Combined effects of replacement of sucrose with D-tagatose and addition of different probiotic strains on quality characteristics of chocolate milk

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Abstract Nowadays, tendency to improve human nutrition and consume new healthful foods such as low-calorie and functional ones has been increased. In this study, effects of ratios of sucrose/D-tagatose (100:0, 0:100, or 50:50) as well as type of commercial probiotic strains (*Lactobacillus acidophilus* LAFTI L10, *Lactobacillus casei* LAFTI L26, *Lactobacillus rhamnosus* HN001, and *Bifidobacterium animalis* subsp. *lactis* LAFTI B94) on biochemical and microbiological characteristics, percent of residual sugar, color, and sensory attributes of synbiotic chocolate milk were investigated during 21 days of refrigerated storage (5 °C). The treatments inoculated with *L. acidophilus*, *L. casei*, *L. rhamnosus*, and *B. lactis* showed significantly higher biochemical and color changes compared to non-probiotic ones. The greatest viability at the end of storage was related to the treatment of D-tagatose with *L. rhamnosus* (T-R) as well as D-tagatose with *L. casei* (T-C). Although *L. acidophilus*, *L. casei*, and *L. rhamnosus* mostly tended to ferment D-tagatose, *B. lactis* did not substantially consume the mentioned sugar. In general, the treatments T-R, ST-R (sucrose and D-

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tagatose with *L. rhamnosus*), T-B (D-tagatose with *B. lactis*), and ST-B (sucrose and D-tagatose with *B. lactis*) were realized as the best ones in terms of probiotic viability, functional property of D-tagatose, and sensory attributes. In conclusion, D-tagatose could be successfully used as a natural sugar substitute with functional properties for probiotic chocolate milks enhancing their health benefits, but the proper selection of ratio of sucrose/D-tagatose and type of probiotic strain is recommended.

Keywords Chocolate milk · D-tagatose · Low-calorie · Probiotic · Synbiotic

1 Introduction

Foods that are not intended only to satisfy hunger and provide humans with necessary nutrients but also to provide medicinal characteristics (prevent nutrition-related diseases and increase physical and mental well-being of consumers) are called “functional foods” (Menrad 2003). One of the most promising ways for development of functional foods is using probiotics, prebiotics, and synbiotics.

Probiotics are special types of live healthful microorganisms which possess favorable impacts on animal and human host mainly via maintaining and/or improving microbial balance between harmful and beneficial microbiota, especially in the intestine (Korbekandi et al. 2011). They have an established role in reducing human illnesses, particularly gastrointestinal infections caused by deficient or compromised gut microbiota. Other therapeutic functions are attributed to probiotics such as anti-cholesterol activity, alleviation of lactose intolerance symptoms, promotion of beneficial immune responses, antimicrobial impact, anti-high blood pressure effect, and anti-carcinogenic and anti-mutagenic activities (Shah 2007). Recently, there have been reports on potential benefits of probiotics for human skin (Krutmann 2009) and against colds and flu (Leyer et al. 2009).

Probiotics should be alive to an adequate number in order to exert their positive effects on health of the host. This attribute is known as “viability,” namely adequate number of live probiotic cells in a food product at time of consumption (Mortazavian et al. 2010). No general agreement has been established on the recommended and suggested levels ranging from 10^6 to over 10^7 cfu.mL⁻¹ (Ferdousi et al. 2013; Korbekandi et al. 2011; Mohammadi et al. 2011a; Mortazavian et al. 2010, 2011). However, it is generally recommended that probiotic culture must be present in the product at minimum numbers of 10^7 cfu.mL⁻¹ (IDF 1992). With respect to dairy products, a therapeutic minimum of 10^9 cfu.day⁻¹ and consumption of 100 g or ml should translate to a food containing at least 10^7 cells per gram or milliliter (Reid 2001), which is in agreement with current Japanese recommendations (Ishibashi and Shimamura 1993). These suggestions have been made to compensate possible decline in concentration of probiotic organisms during processing and storage of a probiotic product as well as passing through upper and lower parts of the gastrointestinal tract.

The most typical food matrices (vehicles) for probiotic bacteria are different kinds of fermented milk products, especially yogurt (Gueimonde et al. 2004). During the past few years, diversity of probiotic foods has increased on the market. Nowadays, probiotics can be found in non-fermented dairy products such as milk (Shin et al. 2000), ice cream (Mohammadi et al. 2011a) and dairy dessert (Mortazavian et al. 2011),

and non-dairy products such as fruit juices, cereal-based foods (Helland et al. 2004), and meat-based products (Rouhi et al. 2013).

In non-fermented liquid flavored milks, chocolate flavor is among the most popular ones (Yanes et al. 2002). Also, chocolate milk is an excellent sports recovery beverage and has a great amount of carbohydrates more than that of plain milk, along with protein, electrolytes, and other key nutrients, in quantities that commercial carbohydrate replacement drinks cannot match (Bayoumi et al. 2011).

D-tagatose, an epimer of D-fructose, has attracted a great deal of attention in recent years (used in nonchronic drugs, tooth paste, mouth wash, dietary supplements, and a wide variety of foods such as beverages, healthy foods, cereals, health bars, chocolate, candy, chewing gum, yogurt, soft drinks, and milk-based drink along with bakery and confectioneries) due to its health benefits and similar properties to sucrose (appropriate sweetness—92% of sucrose in 10% solutions—with a sucrose-like taste and no cooling effect or aftertaste). It has been also known as a flavor enhancer and a low caloric value sugar with tooth-friendly property (because of not being fermented by oral microflora) (FDA 2003; Kim 2004; Levin 2002). In the USA, tagatose is qualified as generally recognized as safe (GRAS) for use in foods under FDA-regulated program (Rulis 2001). Joint FAO/WHO Expert Committee on Food Additives (JECFA) stated that there is no need to limit the allowed daily intake (ADI) of tagatose (JECFA 2004). D-tagatose naturally occurs in *Sterculia setigera* gum and is found in small quantities in various foods such as sterilized and powdered cow milk, hot cocoa, and a variety of cheese, yogurt, and other dairy products. It is a highly soluble sugar and stable at pH 2–7 (Levin 2002).

One of the numerous health benefits attributed to D-tagatose is prebiotic effect (Bertelsen et al. 1999). While D-tagatose is not fermented by bacteria of the dental plaque, not even after repeated exposure, certain members of the more varied microbiota of the intestinal tract are able to utilize D-tagatose as a substrate. D-tagatose fermentation appears to be particularly widespread among lactobacilli, enterococci, and other lactic acid bacteria (Bertelsen et al. 2001). Studies on the prebiotic activity of D-tagatose confirmed that D-tagatose promotes the growth of lactobacilli and leads to an increased production of butyrate which is considered to have a beneficial trophic effect on the colonic mucosa (Jensen BB, Buemann B 1998; Venema et al. 2005).

No study was found on investigating survival of probiotic strains added to chocolate milk, especially prebiotic chocolate milk, during storage and its impact on attributes of the product. The hypothesis was that formulation of probiotic chocolate milk containing D-tagatose on the one hand would lead to a highly functional dairy product (synbiotic food with proper impact on probiotics viability); on the other hand, it could provide an acceptable vehicle for probiotic bacteria (with acceptable sensory properties).

2 Materials and methods

2.1 Starter culture

The DVS pouches of commercially lyophilized single probiotic cultures including *Lactobacillus acidophilus* LAFTI L10 (DSM, Australia), *Lactobacillus casei* LAFTI

L26 (DSM, Australia), *Lactobacillus rhamnosus* HN001 (Danisco, Denmark), and *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) LAFTI B94 (Danisco, Denmark) were used. These starter cultures are widely used in the dairy industry. The cultures were maintained according to the manufacturer's instructions until used.

2.2 Sample preparation

Milk with 15.0% dry matter was formulated using reconstituted skim milk powder (Anchor, New Zealand) and sterilized distilled water. Other ingredients including 1.0% alkalized cocoa powder with fat content of 10–12% (Schokinag, Germany) and 0.03% κ -carrageenan (Sigma, USA) were added. Then, three treatments with different ratios of sucrose/D-tagatose (100:0, 50:50, and 0:100) were produced. In 100:0 treatments, 6.5% of sucrose (Chem-Lab, Belgium) was added to chocolate milk. Considering relative sweetness of D-tagatose (0.92), 7.06% of D-tagatose (Damhert, Belgium) was added to 0:100 treatments and 3.25% of sucrose and 3.53% of D-tagatose were added to 50:50 treatments. After heat treatment (90 °C, 15 min), homogenization (2 stages, 200 and 50 bars at 90 °C), and cooling to 15 °C, the treatments were inoculated in a hygienic condition with one of the probiotic single strain cultures (*L. acidophilus* L10, *L. casei* L26, *L. rhamnosus* HN001, or *B. lactis* B94) at initial concentration of 10^8 cfu.mL⁻¹. Also, the treatments without inoculated probiotic were produced as control. Chocolate milk was packed in a hygienic condition and stored for 21 days at refrigerated temperature (5 °C). The treatments with 100:0, 0:100, and 50:50 ratios of sucrose/D-tagatose were nominated as S, T, and ST, respectively. Those inoculated with *L. acidophilus* L10, *L. casei* L26, *L. rhamnosus* HN001, and *B. lactis* B94 were identified as A, C, R, or B, respectively. Therefore, 15 treatments were produced (S, T, and ST as controls, S-A, T-A, ST-A, S-C, T-C, ST-C, S-R, T-R, ST-R, S-B, T-B, and ST-B). pH, titrable acidity, redox potential, and viability of probiotics were monitored at day 0 as well as per 7-day intervals during 21 days of the refrigerated storage. Percent of the residual sugars, color, and sensory properties was studied only at days 0 and 21. The residual sugars and biochemical changes were indicators for monitoring metabolic activity of probiotics in chocolate milk.

2.3 Microbiological analysis

Serial decimal dilutions were prepared with Ringer's solution (Ringer's tablets from Merck, Germany). MRS agar medium (Merck, Germany) was used for enumeration of probiotics. The plates were incubated aerobically (for lactobacilli) and anaerobically (for bifidobacteria) at 37 °C for at least 72 h (Mortazavian et al. 2010). Anaerobic conditions were produced using the GasPac system (Merck, Germany) in anaerobic jars.

2.4 Biochemical analysis

pH values and redox potential of the samples were measured at room temperature using a pH meter (MA235, Mettler, Toledo, Switzerland). Titrable acidity was determined according to the modified method of Mortazavian et al. (2010) after mixing 10 mL of the sample with 40 mL of distilled water and titrating with 0.1 N NaOH using 0.5% phenolphthalein.

Parameters of mean pH drop rate, mean acidity increase rate, and mean redox potential increase rate during storage time were calculated as follows (Mohammadi et al. 2011b):

$$\text{pH drop rate} = (\text{final pH value} - \text{initial pH value}) / \text{storage time} [\text{pH value} \cdot \text{day}^{-1}]$$

Acidity increase rate

$$= (\text{final acidity value} - \text{initial acidity value}) / \text{storage time} [\text{Dornic degree} \cdot \text{day}^{-1}]$$

$$\text{Redox potential increase rate} = (\text{final value} - \text{initial value}) / \text{storage time} [\text{mV} \cdot \text{day}^{-1}]$$

Time range of maximum pH drop ($t_{\text{max-pH-D}}$) : 7-day time interval during refrigerated storage, in which the greatest pH decline is observed [day-day]

2.5 Chemical analysis

Lactose, sucrose, and D-tagatose were determined according to the method of Bogdanov and Baumann (1988). After filtering the solution, sugar content was determined by HPLC with a column of 4.6 mm internal diameter and 250 mm length, containing amine-modified silica gel with 5–7 μm particle size, Develosil (Nomura Chemical Co., Japan). Column and detector temperature were 30 °C, mobile phase was degassed acetonitrile:water (80:20, v/v), and flow rate and sample volume were 1.3 mL.min⁻¹ and 10 μL , respectively. Peaks were identified on the basis of their retention times. Standards of lactose, sucrose, and D-tagatose were supplied from Sigma (USA). Quantification was performed according to the external standard method on peak areas or peak heights. Determinations were performed on a Waters HPLC (USA) using Waters Refractive Index detector (USA). Percent recoveries were regarded in calculating percentage of sugars. Percent decrease in each type of sugar (X) during the storage was calculated as follows:

$$\text{Percent decrease of } X = \% X_{\text{day0}} - \% X_{\text{day21}}$$

2.6 Color analysis

Color was measured using a Hunter Lab colorimeter (ColorFlex, Model no. 45/0, Hunter Lab Reston, Virginia, USA). 'L'=lightness is expressed as dark to light color. Green to red is expressed by 'a' value, and blue to yellow is expressed by 'b' value. Three readings of each parameter were taken for each sample. C values, which were calculated using $C = (a^2 + b^2)^{1/2}$ denote cumulative measurement of chromaticity or saturation, as determined by a and b values. ΔE (total difference) values were

computed using $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$ where $\Delta L = L_{\text{day21}} - L_{\text{day0}}$ and the same was for Δa and Δb . ΔE values provide cumulative measurement of total color changes as determined by changes in L , a , and b values during 21 days of storage.

2.7 Sensory analysis

Sensory testing was conducted in compliance with “Committee of Morality in Research” in “National Nutrition and Food Technology Research Institute= NNFTRI” (Iran) and the panelists were requested to complete the human subjects’ consent form. Twelve panelists were selected based on interest, time availability, and knowledge of dairy associated with sensory parameters. The panelists were selected from among faculty members, staff, and students of the NNFTRI. The panel was composed of eight male and four female individuals with mean age of 27 years old. The samples (100 mL) were served at 5 ± 2 °C in 150 mL crystalline plastic cups fitted with white plastic lids and labeled with 3-digit codes to remove bias. The packages were shaken uniformly before the analysis in order to re-suspend any settled cocoa particles in the bottom. The panelists individually evaluated five chocolate milks per session individually in an odor-free room under white fluorescent lights (70 ft.c^{-1} intensity) on a white Lab bench dedicated to sensory analysis.

Sensory evaluation was carried out at ambient temperature about 2 h before or after meals. Potable water was available for palate cleansing. The panelists received a tray containing five samples, a glass of water, an evaluation form, and a pencil. They were allowed to rest between trays to prevent fatigue. Chocolate milk formulations were compared using “scoring methodology” (Drake 2009). Sensory attributes included flavor (taste and odor), mouthfeel, appearance (color, visible purity, presence of foreign matter, spots of mold, and phase separation), and total score. The panelists scored the samples using a five-point hedonic scale, where 0=unconsumable, 1=unacceptable, 2=acceptable, 3=satisfactory, and 4=excellent. To calculate total score with weighted average, the given numbers for each sensory parameter were multiplied by the relevant coefficients, namely 6 for flavor, 3.5 for mouthfeel, and 2 for appearance.

2.8 Statistical analysis

The experiments were performed in triplicate and interactive effect of the variables and the ranked orders of means were determined using two-way “Analysis of Variance (ANOVA test)” at significance level of “ $P < 0.05$ ” from Minitab software. The design of study was “completely randomized design (Full Factorial).”

3 Results and discussion

3.1 Biochemical characteristics during refrigerated storage

According to Fig. 1a–c, redox potential was very low and negative at the beginning of storage, which could be attributed to discharging much oxygen content from chocolate milk due to severe heat treatment as well as dissolving sugars.

Table 1 shows mean pH drop rate, mean acidity increase rate, mean redox potential increase rate, time range of maximum pH drop, and initial and final titrable acidity in different treatments during the refrigerated storage. As shown in Fig. 1a–c and Table 1, non-probiotic treatments had no significant biochemical changes during the refrigerated storage ($P>0.05$). In contrast, addition of probiotics made significant changes in the mentioned parameters. The amount of changes depended on strain of inoculated probiotic. The chocolate milk inoculated with *L. acidophilus* showed the greatest biochemical changes (mean pH drop rate, mean titrable acidity increase rate, and mean redox potential increase rate) during the storage ($P<0.05$). Afterward, *L. casei*, *L. rhamnosus*, and *B. lactis* were placed, respectively. *L. acidophilus* and *L. casei* have high proteolytic activities (Sasaki et al. 1995) that improve their acidification rate. Proteolytic activities of probiotics are directly proportional to their acidification rate (Azcarate-Peril et al. 2005). Bifidobacteria are generally very poor fermentative probiotics, particularly at refrigerated temperatures (Lankaputhra et al. 1996) and cause slow pH decrease.

Fig. 1 a Changes in biochemical characteristics of treatments with no substitution of sucrose during 21 days of refrigerated storage. b Changes in biochemical characteristics of treatments with full substitution of sucrose with D-tagatose during 21 days of refrigerated storage. c Changes in biochemical characteristics of treatments with partial substitution of sucrose with D-tagatose (50:50) during 21 days of refrigerated storage

Compared with sucrose, D-tagatose caused greater biochemical changes in all probiotic-containing treatments (lactobacilli), except bifidobacteria. The highest biochemical changes as well as highest final titrable acidity were related to T-A and the lowest ones were attributed to T-B. Therefore, D-tagatose stimulated growth and/or activity of lactobacilli probiotics more than sucrose. *L. acidophilus* was the most active probiotic in chocolate milk during the storage time. Time range of maximum pH drop ($t_{\max\text{-pH-D}}$) was different among probiotic chocolate milk during the storage. This parameter was day 14–21 of storage in treatments inoculated with *L. acidophilus* and *L. casei*, whereas day 0–7 in those with *L. rhamnosus* and *B. lactis*, meaning that *L. rhamnosus* and *B. lactis* showed peak of activity during 7 days of storage, followed by lack of activity until the end of this period. The aforementioned observations are illustrated in Fig. 1a–c.

3.2 Viability of probiotic bacteria during the refrigerated storage

Table 2 shows viability of probiotic microorganisms in different treatments during 21 days of the refrigerated storage. As represented by this table, viability of all probiotic

Fig. 1 continued.

lactobacilli was significantly greater in tagatose-containing treatments, followed by mixture of sugars, which was in line with data of Table 1, in which D-tagatose caused greater pH drop rate and acidity increase rate in lactobacilli-containing treatments compared to sucrose (Section 3.1). For bifidobacteria, the highest and lowest viability was related to sucrose-containing and tagatose-containing treatments, respectively, which revealed that bifidobacteria was not active in the presence of D-tagatose compared to sucrose (Section 3.3). Therefore, the greatest viability throughout the storage time was related to D-tagatose with *L. acidophilus* (T-A), D-tagatose with *L. casei* (T-C), D-tagatose with *L. rhamnosus* (T-R), and sucrose with bifidobacteria (S-B).

From day 14 onwards, probably due to increase in titrable acidity and reduction in pH and sensitivity of probiotics to acidic media (Ventura et al. 2011), viability of lactobacilli in the presence of D-tagatose as well as bifidobacteria in the presence of sucrose considerably decreased. According to Table 1, this might be because time range of the maximum pH drop for *L. acidophilus* was day 14–21. Within this time interval, *L. acidophilus* showed the greatest biochemical changes during storage that led to harsher conditions and the highest decrease in viability after day 14. The present results were in agreement with those by Nighswonger et al. (1996) who reported that

Fig. 1 continued.

Table 1 Biochemical parameters of different treatments during 21 days of refrigerated storage

Treatments ^a	M-pH-DR (1.day ⁻¹)	M-A-IR (°D.day ⁻¹)	M-RP-IR (mV.day ⁻¹)	<i>t</i> _{max-pH-D} (day-day)	Initial titrable acidity (°D)	Final titrable acidity (°D)
S	0.000k	0.00k	0.00l	—	15.4ab	15.1i
T	0.000k	0.00k	0.00l	—	16.3a	14.4i
ST	0.000k	0.00k	0.00l	—	15.6ab	15.5hi
S-A	0.095b	2.39b	5.00b	14–21	15.4ab	65.6b
T-A	0.099a	2.56a	5.42a	14–21	16.3a	70.2a
ST-A	0.094b	2.35c	4.90c	14–21	15.6ab	65.0b
S-C	0.051d	0.85e	2.80e	14–21	15.4ab	33.3d
T-C	0.054c	1.02d	3.23d	14–21	16.3a	37.8c
ST-C	0.050d	0.83e	2.76e	14–21	15.6ab	33.2d
S-R	0.016g	0.45g	0.95h	0–7	15.4ab	24.9ef
T-R	0.025e	0.53f	1.61f	0–7	16.3a	27.6e
ST-R	0.019f	0.44g	1.04g	0–7	15.6ab	25.0ef
S-B	0.012h	0.26h	0.85i	0–7	15.4ab	21.0g
T-B	0.002j	0.12j	0.23k	0–7	16.3a	18.9gh
ST-B	0.010hi	0.16i	0.52j	0–7	15.6ab	19.1g

Means in the same column shown with different letters are significantly different ($P < 0.05$)

^a S treatments with ratio of the sucrose/D-tagatose: 100:0, T those with ratio of the sucrose/D-tagatose: 0:100, ST those with ratio of the sucrose/D-tagatose: 50:50, A *L. acidophilus* L10, C *L. casei* L26, R *L. rhamnosus* HN001, B *B. lactis* B94, M-pH-DR mean pH drop rate, M-A-IR mean acidity increase rate, M-RP-IR mean redox potential increase rate, *t*_{max-pH-D} time range of maximum pH drop

refrigerated storage stability of *L. casei* as an adjunct in fermented products was equal to or greater than that of strains of *L. acidophilus* tested on day 21 or 28 of refrigerated storage at 5 to 7 °C. Gilliland and Lara (1988) reported that some cells of *L. acidophilus* which failed to form colonies on the enumeration medium still possessed β-galactosidase activity. The lactobacilli contain either β-galactosidase and/or β-phosphogalactosidase as the enzyme responsible for enabling the organism to utilize lactose (Premi et al. 1972). Therefore, metabolic enzymes yielded from dead probiotics might increase fermentation ability of residual adapted and viable cells resulting in higher biochemical changes, which could cause more continuous declines in probiotics viability.

According to Fig. 1a–c and Table 1, time range of the maximum pH drop for biochemical changes in the treatments containing *L. rhamnosus* or *B. lactis* was on days 0–7 of storage. Antimicrobial effects of the organic acids produced during initial week of storage could be responsible for the afterward decline in viability of the mentioned probiotics. As can be observed in Table 2, the most decrease in viability of *L. rhamnosus* and *B. lactis* on days 14–21 of storage was related to D-tagatose- and sucrose-containing treatments, respectively. Another probable reason for considerable decrease of bifidobacteria viability could be increase in oxygen level of the product via permeation through the package during storage. If a minimum of 10^7 cfu.mL⁻¹ viable counts of probiotics were considered standard level, all the treatments would have viability of higher than this level through 21 days of refrigerated storage.

Table 2 Viable counts (log cfu.mL⁻¹) of probiotic microorganisms in different treatments during 21 days of refrigerated storage

Treatments ^a	Storage time (day)			
	0	7	14	21
S-A	8.02aD	9.49bB	9.97dA	8.92dC
T-A	8.02aD	9.89aB	10.93aA	9.04bC
ST-A	8.00aD	9.51bB	10.66cA	8.94cdC
S-C	8.01aD	8.88deC	9.54eA	8.99bcB
T-C	7.98aD	9.85aB	10.83bA	9.23aC
ST-C	8.02aD	9.36cB	9.99dA	8.86deC
S-R	7.99aC	8.49fgB	8.75hiA	8.79eA
T-R	8.02aD	8.85eC	9.32fA	9.27aAB
ST-R	8.01aC	8.55fB	8.81hA	8.82eA
S-B	7.99aB	8.97dA	8.95gA	7.47fC
T-B	7.98aB	8.44ghA	7.78kC	6.89hD
ST-B	8.00aC	8.95dA	8.51jB	7.36gD

Means shown with different small and capital letters represent significant differences ($P < 0.05$) in the same columns (among the treatments) and rows (between the days of storage), respectively

^a S treatments with ratio of the sucrose/D-tagatose 100:0, T those with 0:100, ST those with 50:50, A *L. acidophilus* L10, C *L. casei* L26, R *L. rhamnosus* HN001, B *B. lactis* B94

3.3 Residual sugars in different treatments during 21 days of refrigerated storage

Percentage of lactose, sucrose, and D-tagatose in different chocolate milk on day 0 and day 21 of storage as well as their decrease percent is shown in Table 3. Non-probiotic treatments in contrast to probiotic ones had no significant change in sugar amounts during the refrigerated storage. Therefore, addition of different probiotic microorganisms to chocolate milk led to significant decrease in the amount of all sugars. It should be noted that consumption pattern was different between the strains. Garro et al. (1999) studied the refrigerated shelf life of soymilk fermented with single cultures of *Lactobacillus fermentum*, *L. casei*, *Streptococcus salivarius* ssp. *thermophilus*, and *Bifidobacterium longum*. They reported that *L. casei* showed a high rate of sucrose utilization during the refrigerated storage at 4 °C for 28 days. According to results of the present study, both *L. acidophilus* and *L. casei* mostly tended to ferment D-tagatose, lactose, and sucrose, respectively. In the research by Garro et al. (1999), lactose and D-tagatose were not present in the environment. Also, *L. casei* CRL 207, different strain from the present study, was used. Moreover, soymilk matrix was very dissimilar to that of chocolate milk.

L. rhamnosus mostly fermented D-tagatose, sucrose, and lactose, respectively, and this order for *B. lactis* was sucrose, lactose, and D-tagatose for *B. lactis*. *B. lactis* did not significantly consume D-tagatose ($P > 0.05$). These results have been confirmed by several studies. Roy and Ward (1990) determined fermentation patterns of 20 *Bifidobacterium* strains by two methods: a carbohydrate utilization test based on acid production from a given range of sugars in the modified MRS broth (micromethod) and

Table 3 Residual sugars in different treatments during 21 days of refrigerated storage

Treatments ^a	Lactose (%)		Sucrose (%)		D-Tagatose (%)		Decrease in total sugar
	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21	
S	7.81aA	7.80aA	6.51aA	6.50aA	—	—	0.02
T	7.79aA	7.79aA	—	—	7.06aA	7.06aA	0
ST	7.82aA	7.80aA	3.24bA	3.24dA	3.51bA	3.51eA	0.02
S-A	7.80aA	7.25fB	6.52aA	6.04cB	—	—	1.03
T-A	7.80aA	7.46deB	—	—	7.07aA	6.31dB	1.1
ST-A	7.82aA	7.53dB	3.25bA	3.11eB	3.53bA	2.87hB	1.09
S-C	7.81aA	7.45deB	6.49aA	6.48aA	—	—	0.37
T-C	7.79aA	7.66bcB	—	—	7.05aA	6.76cB	0.42
ST-C	7.79aA	7.72abAB	3.26bA	3.23dA	3.54bA	3.29gB	0.35
S-R	7.82aA	7.71bB	6.50aA	6.39bB	—	—	0.22
T-R	7.80aA	7.71bB	—	—	7.08aA	6.91bB	0.26
ST-R	7.82aA	7.76aAB	3.23bA	3.20dA	3.53bA	3.42fB	0.2
S-B	7.78aA	7.75abA	6.50aA	6.43abB	—	—	0.1
T-B	7.81aA	7.75abAB	—	—	7.06aA	7.05aA	0.07
ST-B	7.80aA	7.77aA	3.25bA	3.17deB	3.55bA	3.52eA	0.14

Means shown with different small and capital letters represent significant differences ($P < 0.05$) in the same columns (among the treatments) and rows (in each type of sugar, between the days of storage), respectively

^a S treatments with ratio of the sucrose/D-tagatose 100:0, T those with 50:50, ST those with 0:100, ST those with 50:50, A *L. acidophilus* L10, C *L. casei* L26, R *L. rhamnosus* HN001, B *B. lactis* B94

gas chromatographic (GC) analysis of fermented modified MRS broth. They reported that sucrose was used by all strains, except *Bifidobacterium bifidum*. Also, lactose present in the culture medium ensured growth of each strain of bifidobacteria. However, tagatose was not fermented by any of the used *Bifidobacterium* strains. Hoyles et al. (2002) reported the results of a polyphasic taxonomic study on some bifidobacteria such as *B. lactis* CCUG 37979^T from human sources. Using API systems, acid was produced from L-arabinose, lactose, mannose, maltose, melibiose, raffinose, ribose, sucrose, and trehalose, but not from D-arabitol, cyclodextrin, pullulan, glycogen, mannitol, methyl β -D-glucopyranoside, sorbitol, and tagatose. Bertelsen et al. (2001) screened a number of 174 normal or pathogenic human enteric bacteria and dairy lactic acid bacteria for D-tagatose fermentation by incubation for 48 h. Only a few normally occurring enteric human bacteria were able to ferment D-tagatose, among which *Enterococcus faecalis*, *Enterococcus faecium*, and *Lactobacillus* strains can be mentioned. D-tagatose fermentation seems to be common among lactic acid bacteria. According to Bertelsen et al. (2001), most of the analyzed dairy lactic acid bacteria fermented D-tagatose, and among which *Lactobacillus*, *Leuconostoc*, and *Pediococcus* strains most strongly fermented this sugar; after them, *Enterococcus*, *Streptococcus*, and *Lactococcus* were placed. None of the analyzed *Bifidobacterium* strains such as *B. lactis* BB-12 fermented tagatose. However, Alander et al. (2001) reported that *B. lactis* BB-12 weakly fermented tagatose.

Among probiotic chocolate milk, the lowest and greatest consumption of total sugars during 21 days of refrigerated storage were in treatments T-B and T-A, respectively. Also, according to Fig. 1a–c and Table 1, these treatments had the lowest and highest biochemical changes among probiotic treatments at the end of storage, respectively. Therefore, there was a correlation between total consumed sugars and produced organic acids, which was in agreement with the findings of Garro et al. (1999). In probiotic-containing treatments, the highest decrease in lactose, sucrose, and D-tagatose was related to treatments S-A, S-A, and T-A, and the lowest decrease of the mentioned sugars was for S-B and ST-B, S-C, and T-B, respectively.

3.4 Color properties during refrigerated storage

Table 4 shows parameters of color scales in all the treatments during the refrigerated storage. As represented by this table, all the probiotic treatments showed more *L*, *b*, and *C* values than non-probiotic ones on day 0 ($P < 0.05$). Therefore, initial inoculation of probiotics at concentration of 10^8 cfu.mL⁻¹ affected these color scales. It would be noted that whiteness of milk results from scattering visible light by suspended particles, mostly casein micelles (Owens et al. 2001). Also, bacterial cells present at high concentrations (e.g., 10^7 cfu.mL⁻¹) might act as light-scattering particles (Owens et al. 2001) and increase *L* value. The present results were in agreement with those of Owens et al. (2001), who showed that both bacteria-treated milks (with *Lactococcus lactis* ssp. *lactis* or *Propionibacterium freudenreichii* ssp. *shermanii*) had significantly higher *b* values than nonfat milk, suggesting a shift from blue to more yellow hues. In the present results, *a* values in the treatments inoculated with each probiotic bacteria showed a special pattern compared to non-probiotic ones at the start of storage. Owens et al. (2001) suggested that bacterial cell characteristics, such as morphology, could influence color attributes of milk, namely *a* values.

Table 4 Parameters of color scales in different treatments during 21 days of refrigerated storage

Treatments ^a	L		a		b		C ^c		ΔE^f
	Day 0	Day 21	ΔL^b	Day 0	Day 21	Δa^c	Day 0	Day 21	
S	43.20bA	42.45cdA	-0.75	7.69bcA	7.24cA	-0.45	10.96	10.85	0.91
T	43.21bA	43.14cA	-0.07	7.67bcA	8.08bcA	0.41	11.35	11.62	0.41
ST	42.28bA	42.34cdA	0.06	7.94bA	8.39bAB	0.45	11.77	11.73	0.66
S-A	45.25aB	49.95aA	4.7	7.01cB	8.07bcA	1.06	11.42	12.28	4.82
T-A	44.85abB	49.13aA	4.28	6.77cB	7.62cA	0.85	11.66	12.59	4.39
ST-A	44.28abB	48.85aA	4.57	7.90bAB	8.46bA	0.56	12.69	13.39	4.62
S-C	45.22aA	42.28cdB	-2.94	7.81bcA	7.37cA	-0.44	12.56	11.79	3.03
T-C	45.28aA	41.87cdB	-3.41	8.35bA	8.75bA	0.4	12.70	13.67	3.56
ST-C	44.47abA	42.68cAB	-1.79	8.11bA	8.13bcA	0.02	12.48	12.78	1.83
S-R	45.19aA	44.65bcA	-0.54	9.67aA	9.01abAB	-0.66	13.21	13.18	1.05
T-R	46.15aA	44.11bcB	-2.04	9.42aAB	9.99aA	0.57	13.35	13.98	2.14
ST-R	44.68abA	43.59cAB	-1.09	9.44aA	9.20abA	-0.24	13.68	12.99	1.32
S-B	46.20aA	45.80bA	-0.4	7.69bcA	7.18cAB	-0.51	12.07	11.70	0.65
T-B	45.21aA	44.24bcA	-0.97	7.67bcA	7.42cA	-0.25	12.33	11.84	1.09
ST-B	45.28aA	45.64bA	0.36	7.85bA	7.63cA	-0.22	12.51	11.83	0.81

Means shown with different small and capital letters represent significant differences ($P<0.05$) in the same columns (among the treatments) and rows (in each color scale, between the days of storage), respectively

^a S Treatments with ratio of the sucrose/D-tagatose 100:0, T those with 0:100, ST those with 50:50, A *L. acidophilus* L10, C *L. casei* L26, R *L. rhamnosus* HN001, B *B. lactis* B94

^b $\Delta L = L_{\text{day21}} - L_{\text{day0}}$

^c $\Delta a = a_{\text{day21}} - a_{\text{day0}}$

^d $\Delta b = b_{\text{day21}} - b_{\text{day0}}$

^e $C = (a^2 + b^2)^{1/2}$

^f $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$

L , a , and b values were stable in non-probiotic chocolate milk and those with *B. lactis* during storage ($P>0.05$). L , a , and C values increased in the treatments inoculated with *L. acidophilus* during the storage and no change occurred in b value. However, those with *L. casei* or *L. rhamnosus* showed decrease in L value with no changes in a and b values. The cause might be different final pH in different treatments during the storage. Rankin and Brewer (1998) compared inoculated and fermented nonfat milks (pH 4.4) to nonfat, 2% milk fat, and whole milk with instrumental and sensory color measurements. They concluded that L and a values for fermented nonfat milks were higher than nonfat milk, but fermentation had no effects on b value. Also, a trained sensory panel scored whiteness of fermented milks as whiter than nonfat milk. Owens et al. (2001) reported that L values in milks at pH 4.0 and 4.6 were higher than the nonfat milk and pH 5.0 and 6.0 samples. Also, nonfat milks acidified to pH 5.0 displayed the lowest L values. According to the present results, pH of all the treatments inoculated with *L. acidophilus* was near 4.6, but those with *L. casei* or *L. rhamnosus* were above 5. At pH of about 4.6, isoelectric point of the casein micelle was reached, resulting in self-association of micelles. Therefore, average particle size sufficiently increased to alter light-reflecting properties (Walstra 1990).

Decrease in L value at pH of about 5.0 is most likely attributable to decreased size of particles contributed by the altered casein micelles. The mentioned range of pH represents the one at which colloidal calcium phosphate from the casein micelle structure is maximally solubilized into aqueous phase, allowing for dissociation of casein micelle protein subunits into serum phase of milk and increases porosity of casein micelles (Guinee et al. 1993). Roefs et al. (1985) demonstrated a casein micelle size minima at pH 5.2, resulting from dissociation of several casein protein fractions from the micelle structure due to increased solubility at this pH. Both a and b values in the treatments with *L. casei* or *L. rhamnosus* were stable and relatively unaffected by dissolution of calcium phosphate from the casein micelle at pH 5.0. However, a value increased in those with *L. acidophilus*, which was probably affected by self-association of the casein micelles at pH near isoelectric point of 4.6. These results were in contrast with the findings by Owens et al. (2001).

Ratio of sucrose/D-tagatose did not show any single effect on color scales during the refrigerated storage and its effects were in combination with effects of type of inoculated probiotics. According to Table 4, the treatments inoculated with *L. acidophilus*, *L. casei*, *L. rhamnosus*, *B. lactis*, and non-probiotic ones showed the highest to lowest ΔE value, respectively. It is interesting to note that this order was similar to that of biochemical changes (Section 3.1). Rankin and Brewer (1998) also reported that ΔE values for the fermented milks were higher than the non-fermented ones.

As shown in Table 4, L and a values showed more changes during the storage than b value. Simultaneous evaluation of L and a values can be accomplished by a lightness-redness diagram. Figure 2 shows relative spatial positions of different chocolate milk on lightness-redness diagram at the start and end of the refrigerated storage. Examination of the present data in that manner (Fig. 2) demonstrated relative spatial positions of chocolate milk samples from color point of view, in which size of arrows indicated changes in L and a values during the storage. Therefore, the treatments inoculated with *L. acidophilus* had the most changes in both L and a values.

Fig. 2 Relative spatial positions of different chocolate milks on the lightness-redness diagram at the start and end of refrigerated storage

3.5 Sensory evaluation

Figure 3 indicates sensory characteristics of different chocolate milk (flavor, mouthfeel, appearance, and total score) at the start and end of the refrigerated storage. The panelists were unable to distinguish differences in color properties among the treatments and accepted all of them from color point of view (data not shown). As shown in Fig. 3, none of the treatments were significantly different in sensory characteristics at the start of storage ($P > 0.05$). Different ratios of sucrose/D-tagatose had no significant effect on sensory characteristics during the storage; however, type of inoculated probiotic had such an effect. The highest sensory scores at the end of storage belonged to control chocolate milk and then to those inoculated with *L. rhamnosus* and *B. lactis*. Therefore, these treatments showed acceptable shelf life for 21 days at 5 °C. However, the treatments inoculated with *L. acidophilus* and *L. casei* had the lowest scores and even were not considered suitable for drinking at the end of storage. High acidity and low pH of these probiotic treatments led to acidic taste, visible coagulated particles, and

Fig. 3 Sensory characteristics of different chocolate milks at the start (a) and the end (b) of refrigerated storage

undesirable mouthfeel. Effect of titrable acidity on perception of sourness taste is more important than pH (Belitz et al. 2009). According to Fig. 3 and Table 1 (final titrable acidity levels), the panelists were able to perceive unpleasant sourness and other unfavorable sensory characteristics related to titrable acidity at value of 33.2 °D at the end of storage (ST-C and S-C treatments), but not at 27.6 °D (T-R treatment). Therefore, if the limit of 0–27.6 °D was considered a recognition limit, treatments T-A and ST-A would be acceptable only for 7 days of refrigerated storage, S-A and all chocolate milk inoculated with *L. casei* for 14 days and S-R, and ST-R and those inoculated with bifidobacteria for 21 days.

4 Conclusion

Results of this study revealed that type of probiotic strains and replacement of sucrose with D-tagatose significantly ($P < 0.05$) affected viability of probiotic bacteria as well as physical and chemical characteristics of chocolate milk during 21 days of the refrigerated storage. Sensory attributes of the samples were affected by only type of probiotic strains. The treatments inoculated with *L. acidophilus*, *L. casei*, *L. rhamnosus*, and *B. lactis* as well as non-probiotic ones showed the most to the least biochemical changes and ΔE value, respectively. The greatest viability throughout the storage time was related to D-tagatose with *L. acidophilus* (T-A) until day 14 of storage and then to D-tagatose with *L. casei* (T-C) as well as D-tagatose with *L. rhamnosus* (T-R) until day 21. Although *L. acidophilus*, *L. casei*, and *L. rhamnosus* mostly tended to ferment D-tagatose, *B. lactis* did not significantly affect its consumption. The highest sensory scores at the end of storage belonged to control chocolate milk and then to those inoculated with *L. rhamnosus* and *B. lactis*. In general, treatments T-R, ST-R, T-B, and ST-B were found to be optimal for industrial production considering total aspects including probiotic viability, functional property of D-tagatose, and sensory attributes. Investigations using other probiotic species and strains as well as other low-calorie and/or prebiotic sweeteners are recommended as complementary studies.

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