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Effects of *Bifidobacterium animalis* and inulin addition on quality characteristics of synbiotic milk chocolate

Auswirkungen der Zugabe von Bifidobacterium animalis und Inulin auf die Qualitätsmerkmale von synbiotischer Milchschokolade

Ayşe Akgül[1]), Durmuş Sert[2]), Emin Mercan[3])

Summary

The aim of this study was to investigate effects of addition of *B. animalis* BB-12 (before tempering, BT or after tempering, AT) and inulin (6, 8, and 10%) on thermal, physico-chemical characteristics, fatty acid composition, textural properties and probiotic viability of milk chocolates during 60 days of storage. The free fatty acid (FFA) value of milk chocolates ranged from 0.66 to 0.89 (% oleic acid), in which higher ratio of inulin addition increased the FFA. Probiotics and inulin addition increased darkness of chocolates when compared to control. D[4,3] value of samples ranged from 9.26-17.97 µm. Higher inulin ratio in chocolates increased viscosity and yield value. These values in the group BT were higher than those of the group AT. BB-12 count of probiotic and synbiotic samples ranged from 7.09–8.06 log cfu/g during the storage. Higher inulin ratio increased the probiotics count in samples. Probiotic addition after tempering increased the count of probiotic and improved viability at the end of storage. The results showed that *B. animalis* BB-12 is capable of surviving in milk chocolate, at an appropriate bacteria count (>10^7 cfu/g), up to 60 days of storage at 18°C.

Keywords: Synbiotic, Milk chocolate, Inulin, *B. animalis* BB-12, Texture

Introduction

Chocolate is a popular food consumed by people of all age groups around the world. The popularity of chocolate is mainly due to its eating pleasure, positive emotions, unique texture, and taste (Toker et al., 2016). Due to the increase in people's awareness of healthy eating and well-being, there is an increasing trend in the development and consumption of functional confectionery products such as functional chocolate (Wollgast and Anklam, 2000; Erdem et al., 2014). The addition of probiotic, prebiotic, and synbiotic is one of the most promising ways to produce functional chocolate (Lalicic-Petronijevic et al., 2017).

Probiotics are defined as viable microorganisms that provide health benefits to the host when administered adequate level (Hill et al., 2014). A large number of studies on addition of probiotic bacteria to dairy (ice cream, cheese, yogurt, dairy desserts, etc.) and non-dairy products (juices, cereal, etc.) are present. However, studies on probiotic chocolate are very few compared to other probiotic foods. On the contrary, the viability of chocolate from encapsulated probiotic bacteria was three times higher than the probiotic dairy product in the small intestine (Maillard and Landuyt, 2008). Previous studied reported that *Lactobacillus helveticus, Bifidobacterium longum* (Maillard and Landuyt, 2008), *Lactobacillus rhamnosus* (Raymond and Champagne, 2015), *Lactobacillus paracasei* (Coman et al., 2012), *Lactobacillus acidophilus, Bifidobacterium lactis* (Laličić-Petronijević et al. 2015), *Bacillus indicus* (Erdem et al., 2014) were used in probiotic chocolate production. However, *Lactobacillus* and *Bifidobacterium* are the most dominant species studied in probiotic formulations for the production of chocolate (Erdem et al., 2014).

Prebiotics can be defined as non-digestible carbohydrate compounds that selectively stimulate one or a limited group of beneficial bacteria in the human gut (Gibson et al., 2017). Inulin, fructooligosaccharide, galactooligosaccharides and polydextrose are widely used prebiotics scientifically and commercially. Inulin is an oligosaccharide derived from certain plants such as chicory, asparagus root, onion, and garlic (Bengmark et al., 2001). Inulin has some important characteristics including prebiotic, bifidogenic, fermentable, fibre-enriched, and low calorie (Konar et al., 2014). Also, inulin has beneficial effects on human health such as improved calcium absorption and possibly a reduced risk of colon cancer as well as improvements to lipid metabolism (Roberfroid, 2000).

Synbiotics is a combination of probiotics and prebiotic to improve the viability of probiotics. Previous studies reported that the matrix and production process of chocolate could be appropriate for the addition of probiotic culture and prebiotic materials, showing that synbiotic chocolate products could be produced. There were very few studies on synbiotic chocolate products. Erdem et al. (2014) conducted a study on the development of synbiotic dark chocolate containing *Bacillus indicus* HU36, maltodextrin, and lemon fiber. In another study, milk chocolates were produced by the addition of *Lactobacillus casei* NCDC 298 and inulin (Mandal et al., 2013).

While chocolate is an appropriate product for the incorporation of both probiotic and prebiotic, there are very limited researches about this topic. Therefore, to the best of our knowledge, this study is the first research attempt to produce synbiotic milk chocolate containing *Bifidobacterium animalis* subsp. *lactis* BB-12 and inulin. This study aimed to investigate the effect of the addition of *B. animalis* BB-12 (before or after tempering stage) and inulin (6, 8, and 10%) to milk chocolates on thermal, physicochemical, fatty acid composition, textural, the viability of probiotic bacteria and sensory characteristics.

Material and methods

Materials

Raw materials used in milk chocolate production was kindly provided by the Research & Development Department of Torku (Konya Şeker Gıda Sanayi A.Ş., Konya, Turkey). The following ingredients were used in the production: Sugar (Konya Şeker, Konya, Turkey), cocoa butter (Altinmarka, Istanbul, Turkey), cocoa mass (Altinmarka), milk powders (Panagro, Konya, Turkey), demineralized whey powder (Panagro), lecithin (Yılmaz Chemistry, İstanbul, Turkey), polyglycerol polyricinoleate (PGPR 4120; Palsgaard A/S, Juelsminde, Denmark), chicory inulin (Orafti GR, Beneo-Orafti S.A, Belgium), vanillin aroma (Borregard, Norway), *Bifidobacterium animalis* subsp. *lactis* BB-12 (nu-trish® BB-12®, Chr. Hansen A/S, Hoersholm, Denmark).

Milk chocolate production

Production of milk chocolates was carried out in the pilot plant of Torku R & D department (Konya, Turkey). A planetary mixer (Varimixer RN10, A/S Wodschow & Co., Brøndby, Denmark), pilot type three-roll refiner (Buhler SDY 200, Uzwil, Switzerland) and pilot conching (ELK'olino single-shaft conche, Bühler, AG, Switzerland) were used for producing samples. Formulations of samples are given in Table 1 for preparing 100 g of milk chocolate. For each formulation, 5 kg of sample was produced according to the production flow chart in Fig. 1. In probiotic or synbiotic samples, *B. animalis* BB-12 culture was added to the chocolate mixture before or after tempering to achieve a minimum of 10^7 cfu/g in the final product. The tempering was carried out manually using two spatulas. During tempering, chocolates were firstly heated to 40°C and cooled to

TABLE 1: *Milk chocolate formulations.*

Ingredient (g/100 g)	C	P	S6	S8	S10
Sugar	38.59	38.59	32.59	30.59	28.59
Cacao butter	26.7	26.7	26.7	26.7	26.7
Cacao mass	11	11	11	11	11
Drum dried whole milk powder	13	13	13	13	13
Skim milk powder	8	8	8	8	8
Demineralized whey powder	2	2	2	2	2
Lecithin	0.5	0.5	0.5	0.5	0.5
PGPR	0.2	0.2	0.2	0.2	0.2
Vanillin aroma	0.01	0.01	0.01	0.01	0.01
Inulin	-	-	6	8	10
Bifidobacterium animalis subsp *lactis* BB-12	-	0.01	0.01	0.01	0.01
Total	100	100	100	100	100

C=Control: not containing probiotic and prebiotic, P=Probiotic: containing probiotic (*B. animalis* BB-12)
S=Symbiotic: containing probiotic and prebiotic (inulin), S6= 6% inulin, S8=%8 inulin, S10=%10 inulin
PGPR: Polyglycerol polyricinoleate

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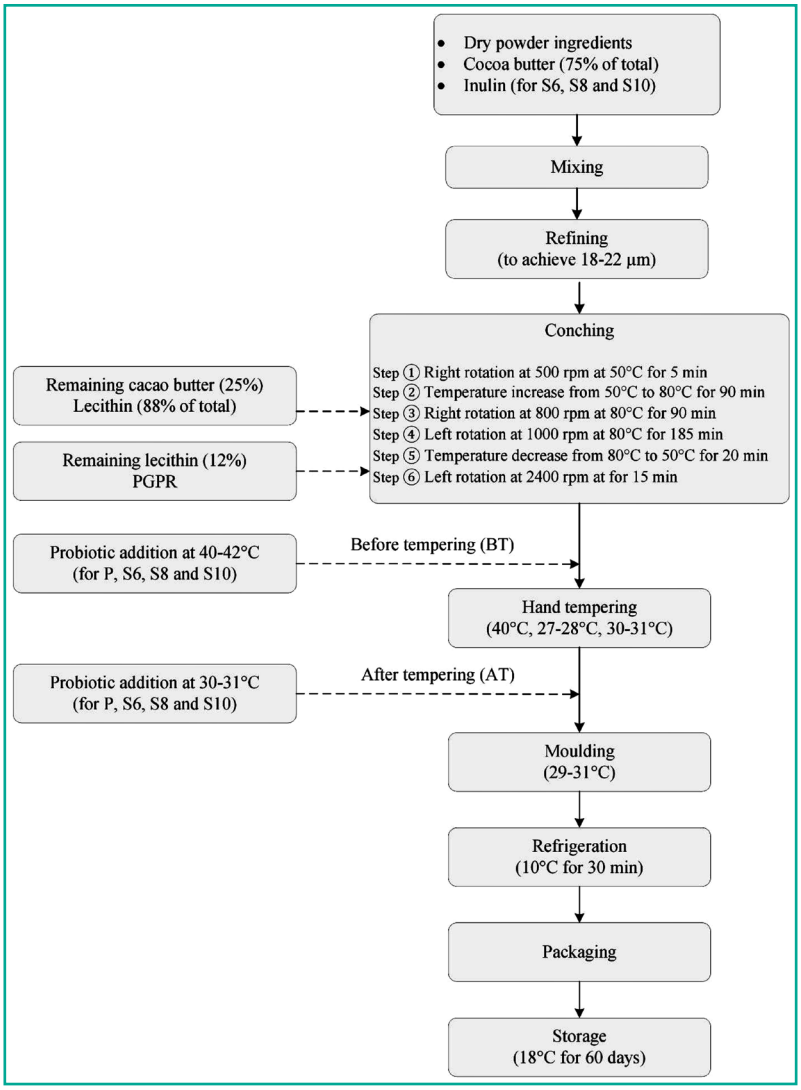

FIGURE 1: *Production flow chart for milk chocolates.*

27°C, and then heated to 30°C. Following the production, chocolates were stored at 18ºC for 60 days. In the following text, before tempering and after tempering are denoted by BT and AT, respectively. Physicochemical characteristics, textural properties, fatty acid composition, and probiotic viability of samples were determined at 1. and 60. days of storage.

Proximate analyses

Moisture, protein, total fat, and ash content of milk chocolates were determined according to the AOAC methods (AOAC, 1995a; AOAC, 1995b). The total sugar content of samples was calculated using the Lane-Eynon titration method. Water activity (aw) of samples were determined using a water activity instrument (Novasina LabMaster-aw, Novasina AG, Lachen, Switzerland) at 25ºC. The pH of the melted chocolates was measured by a WTW 315i/SET pH meter (Weilheim, Germany).

Fat extraction and determination of chemical oxidation markers

The fat extraction was done according to Mexis et al. (2010). In summary, 5 g of crushed milk chocolate was put into a separatory funnel containing 100 mL of diethyl ether and 10 mL of distilled water. The separating funnel was shaken for 2 min and left to equilibrate for 24 hours. The supernatant was transferred to a flask and diethyl ether evaporated in a water bath at 40°C. The extracted oil was dried at 105°C for 3 min. The peroxide value (meq O_2/kg) of samples was determined according to ISO 27107:2008 potentiometric end-point determination method (ISO, 2008). The free fatty acid (FFA) content of samples was measured by ISO 660:2009 method (ISO, 2009) and expressed as oleic acid %.

Colour

A colorimeter (Minolta Chroma Meter CR-400, Osaka, Japan) was used to determine colour parameters of chocolates according to the CIELab colour space system. Colour parameters were given as L*: brightness, a*: ±redness-greenness, and b*: ±yellowness-blueness.

Particle size measurement

To determine particle size parameters of chocolate samples, a Malvern MasterSizer 3000 equipped with a Hydro EV dispersion unit (Malvern Instruments Ltd., Worcestershire, UK) was used. Refined milk chocolate samples were dispersed in isobutanol (refractive index 1.39, (Fisher Scientific, Pittsburgh, PA) at 22 ± 2°C. Particle size parameters were expressed as span, D[4,3], D[3,2], d(0.1), d(0.5) and d(0.9).

Determination of fatty acid compositions

Fatty acids methyl esters (FAME) were prepared as described in AOCS Official Method Ce 2-66 using BF3-methanol reagent (AOCS, 2009). FAMEs analysis was carried out using a capillary gas chromatography instrument (Perkin-Elmer 8320B, Norwalk, CT) equipped with a flame ionization detector. A capillary column coated with a 100% Cyanopropyl Polysiloxane Film (CP™ Sil 88, 50 m × 0.25 mm i.d., 0.20 film thickness, Chrompack, Middelburg, The Netherlands). 0.5 µL of the sample was injected into injection block at 250°C. 1 mL/min of flow rate was used for helium (carrier gas). The temperature of the detector and column were 250 and 177°C, respectively. The obtained peaks were defined according to retention times, and the areas were calculated according to the relative intensity.

Rheological analysis

The rheological analysis of milk chocolates was performed using a Haake VT-550 rheometer equipped with an SV DIN rotor at 40ºC as described in IOCCC (2000). Casson plastic viscosity and Casson yield values were calculated by Haake RheoWin 4.63.0004 software using the Casson model.

Texture analysis

To determine textural properties of milk chocolate bars, penetration test was carried out using a texture analyser (TA.XTPlus, Stable Micro Systems, Godalming, Surrey, UK). In this analysis, the hardness value of chocolate bars was determined. Samples were penetrated by a 1" spherical probe (P/1S) using a 5 kg load cell at 22°C. During the analysis, pre-test, test, and post-test speeds were 1.5 mm/s, 2.0 mm/s, and 10 mm/s, respectively. The mean area under the force vs. time curve was expressed as hardness (N) value.

Viability of probiotic bacteria

Firstly, serial dilutions of samples were prepared using peptone water (0.1 g/100 g) in the enumeration of *B. animalis* BB-12. Then, serial dilutions were plated on modified MRS agar containing cysteine hydrochloride (0.25%). Plates were incubated anaerobically at 37°C for 72 h. After that, colonies as viable probiotic cells were enumerated, and results were expressed as log cfu/g (Chr. Hansen, 2007).

Melting characteristics

Differential scanning calorimetry (DSC; DSC 25, TA Instruments, New Castle, DE, USA) was used to determine melting characteristics of milk chocolates as described by Afoakwa et al. (2008a) with some modifications. Approximately 5 mg of sample was placed into pans. Then, the pan was scanned from 0 to 190°C at a rate of 10°C/min, under nitrogen gas. For each peak obtained from the thermogram, onset (T_{onset}), peak (T_{peak}), end (T_{end}) temperatures, and enthalpy of melting (ΔH) were determined. Also, the melting index (T_{index}) was calculated as ($T_{end} - T_{onset}$).

Statistical analysis

To analyse the data, a one-way ANOVA was performed using SPSS software (version 22.0, IBM Corp., Armonk, NY). Results are presented as mean ± standard deviation.

Results and discussion

Physicochemical characteristics of milk chocolates

Physicochemical characteristics of milk chocolates are given in Table 2. These characteristics were determined at the beginning of the storage. The moisture content of chocolates was not affected by probiotic and inulin addition at different levels in groups of both before tempering (BT) and after tempering (AT) ($P > 0.05$). The moisture content of all samples ranged from 1.00 to 1.06%. The moisture content of chocolate is usually between 0.5–1.5%, and the viscosity of chocolate increases by higher moisture (Afoakwa et al., 2007).

In groups of both before and after tempering, inulin addition to chocolates increased the protein content of milk chocolates compared to the control sample. However, this increase was not statistically significant ($P > 0.05$). The protein content of all samples was higher than the average protein content of milk chocolates reported by previous studies (Becket, 2008; Chan et al., 1994). The protein of chocolate is composed of non-fat cacao and milk particles.

The total fat content of milk chocolates ranged from 34.50–35.55%. The fat content of probiotic and synbiotic chocolates was lower than the control. However, effects of probiotic and synbiotic on fat content was not significant ($P > 0.05$). As expected, the fat content of samples decreased with increasing inulin addition. Also, the fat content of chocolates in group BT was higher than the same sample in group AT. The fat content of most chocolates ranges from 25 to 35% depending on process conditions, and it affects the textural properties of chocolate (Afoakwa, 2010).

The ash content of samples changed from 1.54 to 1.61%. Botelho et al. (2014) reported that the ash content of dark chocolate was between 1.33–1.38%.

The water activity values of milk chocolates ranged from 0.177 to 0.186. Similar to this study, lower water activity values in milk chocolates were also reported by previous studies (Konar, 2013; Konar et al., 2015) in which water activity was between 0.19–0.24.

The pH value of the control sample was 6.51. In the group of BT, higher ratio of inulin addition slightly decreased pH of chocolates ($P > 0.05$). However, pH values of P and S6 in the group of AT were similar to control. The pH of S8 and S10 in AT was lower than control. Higher pH values with a range of 7.28–7.30 in milk chocolates were reported by Farzanmehr and Abbasi (2009). A pH range of 6.18–7.36 in chocolate spreads was reported by a previous research (Kara et al., 2014). pH is between 5.5–6.0 in natural chocolate products, whereas the pH of alkaline treated chocolates ranged from 6.0 to 7.8 (Altan, 2009).

In chocolate samples, the peroxide was not detected. In other words, there was no lipid oxidation in milk chocolates at the beginning of the storage. This situation in chocolate may be related to cacao butter, which has high ratio saturated and monounsaturated fatty acids (Botelho et al., 2014).

The free fatty acid (FFA) value of milk chocolates ranged from 0.66 to 0.89 (% oleic acid). In the group of BT, the FFA value of all samples was higher than the control. However, in the group of AT, the FFA value of samples excluding S10 was lower than control. In both group BT and AT, higher ratio of inulin addition increased the FFA value of chocolates ($P < 0.05$). Also, this increase was higher in group BT.

TABLE 2: *Physicochemical properties of milk chocolates.*

		C	BT				AT			
			P	S6	S8	S10	P	S6	S8	S10
Moisture (%)		1.00±0.03	1.03±0.01	1.03±0.01	1.02±0.06	1.03±0.05	1.01±0.01	1.02±0.06	1.06±0.01	1.04±0.07
Protein (%)		8.90±0.06	8.96±0.00	9.03±0.01	9.04±0.02	9.04±0.03	8.92±0.14	9.01±0.05	8.97±0.01	9.00±0.02
Total fat (%)		35.55±0.38	35.15±0.07	35.05±0.49	34.80±0.85	34.75±0.64	35.00±0.28	34.90±0.14	34.60±0.14	34.50±0.14
Ash (%)		1.61±0.02	1.58±0.04	1.56±0.02	1.55±0.01	1.54±0.01	1.57±0.02	1.59±0.06	1.56±0.06	1.56±0.06
Total sugar (%)		40.46±0.30	40.85±0.52	40.08±1.24	40.48±0.37	41.06±0.28	40.25±0.14	40.35±0.30	40.42±0.05	40.51±0.33
Water activity		0.181±0.001	0.180±0.000	0.181±0.004	0.183±0.004	0.186±0.009	0.177±0.001	0.181±0.001	0.181±0.002	0.180±0.006
pH		6.51±0.01	6.50±0.01	6.47±0.01	6.45±0.05	6.40±0.08	6.51±0.01	6.52±0.03	6.48±0.01	6.41±0.04
Peroxide (meq O_2/kg)		0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Free fatty acid (oleic acid %)		0.66±0.04	0.67±0.02	0.72±0.03	0.83±0.06	0.89±0.04	0.58±0.06	0.60±0.01	0.63±0.07	0.69±0.06
Colour	L*	46.11±0.08	44.37±0.74	45.79±0.37	43.78±0.71	43.86±0.92	43.29±2.20	44.89±0.42	43.67±2.09	45.93±0.69
	a*	10.68±0.10	10.83±0.43	10.77±0.23	11.31±0.31	11.32±0.40	11.21±0.48	11.11±0.23	11.18±0.71	10.70±0.32
	b*	14.80±0.09	14.31±0.75	14.59±0.40	15.09±0.58	15.29±0.30	15.31±1.14	15.38±0.37	14.74±1.16	14.42±0.43
Particle size	Span	2.67±0.27	2.61±0.07	2.71±0.04	2.30±0.19	2.35±0.04	2.69±0.02	2.87±0.03	2.74±0.02	2.41±0.01
	D[4,3] (µm)	10.73±0.22	12.88±0.25	17.97±1.02	9.26±0.23	11.06±0.12	15.11±0.67	15.19±0.05	16.02±0.03	11.80±0.80
	D[3,2] (µm)	3.95±0.47	4.74±0.15	5.42±0.02	4.04±0.39	4.44±0.11	5.13±0.23	4.74±0.05	4.86±0.04	4.42±0.10
	d(0.1) (µm)	1.68±0.30	2.07±0.08	2.42±0.01	1.77±0.24	1.93±0.06	2.24±0.13	1.97±0.04	2.05±0.02	1.90±0.06
	d(0.5) (µm)	8.15±0.55	9.84±0.29	12.82±0.13	7.59±0.47	8.93±0.16	11.38±0.46	10.76±0.13	11.74±0.11	9.12±0.22
	d(0.9) (µm)	23.38±0.43	27.69±0.18	37.11±0.94	19.21±0.20	22.92±0.05	32.86±1.10	32.83±0.05	34.20±0.14	23.91±0.50

$\bar{X}$ ± standard deviation, n = 3

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Colour is one of the important factors which affect consumer acceptance. Composition and processing parameters can affect colour of chocolates. L* value of the control was 46.11. In groups of BT and AT, the L* value of probiotic and synbiotic chocolates was lower than control. In other words, probiotics and inulin addition to chocolate increased darkness when compared to control a* value of all samples ranged from 10.68–11.32. There was a slight increase in the a* value of chocolates depending on the addition of probiotic culture and inulin ($P > 0.05$). It was found that b* value of milk chocolate samples ranged from 14.31–15.38. Chocolates with different particle sizes differ in their structural and particle arrangements that affect light scattering coefficients and hence, their appearance (Afoakwa et al., 2008b).

Particle size is a crucial factor of the rheological characteristics in chocolates, which have a direct effect on sensory perception. Span, D[4,3], D[3,2], d(0.1), d(0.5) and d(0.9) values are given in Table 2. Span reflects the magnitude of particle size distribution. Span value of milk chocolates was between 2.41–2.87. D[4,3] value of samples ranged from 9.26–17.97 µm. Except for S8-BT, D[4,3] value of probiotic and synbiotic chocolates was higher than control ($P < 0.05$). Differences were observed in D[4,3] value between groups of BT and AT. Switzerland is known as the home of the finest milk chocolate because of the particle size, which is always approximately below 20 µm. This particle size with the conching process makes very smooth chocolate (Becket, 2008). Previous studies showed that chocolate with 30–35 µm particle size senses as coarse, and it has a significant effect on the sensorial quality of chocolates (Afoakwa, 2010). It was found that the D[3,2] value of samples ranged from 3.95–5.42 µm. D[3,2] value of probiotic and synbiotic chocolates in the group of BT and AT was higher than the control sample. It was reported that D[3,2] value of dark chocolates with different particle sizes ranged from 2.56 to 4.54 µm (Afoakwa et al., 2008a). d(0.1) and d(0.5) values of milk chocolates samples were between 1.68–2.42 µm and 7.59–12.82 µm, respectively. d(0.9) value of samples ranged from 19.21 to 37.11 µm. Except for S6-BT, the d(0.9) value of chocolates was lower than 35 µm ($P < 0.05$). This was a good achievement because the particle size of good chocolate should be below 35 µm (Awua, 2002). Also, smaller particles can develop sensorial characteristics of chocolates. In chocolate production, d(0.9) and specific surface area are crucial parameters. d(0.9) value affects textural properties and coarseness of chocolates, whereas specific surface area determines fat content for required flow characteristics (Becket, 2008). Afoakwa (2010) reported that d(0.1), d(0.5), d(0.9), and specific surface area of chocolates affect particle interactions and packing ability.

TABLE 3: *Fatty acid composition (% peak area) of milk chocolates at 1. day of storage.*

		C	BT				AT			
			P	S6	S8	S10	P	S6	S8	S10
Saturated fatty acids	C 4:0	0.18	0.24	0.20	0.23	0.21	0.20	0.19	0.20	0.21
	C 6:0	0.12	0.15	0.13	0.14	0.13	0.13	0.12	0.13	0.13
	C 8:0	0.08	0.10	0.08	0.08	0.07	0.07	0.08	0.07	0.08
	C 10:0	0.24	0.25	0.22	0.20	0.22	0.20	0.23	0.20	0.28
	C 12:0	0.24	0.31	0.24	0.25	0.25	0.24	0.25	0.24	0.24
	C 13:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 14:0	0.92	1.14	0.91	0.94	0.79	0.90	0.92	0.91	0.85
	C 15:0	0.13	0.14	0.11	0.13	n.d.	0.14	0.11	0.14	n.d.
	C 16:0	25.87	26.30	25.73	26.00	25.82	25.83	25.70	25.88	24.03
	C 17:0	0.24	0.31	0.24	0.25	0.20	0.24	0.24	N/A	N/A
	C 18:0	33.72	32.31	34.14	34.06	34.86	34.05	34.11	34.43	32.87
	C 19:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 22:0	0.01	0.01	0.01	0.02	0.03	0.01	0.02	0.02	0.02
	C 24:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 11:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 21:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 23:0	n.d.	n.d.	0.01	n.d.	n.d.	n.d.	n.d.	n.d.	0.02
Monounsaturated fatty acids	C 14:1	0.08	0.10	0.09	0.08	n.d.	0.08	0.08	0.08	0.08
	C 15:1	0.02	n.d.	n.d.	0.02	n.d.	n.d.	0.02	n.d.	n.d.
	C 16:1	0.34	0.47	0.37	0.36	0.25	0.37	0.36	0.38	0.28
	C 16:1 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 16:1 t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 17:1	0.03	n.d.	n.d.	0.04	n.d	n.d	0.03	n.d	n.d
	C 18:1	32.78	32.33	32.95	32.76	32.28	32.94	32.85	32.90	34.61
	C 18:1c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:1t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:1	1.11	1.39	1.11	1.12	1.85	1.11	1.13	1.13	1.27
	C 22:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 22:1c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 22:1t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 24:1c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Polyunsaturated fatty acids	C 16:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2	3.54	4.05	3.22	2.97	2.78	3.22	3.24	2.99	4.61
	C 18:2 n-6 c,c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2 c,t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2 t,c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2 t,t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2 i	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2 t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:3 n-3 c,c,c	0.04	n.d	n.d	0.02	0.21	n.d	0.04	n.d	n.d
	C 18:3 n-6 c,c,c	0.21	0.26	0.20	0.21	n.d	0.20	0.21	0.21	0.28
	C 18:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:2 n-6 c,c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:3 n-3	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.04
	C 20:3 n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:4 n-3	0.05	0.09	0.01	0.10	0.02	0.02	0.04	0.06	0.07
	C 20:4 n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:5 n-3	0.01	0.01	0.02	0.01	0.02	0.01	0.01	n.d.	n.d.
	C 22:2	0.01	0.01	0.01	n.d.	n.d.	0.01	0.01	0.01	0.03
	C 22:5 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 22:6 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Saturated	61.75	61.26	62.02	62.30	62.59	62.01	61.97	62.22	58.73
	Monounsaturated	34.36	34.29	34.52	34.38	34.38	34.50	34.47	34.49	36.24
	Polyunsaturated	3.87	4.43	3.47	3.32	3.04	3.47	3.57	3.28	5.03
	Trans	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

n.d: not determined

Fatty acid composition of milk chocolates

Tables 3 and 4 show the fatty acid composition of samples at 1. and 60. days of storage, respectively. During the storage period, 26 different fatty acids were determined. Major fatty acids of samples were C16:0 (palmitic acid, >24%), C18:0 (stearic acid, >32%), C18:1 (oleic acid, >32%) and C18:2 (linoleic acid, >2.5%). Other 22 fatty acids were determined at trace level. Afoakwa (2010) reported that chocolate fatty acids consist of stearic (34%) and palmitic (27%) and oleic acids (34%). When considering the total percentage of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), SFA content was the highest, up to 62.59%, followed by MUFA > 34% and, finally, a small quantity of PUFA < 5% were found. In chocolate samples, stearic acid was the major component of the SFA. Also, oleic acid was the major fatty acid between MUFA. Linoleic acid, alpha-linolenic acid, and gamma-linolenic acid were determined at very small amounts compared to SFA and MUFA. However, these fatty acids have a protective effect on health (Tapiero et al., 2002).

Rheological and textural characteristics of milk chocolates

Table 5 shows the rheological and textural characteristics of milk chocolates. The rheological characteristics of the molten chocolate samples were characterized using the Casson model. R^2 values were between 0.990–0.999. The-

TABLE 4: *Fatty acid composition (% peak area) of milk chocolates at 60. days of storage.*

		C	BT				AT			
			P	S6	S8	S10	P	S6	S8	S10
Saturated fatty acids	C 4:0	0.23	0.16	0.18	0.21	0.20	0.21	0.30	0.21	0.21
	C 6:0	0.14	0.11	0.12	0.13	0.13	0.13	0.19	0.14	0.14
	C 8:0	0.09	0.07	0.08	0.08	0.08	0.08	0.12	0.09	0.08
	C 10:0	0.22	0.17	0.20	0.20	0.20	0.19	0.52	0.21	0.20
	C 12:0	0.26	0.23	0.25	0.26	0.25	0.24	0.39	0.26	0.25
	C 13:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 14:0	0.97	0.86	0.93	0.91	0.95	0.91	1.15	0.95	0.95
	C 15:0	0.11	0.11	0.11	0.11	0.11	0.11	n.d.	0.11	0.13
	C 16:0	26.37	25.27	25.78	25.78	25.93	25.74	25.29	26.03	25.93
	C 17:0	0.24	0.25	0.25	0.24	0.25	0.26	0.31	0.25	0.25
	C 18:0	33.12	34.48	33.78	34.24	33.71	34.00	33.04	33.78	33.90
	C 19:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 22:0	n.d.	n.d.	0.01	0.01	n.d.	n.d.	0.01	n.d.	n.d.
	C 24:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 11:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 21:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 23:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Monounsaturated fatty acids	C 14:1	0.09	0.08	0.09	0.08	0.09	0.09	n.d.	0.09	0.09
	C 15:1	n.d.	0.02	0.02	n.d.	0.02	0.02	n.d.	n.d.	0.02
	C 16:1	0.36	0.34	0.37	0.34	0.36	0.36	0.51	0.36	0.36
	C 16:1 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 16:1 t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 17:1	n.d.	0.03	0.04	n.d.	n.d.	0.03	n.d.	n.d.	n.d.
	C 18:1	32.93	33.14	33.18	33.05	33.25	32.99	32.09	33.14	33.11
	C 18:1c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:1t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:1	1.04	1.17	1.13	1.08	1.14	1.11	2.31	1.09	1.13
	C 22:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 22:1c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 22:1t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 24:1c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Polyunsaturated fatty acids	C 16:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2	3.56	3.18	3.18	2.98	3.00	3.20	3.13	2.98	2.99
	C 18:2 n-6 c,c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2 c,t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2 t,c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2 t,t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2 i	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:2 t	n.d.	n.d.	n.d.	0.03	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 18:3 n-3 c,c,c	n.d.	0.03	0.03	n.d.	0.03	0.06	0.22	n.d.	n.d.
	C 18:3 n-6 c,c,c	0.20	0.20	0.21	0.20	0.20	0.21	0.26	0.20	0.20
	C 18:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:2 n-6 c,c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:3 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01	n.d.	n.d.
	C 20:3 n-6	0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:4 n-3	0.05	0.08	0.05	0.07	0.07	0.07	0.11	0.05	0.05
	C 20:4 n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 20:5 n-3	0.00	0.00	0.01	0.01	n.d.	n.d.	0.01	0.04	n.d.
	C 22:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01	n.d.	n.d.
	C 22:5 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C 22:6 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Saturated	61.75	61.71	61.69	62.17	61.81	61.87	61.33	62.03	62.04
	Monounsaturated	34.42	34.78	34.83	34.55	34.86	34.60	34.91	34.68	34.71
	Polyunsaturated	3.82	3.49	3.48	3.29	3.30	3.54	3.75	3.27	3.24
	Trans	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

n.d: not determined

refore, the Casson model was appropriate to describe the rheological characteristics of the milk chocolates. In both group BT and AT, the viscosity of probiotic and synbiotic samples was higher than control ($P < 0.05$). Concerning higher inulin ratio increased the viscosity value of samples. Similarly, Rezende et al. (2015) reported that increasing the inulin content of chocolate caused plastic viscosity to increase. Viscosity increase based on inulin ratio was higher in group BT as compared to group AT. Except for sample P, the viscosity value of S6, S8, and S10 in group BT was higher than those of samples in group AT. When probiotic bacteria were added to the chocolate mixture before tempering, it could cause interaction between ingredients during tempering. This interaction may have led to a viscosity increase in chocolates. The viscosity value of samples in group BT decreased at the end of storage, whereas there was an increase in samples of group AT. In other words, whether the probiotic addition into chocolate before or after the tempering step had a significant effect on viscosity values depending on inulin ratio and storage time.

The probiotic and increasing ratio of inulin addition increased yield value when compared to control ($P < 0.05$). Also, the yield value of samples in group BT was higher than those of in the group of AT. As in viscosity, this may relate to the probiotic interaction effect on other ingredients during the tempering phase. During the storage, the yield value of samples increased, and this increase was higher in group AT compared to BT.

Hardness defines the physical rigidity of samples and directly affects sensory perception during eating. The hardness value of milk chocolate bars ranged from 91.25–143.84 N.s during the whole storage period. The hardness of all samples in group BT was higher than in control ($P < 0.05$). However, the value of P and S6 in group AT was lower than that of control. The hardness value of samples in group BT was higher than those of group AT. In both group AT and BT, an increase in inulin content of chocolates resulted in a higher hardness value. This may occur due to the water absorption characteristic of inulin (Shourideh et al., 2012). Depending on the storage, the hardness value of samples increased. This increase was higher in group AT as compared to group BT.

Viability of probiotic bacteria

Viability of *B. animalis* BB-12 in milk chocolates during 60 days of storage at 18°C is given in Figure 2. The probiotic count of probiotic and synbiotic samples ranged from 7.09–8.06 log cfu/g and was higher than 10^7 cfu/g at the end of the storage. This viable bacteria level was higher than the recommended count (≥ 10^6 cfu/g) for probiotic products. Higher viability may remain due to the protective effect of cocoa butter lipid fraction on bifidobacteria in chocolate (Burgain et al., 2011). Also, the BB-12 count of synbiotic samples increased with related to increasing ratio of inulin addition ($P < 0.05$). Because inulin is prebiotic and is a suitable substrate (bifidogenic) for bifidobacteria (Konar et al., 2014). Besides the

TABLE 5: *Rheological and textural characteristics of milk chocolates during the storage.*

				BT				AT			
Method	Parameters	Day	C	P	S6	S8	S10	P	S6	S8	S10
Rheology	Yield values (Pa)	1	3.025±0.003	3.096±0.008	4.454±0.005	6.076±0.004	8.071±0.016	3.270±0.022	3.896±0.008	3.867±0.016	4.238±0.017
		60	3.037±0.005	3.126±0.002	4.642±0.002	6.280±0.012	8.246±0.022	3.420±0.030	4.235±0.025	4.341±0.042	5.120±0.020
	Viscosity (Pa.s)	1	2.08±0.07	2.84±0.08	3.95±0.07	5.46±0.01	7.40±0.20	2.54±0.03	2.93±0.06	3.44±0.02	3.70±0.01
		60	2.10±0.03	2.73±0.07	3.23±0.04	3.90±0.01	4.18±0.04	2.78±0.04	3.17±0.06	3.54±0.00	3.91±0.01
	R^2	1	0.992±0.002	0.997±0.001	0.990±0.002	0.990±0.003	0.994±0.000	0.997±0.001	0.992±0.003	0.990±0.003	0.999±0.000
		60	0.996±0.000	0.995±0.002	0.993±0.003	0.996±0.002	0.998±0.002	0.998±0.000	0.996±0.001	0.995±0.002	0.992±0.003
Penetration	Hardness (N.s)	1	107.68±3.61	115.84±0.86	121.29±1.40	129.44±1.12	133.83±1.99	91.25±1.39	98.00±0.92	114.98±0.63	126.93±1.84
		60	113.40±1.19	116.61±1.34	121.87±2.35	131.32±0.98	133.59±2.13	95.31±1.27	104.88±0.50	126.43±1.14	143.84±1.96

$\bar{X}$ ± standard deviation, n = 3

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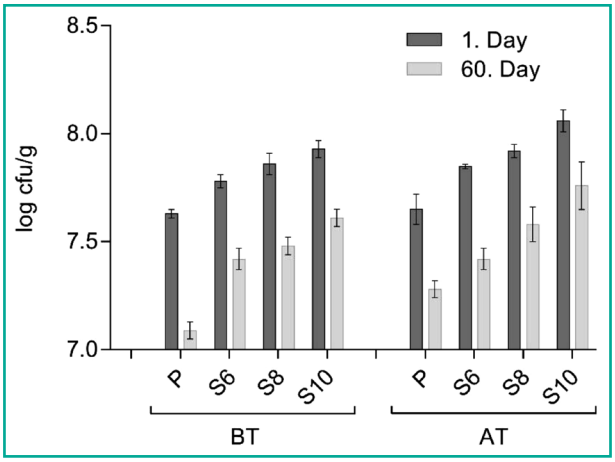

FIGURE 2: *Viability of B. animalis BB-12 in milk chocolates during 60 days of storage at 18°C.*

prebiotic effect of inulin, the sugar content of samples was decreased based on the inulin ratio. This is important for human health due to the lowering of calories. Probiotic addition after tempering slightly increased the viability of probiotic bacteria and improved viability at the end of storage. This can be related to tempering temperature and process. Therefore, probiotic addition to chocolate mixture after the tempering stage could be suitable in terms of the viability of probiotic bacteria. However, viable bacteria count decreased depending on the storage period as expected. The obtained results indicated that *B. animalis* BB-12 is capable of surviving in milk chocolate, at an appropriate bacteria count (>10^7), up to 60 days of storage at 18°C. To achieve and maintain functional properties in synbiotic chocolate, the viability of probiotic bacteria is the crucial factor.

Melting characteristics of milk chocolates

Table 6 illustrates the melting parameters of milk chocolates at the end of storage. At T_{onset} a specific crystal form starts to melt; melting is completed at T_{end} and ΔH indicates the amount of energy required to complete the liquefaction (Afoakwa et al., 2008a). The composition and crystalline state of samples affect peak height, position, and resolution (Becket, 2008). As evaluated values obtained from the fat peak, the T_{onset} value of samples ranged from 31.91–34.17°C. T_{onset} of the sample P in group BT and AT was higher than synbiotic chocolates. Similar results were obtained for T_{peak} values. However, the T_{end} of sample P in group BT and AT was lower than synbiotic chocolates. In other words, inulin addition increased T_{end} as compared to probiotic chocolate. In synbiotic chocolates, T_{index} was higher than control and probiotic samples. ΔH values of samples ranged from 11.72–19.87 J/g. The highest ΔH value was obtained in the control sample. Afoakwa et al. (2008a) reported that in dark chocolates, T_{onset} and T_{end} ranged from 26.0–26.9°C and 33.0–34.6°C, respectively. These values were lower than those of this study. These differences may be caused by milk fat content.

As evaluated values obtained from sugar peak, the T_{onset} value of samples ranged from 133.53–145.57°C. Probiotic addition before or after tempering did not significantly affect T_{peak} and T_{end}. Glicerina et al. (2013) reported that T_{onset} and T_{end} values of sugar melting of dark chocolate ranged from 174.7–181.0 and 189.7–192°C, respectively. The cristallinity of sugar can affect the melting properties of chocolates. The T_{index} value of samples ranged from 20.64–28.84°C. The highest T_{index} value was obtained in the control sample. ΔH values of control and probiotic samples were higher than synbiotic chocolates ($P < 0.05$). This may be due to the sugar content of samples. Because sugar content decreased with increasing addition ratio of inulin. Also, the presence of a solvent in chocolate samples including water affects the melting and crystallization characteristics of sugar. Therefore, it alters the melting properties of chocolates (Beckett et al., 2006; Bhandari and Hartel, 2002).

Conclusions

In this study, different ratios (6, 8, and 10%) of inulin and *B. animalis* BB-12 (before tempering, BT or after tempering, AT) were added to the chocolate mixture to produce synbiotic milk chocolate. Probiotic and/or inulin addition decreased L* value of samples. Higher inulin ratio increased viscosity and yield value of samples. The results revealed that symbiotic milk chocolate is a potential vehicle for BB-12 by keeping bacteria viable for up to 60 days at 18°C. Also, higher inulin content resulted in increased viable probiotic count in chocolates. Based on the findings of the present study, it can be concluded that milk chocolate mass can be successfully enriched with probiotic strains *B. animalis* and prebiotic inulin. It was concluded that probiotic addition to chocolate mixture after the tempering stage could be suitable in terms of the viability of probiotic bacteria. Addition of probiotic at this stage of industrial production facilitates the manufacturing process, improves the overall quality of milk chocolate and maintains viability of the probiotics as crucial ingredient of symbiotic or probiotic chocolates.

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TABLE 6: *Melting characteristics of milk chocolates at the end of storage.*

			BT				AT			
Method	**Parameters**	**C**	**P**	**S6**	**S8**	**S10**	**P**	**S6**	**S8**	**S10**
Fat	T_{onset} (°C)	33.29	34.17	31.91	33.32	32.24	33.30	31.95	32.50	31.72
	T_{peak} (°C)	37.39	37.35	36.22	36.41	36.95	37.39	36.17	36.11	35.87
	T_{end} (°C)	39.68	38.30	39.79	39.72	40.95	39.69	40.18	39.50	39.71
	T_{index} (°C)	6.39	4.13	7.88	6.40	8.71	6.39	8.23	7.00	7.99
	ΔH (J/g)	19.87	11.72	18.70	18.80	15.66	19.55	16.44	18.60	18.04
Sugar	T_{onset} (°C)	135.97	142.20	140.17	137.45	141.84	138.81	145.57	133.53	143.09
	T_{peak} (°C)	159.75	160.17	157.37	155.02	155.67	160.07	160.33	155.34	157.53
	T_{end} (°C)	164.81	165.30	163.31	162.02	162.73	166.05	166.21	161.37	165.26
	T_{index} (°C)	28.84	23.10	23.14	24.57	20.89	27.24	20.64	27.84	22.17
	ΔH (J/g)	35.86	22.78	20.06	15.16	14.48	29.77	18.20	18.96	13.34

Conflict of interest

The authors declare that there are no conflicts of interest.

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