



Semisweet chocolate as a vehicle for the probiotics *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1: Evaluation of chocolate stability and probiotic survival under *in vitro* simulated gastrointestinal conditions

Marluci P. Silva ^a, Fabricio L. Tulini ^a, Júlia F.U. Marinho ^a, Marcella C. Mazzocato ^a, Elaine C.P. De Martinis ^b, Valdecir Luccas ^c, Carmen S. Favaro-Trindade ^{a,*}

^a Departamento de Engenharia de Alimentos (ZEA), Faculdade de Zootecnia e Engenharia de Alimentos (FZEA), Universidade de São Paulo (USP), Pirassununga, São Paulo, Brazil

^b Departamento de Análises Clínicas, Toxicológicas e Bromatológicas (DACTB), Faculdade de Ciências Farmacêuticas de Ribeirão Preto (FCFRP), Universidade de São Paulo (USP), Ribeirão Preto, São Paulo, Brazil

^c Centro de Tecnologia de Cereais e Chocolates (CEREAL CHOCOTEC), Instituto de Tecnologia de Alimentos (ITAL), Campinas, São Paulo, Brazil

ARTICLE INFO

Article history:

Received 12 May 2016

Received in revised form

2 August 2016

Accepted 8 October 2016

Available online 11 October 2016

Keywords:

Viability

Cocoa

Phenolics

Sensorial acceptance

Hardness

ABSTRACT

Semisweet chocolate pleases a broad range of consumers, but it is an underexplored food matrix among probiotic products. Thus, this study aimed to evaluate semisweet chocolate as a vehicle for probiotics (*Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1). The chocolates were evaluated by a_w , pH, surface color and morphology, hardness, microbiological quality, sensorial acceptance and probiotic viability. Besides, probiotic survival in simulated gastric and intestinal fluids (SGF/SIF) was evaluated before and after application in chocolate. Samples presented pH values around 6 and a_w below 0.6. Free probiotics populations were reduced after exposure to SGF/SIF, while no significant reduction was detected in probiotic populations incorporated into chocolate. After 120 days of storage at 25 °C, probiotic populations in chocolate were reduced by only 1.4 and 0.7 logarithmic cycles, respectively. Considering these results and that all samples were very well accepted by panelists, semisweet chocolate can be considered a good vehicle for probiotics.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) define probiotics as “live microorganisms that when administered in adequate amounts confer health benefits to the host” (Food and Agriculture Organization & World Health Organization, 2002). However, probiotics have to survive to food processing and throughout the gastrointestinal tract, challenging the food industry to find new alternatives for incorporating these microorganisms in food. Several parameters such as high temperatures, pH variation and oxygen may affect probiotic survival (Favaro-Trindade, Heinemann, & Pedroso, 2011; Tripathi & Giri, 2014).

Probiotic functionality may be enhanced when these microorganisms are incorporated in food, since interaction with food ingredients can protect microbial cells during the passage through the gastrointestinal tract (Ranadheera, Baines, & Adams, 2010; Vinderola, Binetti, Burns, & Reinheimer, 2011). In this context, dairy products are considered a good vehicle for probiotics, and they have been widely used by the food industry. However, part of the world population do not consume those products due to lactose intolerance, milk allergy or even due to diets that restrict the use of animal protein. To overcome this problem, semisweet chocolate may be an alternative to dairy products. Furthermore, chocolate has other beneficial properties such as recognized antioxidant activity (Gadhiya, Patel, & Prajapati, 2015). Moreover, phenolic compounds in chocolate may also play an important role in delaying oxidative stress in probiotics, which is one of the main causes of probiotic death in food, leading to improved viability and extended shelf life (Bialonska et al., 2010; Curriel, Munoz, & Lopez de Felipe, 2010; Pereira, Almeida, de Jesus, da Costa, & Rodrigues, 2013). According to Gadkari and Balaraman (2015), chocolate is a potential source

* Corresponding author. Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Avenida Duque de Caxias Norte, 225, 13635-000 Pirassununga, São Paulo, Brazil.

E-mail address: carmenft@usp.br (C.S. Favaro-Trindade).

of catechins, epicatechins and type B procyanidins, which may contribute to the antiplatelet effects of this product.

Chocolate is a suspension of particles, mainly sugar and cocoa, in the lipid matrix of cocoa butter. One of the main properties that makes chocolate very pleasant to consumers is its ability to melt in the mouth, despite being a solid product during storage (Luccas, Bonomi, & Kieckbusch, 2014).

The preparation of chocolate involves several steps that may have deleterious effects on probiotic viability. Thus, the incorporation of probiotics into chocolate is performed after the tempering step to avoid any deleterious effect of temperature on bacterial cells. Chocolate storage is another critical point, since semisweet chocolates are stored at room temperature for up to 6 months, depending on the type of packaging and processing, which may interfere with probiotic viability in the product.

According to Lahtinen, Ouwehand, Salminen, Forsell, and Myllarinen (2007) and Pedroso, Dogenski, Thomazini, Heinemann, and Fávoro-Trindade (2013), cocoa butter can protect probiotic microorganisms. Furthermore, the lipid matrix probably protects bacterial cells from water and H⁺ ions. Other studies have also verified the potential of milk and dark chocolate, under refrigeration temperature, for incorporating probiotics (Foong, Lee, Ramli, Tan, & Ayob, 2013; Lalicic-Petronijevic et al., 2015; Mandal, Hati, Puniya, Singh, & Singh, 2013; Nebesny, Zyzelewicz, Motyl, & Libudzisz, 2007; Possemiers, Marzorati, Verstraete, & Vand de Wiele, 2010). However, until 2015, no data was published about probiotic viability in semisweet chocolates stored at room temperature.

Chocolates containing probiotics may be potential candidates for new functional foods due to the combined health benefits of probiotics and chocolate phenolic compounds. Thus, the aim of the present study was to incorporate the probiotics *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1, into semisweet chocolate. Moreover, the influence of probiotics on chocolate quality, the influence of chocolate on probiotic viability during storage and survival under *in vitro* simulated gastrointestinal conditions were also evaluated.

2. Material and methods

2.1. Materials

L. acidophilus LA3 and *B. animalis* subsp. *lactis* BLC1, in freeze-dried form, were kindly provided by Sacco Brasil (Campinas, Brazil).

Aside from probiotics, for semisweet chocolate preparation, the following materials were used: sugar (União, Brazil), cocoa liquor (Barry Callebaut, Brazil), cocoa butter (Barry Callebaut, Brazil), soy lecithin (Bunge, Brazil) and polyglycerol polyricinoleate (PGPR, Danisco, Brazil).

2.2. Production of probiotic chocolate

2.2.1. Probiotic inoculum

The probiotic inoculums were prepared as described by Okuro, Thomazini, Balieiro, Liberal, and Fávoro-Trindade (2013), with modifications. LA3 and BLC1 were cultivated three times in MRS broth for 18 h at 37 °C. Cells were harvested by centrifugation (5752×g for 10 min at 10 °C), washed with 2% sodium citrate and adjusted to ca. 10¹⁰ cfu/ml with the same solution by measuring the absorbance at 600 nm (correlated with agar plate counts). Next, the bacterial suspension was centrifuged (5752×g for 10 min at 10 °C) to remove the sodium citrate solution, and the cell pellet (containing 10¹⁰ cfu/g) was used to prepare the semisweet chocolate.

2.2.2. Semisweet chocolate preparation

The chocolate was prepared at the Cereal Chocotec pilot plant (Instituto de Tecnologia de Alimentos, Campinas, Brazil) using a solution of 47% sugar (w/w), 43% cocoa liquor, 10% cocoa butter, 0.3% soy lecithin and 0.2% PGPR. The cocoa liquor and sugar were mixed, and the mass was refined using a roller mill (Draiswerk, GMBH, Germany). After this step, samples were collected to evaluate maximum particle size (µm), as described by Luccas and Kieckbusch (2006). This control is essential because chocolates with large particles may cause a gritty sensation in the mouth. Next, the mixture was submitted to a conching process in a jacketed mixer (INCO, Germany) for 16 h at 65 °C. The cocoa butter and PGPR were added, respectively, 2 and 1 h before ending the conching step. The tempering of chocolate was performed manually using spatulas in a room with controlled temperature. During this step, the tempering index was constantly checked using the tempering index device, and the tempering process was repeated if index values were not within the range of 4–6.

Probiotic cells were prepared as described in Section 2.2.1 and added to chocolate after the tempering step at a ratio of 10¹⁰ cfu for each 100 g, resulting in a product with 10⁸ cfu/g. At this step, three formulations of chocolate were prepared: (i) LA3, containing only *L. acidophilus* LA3; (ii) BLC1, containing only *B. animalis* subsp. *lactis* BLC1; (iii) control, without probiotics. The pre-crystallized chocolate was then poured into polyethylene molds and placed in a cooling tunnel adjusted to 10 °C for 30 min. The chocolate bars were manually demolded, wrapped in aluminum foil and kept at 20 °C for 15 days to stabilize the crystal lattice. After this step, the chocolate bars were kept at 25 °C for up to 120 days.

2.3. Characterization of probiotic chocolates and evaluation of their stability

2.3.1. Physicochemical characterization

2.3.1.1. Water activity (a_w) and pH. Chocolate bars were finely divided and analyzed using an Aqualab device (Decagon Devices, USA) to measure a_w, and expressed as values from zero to one (maximum a_w). To evaluate final chocolate pH, 10 g of chocolate bars were finely divided, stirred for 20 min in 100 ml of deionized water and analyzed using a potentiometer. These analyses were performed monthly for 120 days.

2.3.1.2. Determination of total phenolic and fat contents. The preparation of defatted chocolate samples was performed as described by Adamson et al. (1999) and Alañón, Castle, Siswanto, Cifuentes-Gómez, and Spencer (2016). Initially, 1 g of chocolate was defatted using 10 ml of *n*-hexane, and the mixture was homogenized by agitation and sonication for 5 min, followed by a centrifugation step at 2465×g for 5 min. This procedure was performed twice and the samples were air-dried to remove the residual of *n*-hexane. Following, to extract the phenolic compounds, 1 g of defatted chocolate was added into 10 ml of ethanol aqueous solution (80% v/v) and homogenized by sonication for 10 min, followed by centrifugation at 4108×g for 5 min. The extraction of phenolic compounds was performed twice. Next, total phenolic content was determined according to Singleton, Orthofer, and Lamuela-Raventos (1999) and Souza et al. (2014). For this, 0.25 ml of the chocolate extract was mixed with 2 ml of distilled water and 0.25 ml of Folin–Ciocalteu reagent. After 3 min at room temperature, the mixture was added of 0.25 ml of saturated sodium carbonate solution (Na₂CO₃) and homogenized. The tubes were placed in a water bath at 37 °C for 30 min to complete the reaction and the absorbance was measured at 750 nm (Hach DR 2800, USA). The total content of phenolic compounds was determined using gallic acid as standard, and the results were expressed as mg of gallic acid

equivalents (GAE) per g of chocolate.

The fat content in chocolates was determined using the [Bligh and Dyer \(1959\)](#) method, which consists in the extraction of lipids with chloroform, methanol and water in a ratio of 1:2:0.8, respectively. The amount of lipids in the samples was determined by removing an aliquot of 5 ml from the chloroform fraction, followed by evaporation of the solvent in a stove at 100 °C for 40 min. The recipient was cooled in a desiccator under vacuum, and then the remaining material was weighed to determine the total fat content.

2.3.1.3. Surface color. The surface color of chocolate was analyzed using a colorimeter (HunterLab Model MiniScan XE, Reston, USA), which can be useful to evaluate the occurrence of the fat bloom phenomenon. The parameters “L”, “a” and “b” were measured and used to calculate the whiteness index (WI) with the [formula \(1\)](#) described by [Lohman and Hartel \(1994\)](#), in which higher values of WI indicate that the chocolate surface is more white than chocolates with lower WI values. This analysis was performed monthly for 120 days.

$$WI = 100 - \left[(100 - L)^2 + a^2 + b^2 \right]^{0.5} \quad (1)$$

2.3.1.4. Hardness. Chocolate hardness was evaluated using a texturometer (TA XT Plus Texture Analyzer, Extralab, Brazil), as described by [Afoakwa, Paterson, Fowler, and Veira \(2008\)](#), using samples prepared as pieces of 40 mm × 25 mm × 7 mm (L × W × H). This analysis was performed monthly for 120 days.

2.3.1.5. Surface morphology assessment by scanning electron microscopy (SEM). Scanning electron microscopy (SEM) was used to evaluate the chocolate surface, as described by [Afoakwa, Paterson, Fowler, and Veira \(2009\)](#) and [Luccas et al. \(2014\)](#). This analysis was performed on the first and last days of storage (120 days).

2.3.2. Microbiological analyses

2.3.2.1. Microbiological quality of chocolate. Enumeration of total and thermotolerant coliforms (*Escherichia coli*) and the detection of *Salmonella* spp. were carried out according to methods previously reported in the literature ([Andrews, Flowers, Silliker, & Bailey, 2001](#); [Kornacki & Johnson, 2001](#), pp. 69–82). This analysis was performed on the first day of storage.

2.3.2.2. Probiotic viability during chocolate storage. Probiotic bacteria may be affected during chocolate processing and storage, so the enumeration of these microorganisms during storage is essential. Five grams of each chocolate sample were diluted in 45 ml of 2% sodium citrate previously heated at 48 °C. After homogenization for 2 min in stomacher, aliquots of 1 ml were withdrawn, serially diluted and inoculated in MRS agar. Next, inoculated

plates were incubated for 48 h at 37 °C, and single colonies were enumerated. This analysis was performed monthly for 120 days.

2.3.2.3. Evaluation of probiotic survival under in vitro simulated gastrointestinal conditions. The incorporation of probiotics in chocolate may change their ability to survive in the gastrointestinal environment. Thus, *L. acidophilus* LA3 and *B. animalis* subsp. *lactis* BLC1 were evaluated with regard to survival under *in vitro* simulated gastrointestinal conditions, as described by [Gbassi, Vandamme, Ennahar, and Marchioni \(2009\)](#), with modifications. One milliliter of probiotic culture or 1 g of probiotic chocolate was added to 9 ml of simulated gastric fluid (SGF: NaCl 9 g/l, pepsin 3 g/l, pH 1.8). After 120 min of incubation at 37 °C under constant agitation (100 rpm), 10 ml of simulated intestinal fluid (SIF: NaCl 9 g/l, pancreatin 10 g/L, trypsin 10 g/L, bile salts 3 g/L, pH 6.5) were added to the samples and incubated for 180 min at 37 °C under constant agitation (100 rpm). Aliquots of 1 ml were withdrawn from the beginning (t = 0) until the end (t = 300 min) of the experiment, at intervals of 60 min, for bacterial enumeration, as previously described in Section 2.3.2.2. All bacterial enumerations were performed in triplicate, and the assay was performed on the first day of storage.

2.3.3. Sensorial acceptance

Probiotic chocolates were submitted to consumer acceptance testing after 20 days of storage, according to [Meilgaard, Civille, and Carr \(1999\)](#), with 100 untrained panelists who evaluated the attributes of taste, aroma, texture and overall acceptance. This sensory evaluation was accomplished in the laboratory using individual booths and under fluorescent light. The samples were placed in plastic plates coded with three-digit random numbers, and served one at a time. Panelists were instructed to rinse the palate after each sample. A 9-point structured hedonic scale was used, with “1” as “dislike extremely” and “9” as “like extremely.”

2.4. Statistical analyses

All experiments were performed as independent triplicates, and the results were evaluated by analysis of variance (ANOVA) followed by Tukey's post-test (95% confidence interval), using the SAS v9.1.3 program (Statistic Analysis Software, SAS Institute Inc., USA).

3. Results and discussion

In the present study, it was evaluated the potential of semisweet chocolate as a new matrix to incorporate probiotics. *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1 were chosen to be applied in semisweet chocolate because they are commercial strains, which means that they were previously studied with regard the beneficial properties and safety. Moreover, both species have been extensively studied by other authors that reported the health benefits and safety of several strains ([Parvez, Malik, Kang, & Kim, 2006](#); [Salminen et al., 1998](#)). In addition,

Table 1

Water activity (a_w) of chocolates stored at 25 °C for up to 120 days (mean ± standard deviation).

Formulation	Days				
	0	30	60	90	120
Control	0.36 ± 0.05 ^{bB}	0.40 ± 0.02 ^{aAB}	0.38 ± 0.01 ^{bB}	0.37 ± 0.01 ^{bB}	0.44 ± 0.03 ^{bA}
LA3	0.51 ± 0.00 ^{aA}	0.44 ± 0.02 ^{aB}	0.43 ± 0.01 ^{aB}	0.41 ± 0.00 ^{bB}	0.52 ± 0.01 ^{aA}
BLC1	0.40 ± 0.03 ^{bC}	0.44 ± 0.03 ^{aBC}	0.43 ± 0.02 ^{aBC}	0.45 ± 0.01 ^{aB}	0.56 ± 0.00 ^{aA}

LA3 chocolate was produced with *Lactobacillus acidophilus* LA3 and BLC1 chocolate was produced with *Bifidobacterium animalis* subsp. *lactis* BLC1. Control chocolate was produced without probiotics.

Values with the same upper case letter in a row and values with the same lower case letter in a column are not statistically different ($p > 0.05$).

Table 2

Values of pH for chocolates stored at 25 °C for up to 120 days (mean ± standard deviation).

Formulation	Days				
	0	30	60	90	120
Control	5.70 ± 0.04	5.74 ± 0.04	5.76 ± 0.02	5.71 ± 0.05	5.75 ± 0.05
LA3	5.66 ± 0.06	5.74 ± 0.03	5.73 ± 0.02	5.74 ± 0.05	5.73 ± 0.04
BLC1	5.72 ± 0.03	5.82 ± 0.02	5.75 ± 0.02	5.72 ± 0.02	5.78 ± 0.03

LA3 chocolate was produced with *Lactobacillus acidophilus* LA3 and BLC1 chocolate was produced with *Bifidobacterium animalis* subsp. *lactis* BLC1. Control chocolate was produced without probiotics.

Values in rows and columns are not statistically different ($p > 0.05$).

these commercial strains have different energetic metabolisms, since *L. acidophilus* LA3 is a microaerophilic microorganism, and *B. animalis* subsp. *lactis* is an anaerobic microorganism, which is interesting to investigate and compare their application in chocolate. The probiotic chocolates were produced and characterized as described below.

3.1. Characterization of chocolates

3.1.1. Physicochemical characterization of chocolates

The a_w is one of the main factors that may affect probiotic viability in food because it indicates the amount of water available to the microorganisms (Vesterlund, Salminen, & Salminen, 2012). Thus, the a_w was measured monthly in all chocolate formulations stored at 25 °C for 120 days, as previously described, and the results are presented in Table 1. All chocolate formulations presented a slight increase in a_w throughout 120 days of storage ($p < 0.05$),

Table 3

Total content of phenolic and fat of chocolate stored at 25 °C (mean ± standard deviation).

Formulation	Total phenolic (mg gallic acid equivalent/g chocolate)	Total content of fat (%)
Control	16.98 ± 0.32	31.34 ± 0.66
LA3	16.69 ± 0.49	31.60 ± 0.46
BLC1	16.52 ± 0.67	31.58 ± 0.58

LA3 chocolate was produced with *Lactobacillus acidophilus* LA3 and BLC1 chocolate was produced with *Bifidobacterium animalis* subsp. *lactis* BLC1. Control chocolate was produced without probiotics.

Values in columns are not statistically different ($p > 0.05$).

although all formulations presented a_w values below 0.6, which may contribute to the microbiological safety of chocolate because very few microorganisms can multiply under such conditions. The control formulation presented lower values of a_w when compared to values detected in probiotic chocolate, which could be attributed to the use of bacterial cells obtained from a liquid suspension. Vesterlund et al. (2012) evaluated the impact of a_w in crushed flaxseed matrix by mixing probiotics in crushed flaxseed followed by drying in a laboratory incubator at 57 °C. Those authors reported that low values of a_w (ca. 0.11) extended the viability of *Lactobacillus rhamnosus* GG, while the same probiotic in a product with 0.43 of a_w was very unstable and reduced approximately 3.7 log cfu/g after 4 months of storage. Although the results were different from the ones in the present study, the main reason for these differences is the complexity of chocolate matrix, which is composed of various ingredients that may protect the probiotics.

Another parameter that may affect chocolate characteristics and the probiotic viability is the pH, which may affect protein function, transport patterns and cellular bioenergetics (Kruhwich, Sachs, & Padan, 2011). Thus, in the present study, the pH of chocolates was evaluated during 120 days of storage at 25 °C, and the results are presented in Table 2. All chocolate formulations presented pH values in a narrow range (5.66–5.82), which were not statistically different ($p > 0.05$). In addition, pH values remained unchanged throughout the storage period ($p > 0.05$). These results revealed that probiotic bacteria did not change the pH, indicating that they were metabolically inactive throughout storage at room temperature, probably due to the low a_w , as previously discussed. According to Lahtinen, Gueimonde, Ouwehand, Reinikainen, and Salminen (2005), probiotics may stay metabolically inactive during the storage of probiotic products, which may even influence bacterial enumeration for these products.

Chocolates produced in this study were also characterized with regard the total phenolic and total fat contents, and the results are presented in Table 3. No differences ($p > 0.05$) were observed among samples with regard the total phenolic and total fat contents, since all chocolate formulations were prepared with same proportions of ingredients, differing only in the incorporation of probiotics cultures. Total fat content was ca. 31.5%, while the total phenolic content was ca. 17 mg GAE/g of chocolate. These values were similar to the results reported by Miller et al. (2006), which analyzed the total phenolic and fat contents of commercial chocolates sold in the United States of America. According to those authors, total fat content of semisweet chocolate ranged from 29.0% to

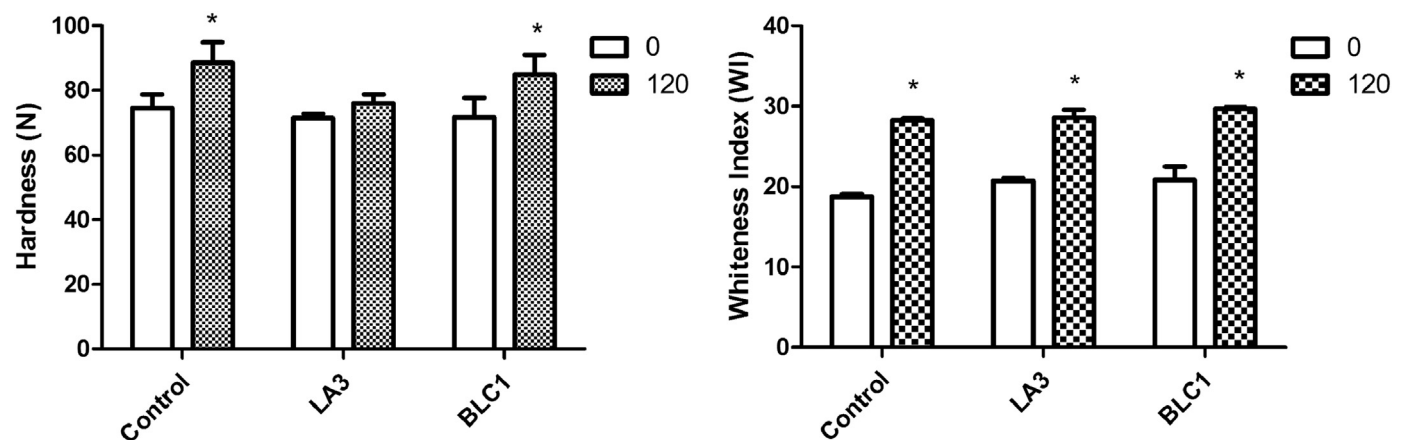


Fig. 1. Hardness (left) and whiteness index (right) of probiotic semisweet chocolates stored at 25 °C for up to 120 days. LA3 chocolate was produced with *Lactobacillus acidophilus* LA3, and BLC1 chocolate was produced with *Bifidobacterium animalis* subsp. *lactis* BLC1. Control chocolate was produced without probiotics. Significant increases ($p < 0.05$) compared to baseline are highlighted with an asterisk.

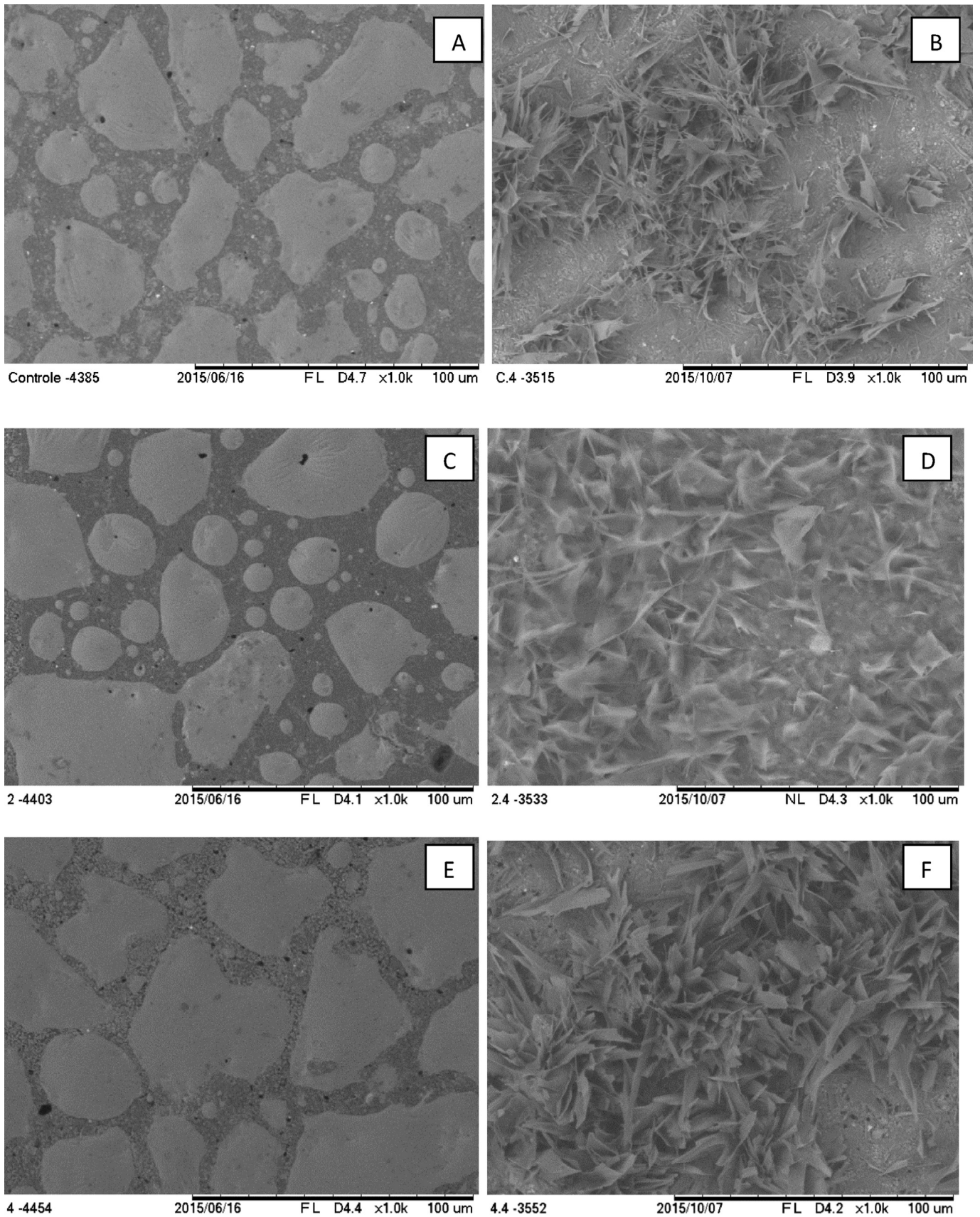


Fig. 2. Micrographs obtained by scanning electron microscopy of chocolates stored at 25 °C for up to 120 days, at 1000× magnification. A, C and E correspond to the 1st day of storage of control (chocolate produced without probiotics), LA3 (chocolate produced with *Lactobacillus acidophilus* LA3) and BLC1 (chocolate produced with *Bifidobacterium animalis* subsp. *lactis* BLC1), respectively. B, D and F correspond to the 120th day of storage of control, LA3 and BLC1 chocolates, respectively.

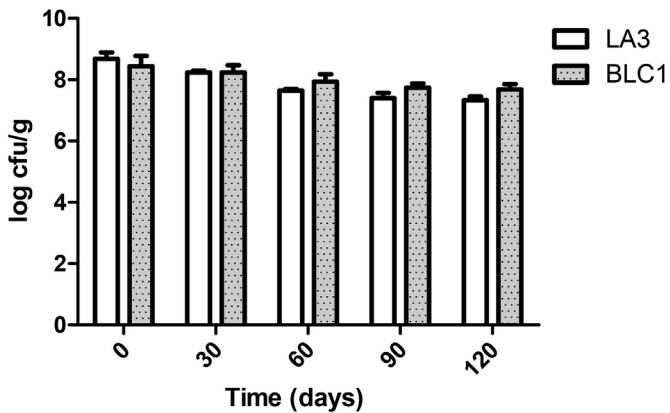


Fig. 3. *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1 populations in chocolates stored at 25 °C for up to 120 days. No significant reduction ($p < 0.05$) was detected in any sample or period.

29.8%, and the total phenolic content was ranged from 11.8 to 12.9 mg GAE/g of product. Similarly, Gültekin-Özgüven, Berktaş, and Özçelik (2016) evaluated the total phenolic content of cocoa powder during the manufacturing steps. Those authors reported that the roasting of cocoa beans was responsible for the loss of about 65% of phenolic compounds. However, despite the loss of phenolic compounds during the process, cocoa powder presented high values of phenolic compounds.

Hardness is a physical parameter in chocolate that may affect the acceptance by the consumers, and it is closely related to the ingredients and tempering process. In the present study, hardness was evaluated throughout the storage period of probiotic chocolates. According to the results presented in Fig. 1, the addition of probiotics did not affect the texture of chocolates when compared to controls in the beginning of the storage period. However, BLC1 and control formulations presented a significant increase in hardness ($p < 0.05$) after 120 days of storage. Recently, Foong et al. (2013) produced dark chocolates with and without probiotics, and reported that chocolate without probiotic was harder than probiotic chocolate. However, tempering processes were different in both preparations, which could have influenced hardness in control samples.

Fat bloom is a phenomenon that may occur in chocolate and it has been studied extensively, although the complete mechanism is not well understood (Sonwai & Rousseau, 2010). Chocolates with fat bloom present a whitish layer on the outer surface, which can be correlated with the whiteness index (WI). In the present study, probiotics chocolates and controls were evaluated with regard to WI throughout 120 days of storage, and the results are presented in Fig. 1. WI increased throughout the storage period in probiotic chocolates and controls, indicating the occurrence of fat bloom probably due to tempering procedures, which is crucial for the development of this phenomenon. In addition to WI, micrographs obtained by SEM also revealed the occurrence of fat bloom in chocolates after 120 days of storage at 25 °C, in all samples, as presented in Fig. 2.

At the beginning of storage, the granulometry of samples presented similar structures. However, at the end of storage, the surface of samples presented fat crystals, which migrated from chocolate matrix to the surface of the product. In chocolate samples containing probiotics, larger crystals were detected when compared to the control sample. The occurrence of this phenomenon may be related to the addition of probiotics during the tempering process, which may influence the recrystallization of lipids. Besides that, the addition of probiotics before tempering to

avoid this defect is not possible, since the previous processes employ high temperatures for long periods, which may affect cell viability.

3.1.2. Microbiological analyses

Before the sensory evaluation of new foods, it is necessary to analyze the microbial quality of samples to guarantee the safety of panelists. In addition, these analyses are important to guarantee that other bacteria will not interfere with the viability of probiotics in chocolate. In the present study, the enumeration of total and thermo-tolerant coliforms (*Escherichia coli*) and the detection of *Salmonella* spp. were performed for chocolates. According to the results, coliform populations were below the detection limit of the method (< 3 MPN/g), and *Salmonella* spp. were not detected in 25 g of sample. These results indicate that the probiotic chocolates produced in this study were safe for consumption.

The viability of probiotics in chocolates was evaluated throughout 120 days of storage at 25 °C and, according to Fig. 3, there was no significant reduction in *L. acidophilus* LA3 or *B. animalis* subsp. *lactis* BLC1 populations. However, *B. animalis* subsp. *lactis* BLC1 presented the highest viability, approximately 7.7 log cfu/g, while *L. acidophilus* LA3 was 7.3 log cfu/g. The presence of high fat content (31%) in chocolate was efficient to protect the *B. animalis* subsp. *lactis* BLC1 of water phase, and possibly of atmospheric oxygen, considering the anaerobic metabolism of this probiotic. Nebesny et al. (2007) produced probiotic dark chocolate with *Lactobacillus casei* and *Lactobacillus paracasei* and stored the samples for one year at 30, 18 and 4 °C. Those authors reported similar results to those presented here: the bacterial populations remained stable (10^6 – 10^7 cfu/g) throughout the storage period. In contrast, Erdem et al. (2014) reported survival of approximately 10^5 cfu/g for *Bacillus indicus* HU36 used to produce synbiotic dark chocolate.

The results from the present study indicate that the pH of approximately 5.7 and the low a_w (ca. 0.44) kept probiotics in a low metabolic state. In addition, high fat content and phenolic compounds (antioxidant compounds responsible for reduced oxidative stress) may also help to maintain the viability of probiotics in chocolates (Pedroso et al., 2013; Tzounis et al., 2011).

Food matrices may provide additional protection to probiotics during passage through the gastrointestinal tract (Ranadheera et al., 2010). In the present study, probiotic survival under *in vitro* simulated gastrointestinal conditions was evaluated before and after incorporation into semisweet chocolate, and the results are presented in Fig. 4. According to the results, *L. acidophilus* LA3 and *B. animalis* subsp. *lactis* BLC1 populations were reduced by 2.9 and 4.1 log cfu/g at the end of the assay, respectively, when evaluated as free cultures. However, no significant reduction was detected for both probiotic population under the same conditions when cells were analyzed after incorporation into the chocolate. The main reason for this increased resistance is the interaction between the probiotics and chocolate ingredients such as fat phenolic compounds, which may protect the cells during the digestion and storage. Possemiers et al. (2010) also reported high survival rates for *Lactobacillus helveticus* (91%) and *Bifidobacterium longum* (80%) incorporated into milk chocolate, when samples were evaluated for survival under *in vitro* simulated gastrointestinal conditions. These data indicate that semisweet chocolate is a potential food matrix to protect probiotic cells during passage through the gastrointestinal tract.

Some mechanisms of interaction between the chocolate matrix and probiotics have been proposed by different authors to explain the protective effects of this product. One of them is the antioxidant activity of phenolic compounds presents in cocoa liquor, which may avoid the oxidative stress, thereby reducing cell death (Maukonen

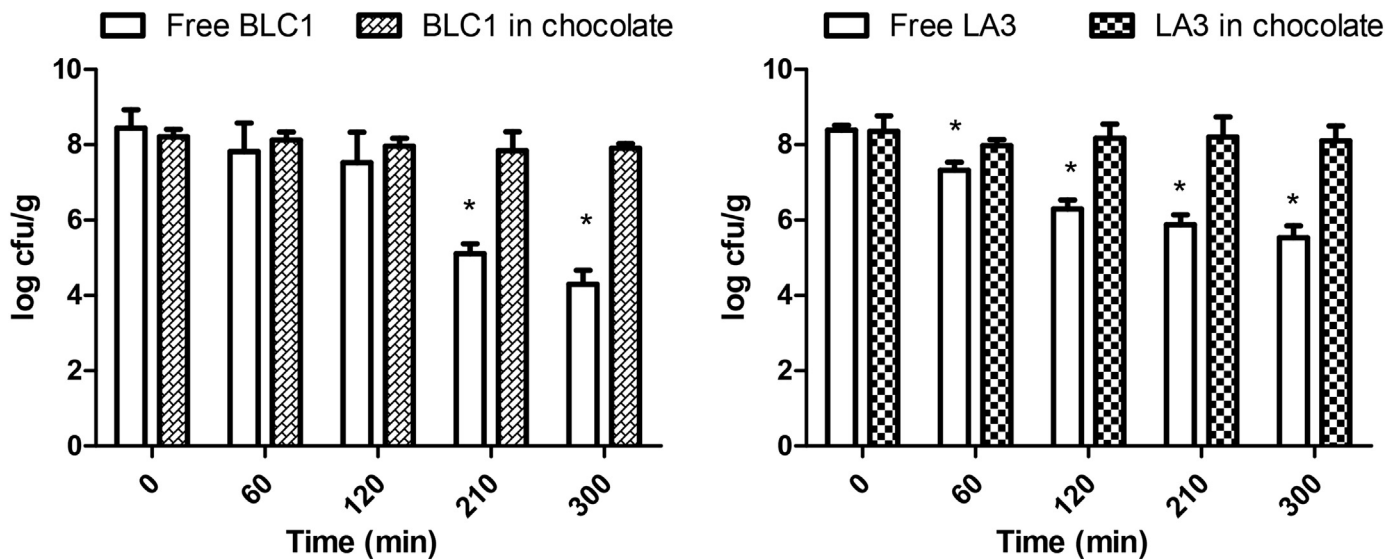


Fig. 4. Survival of *Bifidobacterium animalis* subsp. *lactis* BLC1 (left) and *Lactobacillus acidophilus* LA3 (right) under *in vitro* simulated gastrointestinal conditions. Cells were evaluated as free cultures or incorporated into semisweet chocolate, and exposed to simulated gastric fluid (0–120 min) followed by simulated intestinal fluid (120–300 min). Significant reductions ($p < 0.05$) on probiotic populations compared to initial populations were highlighted with an asterisk. No significant reduction was detected on probiotic populations when they were incorporated into the chocolates.

& Saarela, 2015; Tzounis et al., 2011). Another possible interaction occurs with probiotics and cocoa butter, depending on the hydrophobicity profile of the cell wall of the probiotics, which may contribute to the release of the microorganisms into the intestine during fat digestion. In this context, cocoa butter was even investigated as encapsulant material to protect probiotics, leading to satisfactory results (Lahtinen et al., 2007; Pedroso et al., 2013). Furthermore, the high content of sugar in chocolate (47%) may buffer the gastrointestinal fluids during the digestion (Ranadheera et al., 2010).

3.2. Evaluation of sensory properties

Probiotic semisweet chocolates and controls were evaluated by 100 consumer panelists with regard to color, taste, aroma, texture and overall acceptance, and the results are presented in Fig. 5. According to the results, no significant difference was detected

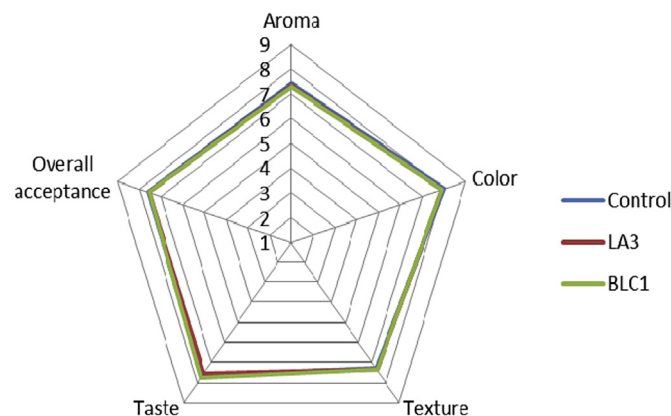


Fig. 5. Radar graphic with scores of probiotic chocolates evaluated by panelists with regard to the color, taste, aroma, texture and overall acceptance, using a hedonic scale ranging from 0 to 9. LA3 chocolate was produced with *Lactobacillus acidophilus* LA3, and BLC1 chocolate was produced with *Bifidobacterium animalis* subsp. *lactis* BLC1. Control chocolate was produced without probiotics. Values were not statistically different ($p > 0.05$).

among all formulations, indicating that the addition of probiotics did not influence the parameters evaluated in the present study. The hedonic scale used by panelists to evaluate the samples ranged from 1 to 9, where “1” meant “dislike extremely” and “9” meant “like extremely”, considering that the lowest score was 7.28 ± 1.38 , it is possible to infer that all products were very well accepted. All formulations presented overall acceptance above to 7.5. Furthermore, no difference statically was observed about the attributes evaluated in each sample.

4. Conclusions

L. acidophilus LA3 and *B. animalis* subsp. *lactis* BLC1 were successfully incorporated into semisweet chocolate, which was revealed to be a potential vehicle for these probiotics by keeping bacteria viable for up to 120 days at 25 °C and by increasing bacterial survival under *in vitro* simulated gastrointestinal conditions. Both strains presented the same behavior ($p > 0.05$) during storage and exposure to *in vitro* simulated gastrointestinal fluids, resulting in high viability at the end of the assays. These results may be partly attributable to the physical and chemical properties of semisweet chocolate such as slightly acidic pH, low a_w , high fat content and the presence of phenolic compounds. In addition, probiotic chocolates were very well accepted by a group of 100 panelists, indicating the potential of these products in the marketing of functional foods.

Acknowledgements

The authors thank the São Paulo Research Foundation for the scholarships awarded (#2014/10754-5 and #2014/14540-0), the Coordination for the Improvement of Higher Education Personnel (CAPES, #23038.0011232/2014-67) and the National Council for Scientific and Technological Development (CNPq, #462493/2014-8) for financial support. Favaro-Trindade C.S. thanks CNPq for the productivity grant (306708/2015-9). The authors also thank the Center of Cereal and Chocolate Technology of the Institute of Food Technology (ITAL, Brazil) for technical assistance and support, Barry Callebaut Brazil for the donation of raw materials and Sacco Brasil for the donation of probiotic cultures.

References

- Adamson, G. E., Lazarus, S. A., Mitchell, A. E., Prior, R. L., Cao, G., Jacobs, P. H., et al. (1999). HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *Journal of Agricultural and Food Chemistry*, 47, 4184–4188.
- Afoakwa, E. O., Paterson, A., Fowler, M., & Vieira, J. (2008). Relationship between rheological, textural and melting properties of dark chocolate as influenced by particle size distribution and composition. *European Food Research Technology*, 227, 1215–1223.
- Afoakwa, E. O., Paterson, A., Fowler, M., & Vieira, J. (2009). Influence of tempering and fat crystallization behaviours on microstructural and melting properties in dark chocolate systems. *Food Research International*, 42, 200–209.
- Alañón, M. E., Castle, S. M., Siswanto, P. J., Cifuentes-Gómez, T., & Spencer, J. P. E. (2016). Assessment of flavanol stereoisomers and caffeine and theobromine content in commercial chocolates. *Food Chemistry*, 208, 177–184.
- Andrews, W. H., Flowers, R. S., Silliker, J., & Bailey, J. S. (2001). Salmonella. In F. P. Downes, & K. Ito (Eds.), *Compendium of methods for the microbiological examination of foods* (4th ed., pp. 357–380). Washington: American Public Health Association (APHA).
- Bialonska, D., Ramnani, P., Kasimsetty, S. G., Muhta, K. R., Gibson, G. R., & Ferreiro, D. (2010). The influence of pomegranate by-product and punicalagins on selected groups of human intestinal microbiota. *International Journal of Food Microbiology*, 140, 175–182.
- Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37, 911–917.
- Curiel, A. J., Munoz, R., & Lopez de Felipe, F. (2010). pH and dose-dependent effects of quercetin on the fermentation capacity of *Lactobacillus plantarum*. *LWT-Food Science and Technology*, 43, 926–933.
- Erdem, Ö., Gültekin-Özgülven, M., Berktaş, I., Erşan, S., Tuna, H. E., Karadağ, A., et al. (2014). Development of a novel synbiotic dark chocolate enriched with *Bacillus indicus* HU36, maltodextrin and lemon fiber: Optimization by response surface methodology. *LWT - Food Science and Technology*, 56, 187–193.
- Fávaro-Trindade, C. S., Heinemann, R. J. B., & Pedrosa, D. L. (2011). Review: Developments in probiotic encapsulation. *Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, 6, 1–8.
- Food and Agriculture Organization of the United Nations and World Health Organization. (2002). *Guidelines for the evaluation of probiotics in food*. Ontario, Canada: Food and Agriculture Organization of the United Nations and World Health Organization.
- Foong, Y. J., Lee, S. T., Ramli, N., Tan, Y. N., & 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, 164–171.
- Gadhiya, D., Patel, A., & Prajapati, J. B. (2015). Current trend and future prospective of functional probiotic milk chocolates and related products – A review. *Czech Journal Food Science*, 33, 295–301.
- Gadkari, P. V., & Balaraman, M. (2015). Catechins: Sources, extraction and encapsulation: A review. *Food and Bioprocess Processing*, 93, 122–138.
- Gbassi, G. K., Vandamme, T., Ennahar, S., & Marchioni, E. (2009). Microencapsulation of *Lactobacillus plantarum* spp. in an alginate matrix coated with whey proteins. *International Journal of Food Microbiology*, 129, 103–105.
- Gültekin-Özgülven, M., Berktaş, I., & Özçelik, B. (2016). Change in stability of procyanidins, antioxidant capacity and in-vitro bioaccessibility during processing of cocoa powder from cocoa beans. *LWT - Food Science and Technology*, 72, 559–565.
- Kornacki, J. L., & Johnson, J. L. (2001). Enterobacteriaceae, coliforms and *Escherichia coli* as quality and safety indicators. In F. P. Downes, & K. Ito (Eds.), *Compendium of methods for the microbiological examination of foods* (4th ed.). Washington: American Public Health Association (APHA).
- Krulwich, T. A., Sachs, G., & Padan, E. (2011). Molecular aspects of bacterial pH sensing and homeostasis. *Nature Reviews Microbiology*, 9, 330–343.
- Lahtinen, S. J., Gueimonde, M., Ouwehand, A. C., Reinikainen, J. P., & Salminen, S. J. (2005). Probiotic bacteria may become dormant during storage. *Applied Environmental Microbiology*, 71, 1662–1663.
- Lahtinen, S. J., Ouwehand, A. C., Salminen, S. J., Forsell, P., & Myllärinen, P. (2007). Effect of starch- and lipid-based encapsulation on the culturability of two *Bifidobacterium longum* strains. *Letters in Applied Microbiology*, 44, 500–505.
- Lalicic-Petronijevic, J., Popov-Rajlic, J., Obradovic, D., Radulovic, Z., Paunovic, D., Petrusic, M., et al. (2015). Viability of probiotic strains *Lactobacillus acidophilus* NCFM® and *Bifidobacterium lactis* HN019 and their impact on sensory and rheological properties of milk and dark chocolates during storage for 180 days. *Journal of Functional Foods*, 15, 541–550.
- Lohman, M., & Hartel, R. (1994). Effect of milk fat fractions on fat bloom in dark chocolate. *Journal of the American Oil Chemists' Society*, 71, 267–276.
- Luccas, V., Bonomi, E. C., & Kieckbusch, T. G. (2014). Comparative characterization between milk chocolates formulated with anhydrous milk fat and with milk fat stearin. *Brazilian Journal of Food Technology*, 17, 130–138.
- Luccas, V., & Kieckbusch, T. G. (2006). Comparative study of the polymorphism of cupuassu fat and cocoa butter using differential scanning Calorimetry. *Brazilian Journal of Food Technology*, 9, 63–68.
- Mandal, S., Hati, S., Puniya, A. K., Singh, R., & Singh, K. (2013). Development of symbiotic milk chocolate using encapsulated *Lactobacillus casei* NCDC 298. *Journal of Food Processing and Preservation*, 37, 1031–1037.
- Maukonen, J., & Saarela, M. (2015). Human gut microbiota: Does diet matter? *Proceedings of the Nutrition Society*, 74, 23–36.
- Meilgaard, M., Civille, G. V., & Carr, B. T. (1999). *Sensory evaluation techniques* (3rd ed.). Boca Raton: CRC Press, 390pp.
- Miller, K. B., Stuart, D. A., Smith, N. L., Lee, C. Y., McHale, N. L., Flanagan, J. A., et al. (2006). Antioxidant activity and polyphenol and procyanidin contents of selected commercially available cocoa-containing and chocolate products in the United States. *Journal of Agricultural and Food Chemistry*, 54, 4062–4068.
- Nebesny, E., Zyzelewicz, D., Motyl, I., & Libudzisz, Z. (2007). Dark chocolates supplemented with *Lactobacillus* strains. *European Food Research Technology*, 225, 33–42.
- Okuro, P. K., Thomazini, M., Balieiro, J. C. C., Liberal, R. D. C. O., & Fávaro-Trindade, C. S. (2013). Co-encapsulation of *Lactobacillus acidophilus* with inulin or polydextrose in solid lipid microparticles provides protection and improves stability. *Food Research International*, 53, 96–103.
- Parvez, S., Malik, K. A., Kang, S. A., & Kim, H. Y. (2006). Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology*, 100, 1171–1185.
- Pedrosa, D. L., Dogenski, M., Thomazini, M., Heinemann, R. J. B., & Fávaro-Trindade, C. S. (2013). Microencapsulation of *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus* in cocoa butter using spray chilling technology. *Brazilian Journal of Microbiology*, 44, 777–783.
- Pereira, A. L. F., Almeida, F. D. L., Jesus, A. L. T., Costa, J. M. C., & Rodrigues, S. (2013). Storage stability and acceptance of probiotic beverage from cashew apple juice. *Food and Bioprocess Technology*, 6, 3155–3165.
- Possemiers, S., Marzorati, M., Verstraete, W., & Vand de Wiele, T. (2010). Bacteria and chocolate: A successful combination for probiotic delivery. *International Journal of Food Microbiology*, 141, 97–103.
- Ranadheera, R. D. C. S., Baines, S. K., & Adams, M. C. (2010). Importance of food in probiotic efficacy. *Food Research International*, 43, 1–7.
- Salminen, S., Wright, A. V., Morelli, L., Marteau, P., Brassarte, D., Vos, W. M., et al. (1998). Demonstration of safety of probiotics — a review. *International Journal of Food Microbiology*, 44, 93–106.
- Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–ciocalteu reagent. *Methods of Enzymology*, 299, 152–178.
- Sonwai, S., & Rousseau, D. (2010). Controlling fat bloom formation in chocolate — Impact of milk fat on microstructure and fat phase crystallisation. *Food Chemistry*, 119, 286–297.
- Souza, V. B., Fujita, A., Thomazini, M., Silva, E. R., Lucon, F., Jr., Genovese, M. I., et al. (2014). Functional properties and stability of spray-dried pigments from Bordo grape (*Vitis labrusca*) winemaking pomace. *Food Chemistry*, 164, 380–386.
- Tripathi, M. K., & Giri, S. K. (2014). Probiotic functional foods: Survival or probiotics during processing and storage. *Journal of Functional Foods*, 9, 225–241.
- Tzounis, X., Mateos, A. R., Vulevic, J., Gibson, G. R., Kwik-Urbe, C., & Spencer, J. P. E. (2011). Probiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. *The American Journal of Clinical Nutrition*, 93, 62–72.
- Vesterlund, S., Salminen, K., & Salminen, S. (2012). Water activity in dry foods containing live probiotic bacteria should be carefully considered: A case study with *Lactobacillus rhamnosus* GG in flaxseed. *International Journal of Food Microbiology*, 157, 319–321.
- Vinderola, G., Binetti, A., Burns, P., & Reinheimer, J. (2011). Cell viability and functionality of probiotic bacteria in dairy products. *Frontiers in Microbiology - Food Microbiology*, 2, 1–6.