawan, Starov, & Stapley, 2006). The modified oil, due to its smaller chains, reduces the number of hydrophobic interactions that the material can make, leading to lower melting points than its analog (Table 5).

Chocolate is a complex matrix of semi-solid suspension of sugar and cocoa particles surrounded by cocoa butter, being fat representing about 70% of the total and its nature significantly determines the properties of the final product. Therefore, the lipid phase is considered responsible for thermal stability, sandy feeling in the mouth, the release of flavor, and overall consumer satisfaction (Ostrowska-Ligęza et al., 2018). Its melting profile is known to have a high and narrow melting range, with rapid melting at body temperature, which causes the release of the flavor (Kadivar et al., 2016).

The DSC was also used to characterize changes in the melting profiles of the chocolate spread added by probiotics and structured TAGs in different proportions (Fig. 2).

All the spread samples showed endothermic transitions with one melting peak and two temperature ranges between −27.24 and 34.02 °C, suggesting that, regardless of the modified oil content used in the formulations, they exhibited similar energy to complete the melt with the same thermal stability. The only exception is the SPT75 sample, which presented only one broader melting peak at a lower temperature of −27.75 °C, not being possible to observe the second melting event on the curve obtained by DSC, since all the fat is incorporated into the spread. This may be explained by the greater amount of modified grape oil found in this sample, suggesting that they required less time to melt than the similar spreads with higher fat content.

The enthalpy values of the melting event (shown as “A” in Fig. 2) demonstrate that the heat released by the samples up to 50% (SPT50) of structured TAG present close values, suggesting that there were no major changes in the spread matrix due to the modified oil to incorporate well in the solid dispersion. In the SPT75 formulation, there is a considerable increase, from that point on the modified oil starts to change the characteristics of the solid spread dispersion, which favors the incorporation of fat in the dispersed phase.

It is observed that the second thermal event (shown as “B” in Fig. 2) has its enthalpy reduced with the addition of the structured TAG, suggesting changes in the characteristics and physicochemical properties of the chocolate spread (Table 6). As seen in the SC formulation, event (B) is due to the solid lipid content, which is in crystalline form and is capable of melting. With the addition of the structured TAG, there is a reduction in the crystalline fat rate, suggesting that it was incorporated into the spread matrix by the fat dispersion, being then incorporated into the lipid phase.

3.6. Microbiological analysis

3.6.1. Microbiological safety

According to the results, the populations of coliforms and *Salmonella* spp. were absent in all the formulations throughout the experimental period, and molds/yeasts and *Staphylococcus aureus* were below the detection limit (<1.0 CFU/g). These results confirm that the chocolate spreads were processed properly and remained stable and safe for at least six months.

The microbiological safety of chocolate spreads is mainly due to the good manufacturing practices adopted during their production, the high concentrations of sugars and fats, the low water activity found in the products and the correct maintenance of this parameter throughout the storage period (Żyżelewicz et al., 2010).

Table 6
Melting onset temperature (T_{onset}), melting peak temperature (T_{peak}), melting enthalpy (ΔH) for chocolate spreads.

Treatments	T_{onset} 1 (°C)	T_{peak}1 (°C)	T_{onset} 2 (°C)	T_{peak}2 (°C)	ΔH 1 (J/g)	ΔH 2 (J/g)
SC	−27.14	−21.71	33.13	38.29	20.68	1.90
SP	−27.23	−22.06	34.34	38.10	26.53	1.80
SPT25	−27.18	−21.55	34.50	36.95	24.89	1.16
SPT50	−27.42	−22.23	34.14	36.27	25.78	1.06
SPT75	−27.75	−22.41	–	–	32.40	–

SC - without the addition of structured TAGs and probiotics; SP - without the addition of structured TAGs and with probiotic; SPT25 – 25% of structured TAGs and probiotics; SPT50 – 50% of structured TAGs and probiotics; SPT75 – 75% of structured TAGs and probiotics.

Table 5
Melting onset temperature (T_{onset}), melting peak temperature (T_{peak}), melting enthalpy (ΔH), or grapeseed oil before and after the reaction.

	T_{onset}1 (°C)	T_{peak}1 (°C)	T_{onset}2 (°C)	T_{peak}2 (°C)	T_{onset} 3 (°C)	T_{peak}3 (°C)	ΔH 1 (J/g)	ΔH 2 (J/g)	ΔH 3 (J/g)
Grape oil	−62.80	−53.83	−38.51	−33.23	−27.41	−23.29	15.78	6.35	38.93
Structured TAG	−40.37	−9.08	–	–	–	–	68.20	–	–

3.6.2. Determination of the cell viability of the probiotic microorganism (E. faecium CRL 183) in spreads

As shown in Table 7, the *E. faecium* probiotic strain remained stable during 180 days of storage at 20 °C, without suffering a significant reduction in viability ($p > 0.05$), except for the SPT75 treatment, which showed a decrease of 0.84 log CFU/g regarding the initial count, without compromising the quality of the product, as the probiotic count remained high (above 8 log CFU/g). It is observed that, after 180 days of storage, no significant differences were found between the samples for this parameter ($p > 0.05$). These results are similar to those found in other studies showing that chocolate is a good matrix for incorporating probiotics, besides to having beneficial health properties and sensory characteristics that attract the consumer (Konar et al., 2016). Nebesny, Żyżelewicz, Motyl, and Libudzisz (2007) used lyophilized strains of *Lacticaseibacillus casei* (formerly *Lactobacillus casei)* and *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*) to produce probiotic chocolate and the product remained stable with the viability of 7.0 log CFU/g during 12 months of storage at 4 °C and 18 °C. Laličić-Petronijević et al. (2015) also observed that the probiotic strains of *Lactobacillus acidophilus* NCFM® incorporated into dark and milk chocolate showed high viability (greater than 7.0 log CFU/g) during 180 days of storage at 4 °C and 20 °C.

The chocolate lipid fraction protects the probiotic strain during production and storage, and from the harsh conditions of gastrointestinal tract during digestion (Aragon-Alegro, Alarcon Alegro, Roberta Cardarelli, Chih Chiu, & Isay Saad, 2007; Konar et al., 2018; Mandal, Hati, Puniya, Singh, & Singh, 2013; Mirković et al., 2018; Possemiers et al., 2010). The viability of the probiotic strain is influenced by other factors such as strain resistance, processing and storage conditions (Markowiak & Ślizewska, 2017). *E. faecium* CRL 183 is resistant to the adverse conditions of the gastrointestinal tract (Cozentino, Luccas, & Cavallini, 2016) and to the technological process for obtaining different products such as chocolate bars (Cozentino et al., 2016), fermented sausage (Roselino et al., 2017) and orodispersible films (Lordello et al., 2021). We emphasize that in the present study, the survival rate of the probiotic *E. faecium* CRL 183 in chocolate spreads was 100% after 180 days of storage at 20 °C, indicating an adaptation of the strain to the food matrix and processing and storage conditions.

4. Conclusion

In this study was developed a new potentially functional spread of chocolate and hazelnut with the addition of *E. faecium* CRL 183 probiotic strain and MLM-type structured TAGs. The addition of MLM-type TAG increased the content of capric acid (medium-chain fatty acid) and did not compromise the chemical composition, thermal stability, microbiological safety, and viability of the probiotic strain. Although some texture parameters of the samples were significantly affected by the incorporation of the structured lipid, the rheological parameters of SPT 25 and SPT 50 showed a good fit of the experimental data to the Casson model without compromising its structure. The sample with 75% replacement of vegetable fat with structured TAG showed a severe reduction in rheological properties, making it impossible to evaluate its texture parameters.

The incorporation of structured TAGs into chocolate spreads can be considered a promising strategy to improve its nutritional profile and diversify the functional food market. Spreads with substitution of 50% of a conventional vegetable fat by structured TAGs have the potential to act in the modulation of the immune system and the lipid profile, obesity control, improvement of the absorption of essential fatty acids, offering health benefits to consumers, due to the presence of the probiotic strain, structured TAGs, and higher content of PUFA. Further studies are needed to prove such effects.

Table 7
Cell viability (log CFU/g) of the probiotic microorganism (*E. faecium* CRL 183) in spreads during storage at 20 °C.

Storage time (days)	Treatments			
	SP	SPT25	SPT50	SPT75
T0	7.91 ± 0.19 aC	8.74 ± 0.13 aB	8.60 ± 0.11 aB	9.25 ± 0.07 aA
T30	8.55 ± 0.13 aA	8.63 ± 0.31 aA	8.62 ± 0.15 aA	8.49 ± 0.20 bA
T60	8.46 ± 0.15 aA	8.46 ± 0.15 aA	8.33 ± 0.35 aA	8.63 ± 0.31 bA
T90	8.40 ± 0.17 aA,B	8.68 ± 0.07 aA	8.57 ± 0.12 aA,B	8.26 ± 0.13 bB
T120	8.03 ± 0.08 aB	8.44 ± 0.18 aA	8.39 ± 0.14 aA	8.23 ± 0.05 bA,B
T150	8.42 ± 0.27 aA	8.72 ± 0.07 aA	8.39 ± 0.15 aA	8.54 ± 0.14 bA
T180	8.38 ± 0.43 aA	8.69 ± 0.09 aA	8.33 ± 0.35 aA	8.41 ± 0.17 bA

Averages (±standard deviation) followed by different lower case letters in the same column differ statistically ($p < 0.05$). Averages (±standard deviation) followed by different capital letters on the same line differ statistically ($p < 0.05$). SP - without the addition of structured TAGs and with probiotic; SPT25 – 25% of structured TAGs and probiotics; SPT50 – 50% of structured TAGs and probiotics; SPT75 – 75% of structured TAGs and probiotics.

CRediT authorship contribution statement

Izabela de Souza Correia Cozentino: Investigation, Visualization, Formal analysis, Data curation, Methodology, Writing – original draft, Writing – review & editing. **Ariela Veloso de Paula:** Supervision, Conceptualization, Methodology. **Clovis Augusto Ribeiro:** Methodology. **Jovan Duran Alonso:** Data curation, Methodology. **Renato Grimaldi:** Methodology. **Valdecir Luccas:** Methodology. **Maria Pia Taranto:** Supervision, Methodology. **Daniela Cardoso Umbelino Cavallini:** Supervision, Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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