Development of Synbiotic Milk Chocolate Enriched with *Lactobacillus paracasei*, D-tagatose and Galactooligosaccharide

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Abstract

Background and Objective: Prebiotics are food ingredients that induce the growth or activity of beneficial bacteria (Bifidobacteria and Lactobacilli). Galactooligosaccharide and tagatose are two main prebiotic compounds which are used in the food industry. Chocolate is widely consumed all over the world and could be used as an excellent vehicle for delivery of prebiotics. Furthermore, the incorporation of probiotics into chocolate, allows broadening the health claims of chocolate. The aim of the current study was to investigate the effect of tagatose and galactooligosaccharide on the physicochemical and sensory properties of milk chocolate and the survivability of *Lactobacillus paracasei* in the optimized formulation.

Material and Methods: Probiotic milk chocolate containing *Lactobacillus paracasei* were formulated by replacing a portion of the sucrose with the galactooligosaccharide powder and tagatose. For this purpose, various concentrations of galactooligosaccharide and tagatose (2.5, 5 and 7.5% w w^{-1}) along with stevia were used in chocolate formulation. Nine formulations were examined to determine some physicochemical, mechanical and sensory properties in order to find the optimum concentrations of these components. The lyophilized *Lactobacillus paracasei* were incorporated in the optimal formulation of prebiotic milk chocolate. The viability of probiotic bacteria in milk chocolate was carried out during storage at 22°C for up to 6 months.

Results and Conclusion: In general, chocolate formulations with high levels of galactooligosaccharide, achieved the highest plastic viscosity and yield stress. The lowest viscosity and yield stress were observed for the samples containing high concentrations of tagatose and in control. In addition, galactooligosaccharide at higher ratios induced the least desirable sensorial effects, whereas tagatose improved the overall acceptability. It can be concluded that the overall acceptability of milk chocolate samples were with (7.5), tagatose: galactooligosaccharide ratios of 2.5%-2.5%, presenting the optimal applicable range as prebiotic compounds. Numbers of live *Lactobacillus paracasei* cells remained above 8.0 log CFU g^{-1} until 6 months under ambient conditions. Milk chocolate was shown to be an excellent vehicle for the delivery of *Lactobacillus paracasei*, and the prebiotic ingredients galactooligosaccharide and tagatose did not interfere in its viability.

Conflict of interest: The authors declare no conflict of interest.

1. Introduction

In recent decades, the beneficial effects of particular dietary ingredients to human health have been stated by many studies. These ingredients have been known as functional components and the foods containing these compounds are known as functional foods [1,2]. Prebiotics are known as non-digestible compounds that improve human health by stimulating the growth or activity of specific bacteria such as bifidobacteria and lactobacilli due

to resistance to digestion in the human small intestine and are fermented by the gut microflora [3-5]. In addition, beneficial microbiota along with prebiotics improve the immune system by transforming the particular nutrients and beneficial phytochemicals into usable compounds. They regulate cholesterol levels and lower inflammation therefore lowering risk markers of cardiovascular diseases [6,7]. Prebiotics include starches, dietary fibers, other non-absorbable sugars, alcohol sugars and oligosaccharides [8]. Moreover, prebiotics are the dominant source of probiotic bacteria in the food [5,9-11]. Probiotics have been proposed as live microorganisms that are used as food supplements with clinical benefits on host health [12-15].

Galactooligosaccharide (GOS) and tagatose as prebiotics, due to their chemical structure are indigestible in the small intestine and also are fermented by the anaerobic bacteria in the colon [16-18]. GOS contains a chain of galactose units with galactose or glucose at the reducing end [19]. Natural food sources of GOS include banana, garlic, onion, artichoke, milk and honey. GOS is not used by the oral microorganisms due to its resistant to salivary degradation and intestinal enzymes.

Tagatose as a functional sweetener is a hexoketose which is present in only small quantities in various foods such as hot cocoa and a variety of processed dairy products such as milk, cheese and yogurts. It naturally occurs in *Sterculia (S.) setigera* gum and is very similar in sweetness to sucrose (92% as sweet) [20]. Tagatose is manufactured from galactose by the chemical or enzymatic procedures. In the first stage, lactose is hydrolyzed to a mixture of glucose and galactose. Then the galactose is isomerized under alkaline conditions to D-tagatose in the presence of calcium hydroxide and calcium chloride acts as a catalyst. The end mixture is purified and crystallization results in the pure tagatose [21].

Lactobacilli and Bifidobacteria species are the most dominant species studied in probiotic formulations. Several attempts were made to develop probiotic chocolate products so far, with the use of prebiotics [22-24]. In addition, Kemsawasd et al. confirmed that milk and dark chocolates were great carriers for protecting immobilized probiotics from the GI injuries [25]. Beards et al. investigated the effects of maltitol, polydextrose and resistant starch addition to chocolate [26]. The obtained results indicated that consumption of samples containing polydextrose-maltitol blend increased the level of Lactobacilli and Bifidobacteria in faces after 6 weeks. Also the increase in the levels of short chain fatty acids such as propionate and butyrate were observed.

During the past two decades, prebiotics have been increasingly used in different types of food products, especially in chocolates. Suter produced a dark chocolate by replacing a portion of the sucrose with the GOS powder [27]. The results indicated that yield stress and apparent viscosity behaviors were increased upon addition of GOS.

The sensory evaluation confirmed no differences between the chocolate samples containing GOS and control. A successful prebiotic chocolate bar was formulated that met commercial quality for taste and texture. Shourideh et al. studied the effect of D-tagatose and inulin on some physicochemical, rheological and sensory properties of dark chocolate [28]. The result indicated that overall acceptability of the chocolate samples increased by rising of the D-tagatose levels.

Taking into account the beneficial characteristics of the aforementioned ingredients, the objectives of the current study were to investigate the possibility of producing synbiotic milk chocolate by a combination of GOS, tagatose and *Lactobacillus (L.) paracasei*; to determine the viability of added strains during storage for 6 months at 22°C; and to evaluate some physicochemical, mechanical and sensory properties of the prebiotic chocolates.

2. Materials and Methods

2.1. Materials

For the preparation of synbiotic milk chocolate, cocoa mass (Delfi, Malaysia), cocoa butter (Cargill, Malaysia), sucrose (Iran sugar Co., Tabriz, Iran), stevia SU200 (Steviol Glycoside, Stevia-pack, Singapore), milk powder (Zarrin-shad, Esfahan, Iran), D-tagatose (Damhert, Belgium), GOS Purimune™ (Galactooligosaccharides, long chain, GO-P 90, 90.5% GOS Dry Basis, highly soluble, stable to high heat and low pH, Product ID 113001-156, Lot 15271, GTC Nutrition, Colorado, Korea), soy lecithin (Cargill, Netherlands), vanillin (Polar Bear, Shang Hal China) and lyophilized concentrated of probiotic bacteria of *L. paracasei* (CHR-Hansen Denmark) were used.

2.2. Preparation of milk Chocolate

The chocolate mass was made in the laboratory ball mill with a capacity of 5 Kg. The diameter of the balls in the mill were 8 mm. Homogenization of chocolate mass was carried out at 50°C at an agitator shaft speed of 40 rpm, recycling the mass through the balls at a medium speed of 10 Kg h^{-1} of the recycling pump, for 5 h. All the ingredients were added to the ball mill at the beginning of the production time [29]. The lyophilized bacteria were added at 40°C in the proportion of 3.33 g 100 g^{-1} which provided the functional level of at least 10^7-10^8 CFU g^{-1} [30]. At the final stage, a three-stage tempering process (33-35, 24-25 and 25-26°C) was implemented (temper index value measured by a temper meter [Chocometer, Aasted Farum, Denmark]: 5.50-6.00). The moulding and vibration process was conducted at 27-30°C [31]. The chocolate samples were cooled at 5°C for 30 min, removed from the moulds, wrapped in aluminum foil and stored at 22°C. Table 1 presents the formulation used in chocolate production.

Table 1. Formulations used for the chocolate samples

Ingredients	Control	Sample								
		1	2	3	4	5	6	7	8	9
Sucrose (g)	34	31.5	29	26.5	31.5	29	26.5	31.5	29	26.5
D-Tagatose(g)	0	0	0	0	2.5	5	7.5	1.25	2.5	3.75
GOS (g)	0	2.5	5	7.5	0	0	0	1.25	2.5	3.75
Stevia (ppm)	0	1	2	3	0	0	0	0.5	1	1.5
Cocoa butter (g)	32	32	32	32	32	32	32	32	32	32
Cocoa mass (g)	8	8	8	8	8	8	8	8	8	8
Milk powder (g)	25	25	25	25	25	25	25	25	25	25
Soy lecithin (g)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vanillin (g)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

2.3. Moisture

The moisture content of chocolate samples was measured by an official standard gravimetric method [32].

2.4. Rheological measurements

Rheological properties of milk chocolate samples were measured using a shear-rate⁄shear stress controlled rheometer (Anton Paar, MCR301, Austria). The samples were melted prior to measurement by incubation at 50°C for 75 min and pre sheared (15 min, shear rate=5 s⁻¹) at 40°C before measurement cycles started. Afterwards, shear stress was measured as a function of shear rate over a wide range of 5 to 50 s⁻¹ [33]. Collected data were fitted with mathematical models including Power law, Bingham, Herschel-Bulkley and Casson [34]. Two statistical indexes of Root Mean Square Error (RMSE) and coefficient of determination (r^2) were calculated to select the best model describing the steady rheological properties of the samples [35]. Furthermore, the rheological parameters including plastic viscosity and yield stress values of the selected models were evaluated.

2.5. Mechanical properties

The hardness of chocolate samples was evaluated using Texture Analyzer (TA.XTplus) with a penetration probe (needle P/2) and a 50 Kg load cell. Hardness was reported as the maximum penetrating force (N) required for the needle to penetrate through a sample (100 × 20 mm, depth 10 mm) at 20°C, over a distance of 5 mm at a constant speed of 2 mm s⁻¹. Mean values from 3 replicate measurements and standard deviations were calculated [36].

2.6. Sensory evaluation

Sensory attributes of milk chocolates including odor, flavor, sweetness, mouth feeling and melting in the mouth were evaluated using a hedonic scale test (5-point eating test, 1= Extremely dislike, 2= dislike, 3= tolerable quality, 4= desired quality, 5= extremely desired quality). Samples were identified with a different three-digit code. 15 trained panelists consumed water and crackers between evaluations [37,38].

2.7. Viability of probiotic bacteria

Live cells of probiotic bacteria were enumerated as colony forming units per gram (CFU g⁻¹). The amount of 10 g of all analyzed samples of milk chocolates with probiotic strain was added to 90 ml of physiological saline solution and homogenized in a Stomacher apparatus (Lab Blender Stomacher 400, Seward, UK) and a decimal dilution series was prepared. The serial dilutions were plated on the appropriate selective media (MRS Agar) and incubated at 37°C for 48 h. The viability of probiotic bacteria in chocolates performed after 1, 7, 14, 21, 28, 60, 90, 120, 150 and 180 days of storage at 22°C [39].

2.8. Statistical analysis

Quantitative data was expressed as mean±SD values of 3 replicates. Statistical analysis was performed using one-way ANOVA with SPSS ver. 17 and the level of significance was at p≤0.05. The significant differences were analyzed using the Tukey's test. Sensory evaluation results were analyzed by Kruskal-Wallis test. Comparisons were considered significantly different if p≤0.05.

3. Results and Discussion

3.1. Moisture content

The effect of various combinations of GOS and tagatose on the mean values of moisture content is shown in Table 2. As it can be seen, there are significant differences (p≤0.05) between the moisture content of prebiotic chocolates and controls, but the lowest value belonged to formulations containing high levels of tagatose. Chocolate formulations containing high ratios of tagatose were not different from the control in terms of moisture content. In contrast, the highest moisture content was observed in samples containing high mass fractions of GOS. Generally, the high moisture content of formulations can be ascribed to high and low hygroscopicity of GOS and tagatose, respectively. The moisture content for the samples ranged from 0.87 to 1.18% which was within the acceptable limit (≤1.5% ww⁻¹). This explains why some prebiotics are popular for replacing some parts of sucrose in chocolate formulations.

Table 2. Physico-chemical analysis of chocolate samples prepared with prebiotics Tagatose and GOS

Samples	Moisture (%)	Casson Viscosity (Pa.S)	Casson Yield (Pa)	Hardness (N)
1	1.05[c]	3.04[c]	6.90[c]	28[a]
2	1.15[a]	3.88[b]	7.77[b]	28[a]
3	1.18[a]	4.51[a]	8.66[a]	27[a]
4	0.97[d]	2.20[d]	6.24[d]	29[a]
5	0.89[e]	2.16[d]	6.13[d]	29[a]
6	0.87[e]	2.00[d]	6.17[d]	27[a]
7	0.98[d]	2.39[d]	6.21[d]	28[a]
8	1.04[c]	2.99[c]	6.87[c]	28[a]
9	1.09[b]	3.70[b]	7.69[b]	28[a]
10	0.90[e]	2.23[d]	6.18[d]	29[a]

Means in same columns shown with different letters are significantly different (p≤0.05).

1: sample containing 2.5% GOS, 2: sample containing 5% GOS, 3: sample containing 7.5% GOS, 4: sample containing 2.5% tagatose, 5: sample containing 5% tagatose, 6: sample containing 7.5% tagatose, 7: sample containing 1.25% GOS and 1.25% tagatose, 8: sample containing 2.5% GOS and 2.5% tagatose, 9: sample containing 3.75% GOS and 3.75% tagatose, 10: control

In a previous study, chocolate formulations possessing high proportions of tagatose (100%) had the lowest moisture content [28]. D-tagatose had fewer tendencies for absorbing and preserving the moisture. Therefore, the findings revealed the lower hygroscopicity of the above ingredient. Furthermore, Gaio reported that moisture content of the dark chocolate samples produced with D-tagatose were lower than the controls containing sucrosev [40].

A general trend emerged that, increases in GOS concentration leads to increases in moisture content. The latter could be due to high hygroscopicity of GOS. GOS possesses numerous hydroxyl groups on its chemical formula which is the cause of the increasing and preserving of the moisture. It may absorb some moisture when released from the ingredients, such as milk powder during conching. Torres et al reported that the GOS ingredient is a highly hygroscopic white powder [41]. Therefore due to the high moisture retaining capacity of GOS, the formulations with high proportions of GOS showed high moisture content.

3.2. Rheological analysis of chocolate samples

In order to explain the influences of various concentrations of GOS and tagatose on the rheological behavior of milk chocolates, their shear stress *vs*. shear rate data were fitted with some reported mathematical models including; Power law (Eq. 1), Bingham (Eq. 2), Herschel-Bulkley (Eq. 3) and Casson (Eq. 4).

$$\sigma = \kappa \gamma^n \qquad \text{Eq. 1}$$

$$\sigma = \mu_{pl}(\gamma) + \sigma_0 \qquad \text{Eq. 2}$$

$$\sigma = \kappa \gamma^n + \sigma_0 \qquad \text{Eq. 3}$$

$$(\sigma)^{0.5} = (\kappa_1)^{0.5}(\gamma)^{0.5} + (\sigma_0)^{0.5} \qquad \text{Eq. 4}$$

Where σ is shear stress (Pa); κ is consistency coefficient (Pa.S)^n; γ is shear rate (s^{-1}); μ_{pl} is plastic viscosity (Pa.S); σ_0 is yield stress (Pa); κ_1 is Casson plastic viscosity (Pa.S); n is flow behavior index (dimensionless). A flow model can be considered to be a mathematical equation because it can characterize rheological data, such as shear rate versus shear stress, in a main shear diagram. It prepares an appropriate and brief manner of describing the data. For some food products, for describing the rheological data, more than one equation may be necessary. In addition, various factors such as temperature, the structure and the composition of foods affect the value of model parameters. Therefore, it is necessary to establish widely applicable relationships that may be called functional models.

The fitting of experimental data with models was assessed on the basis of the r^2 and RMSE. Statistical evaluation of the models indicated that the Casson model was the best at describing the rheological behavior of milk chocolate samples (Table 3). Proper models were chosen based on the highest r^2 value and the lowest RMSE value [35]. Results illustrated that using GOS and tagatose in milk chocolate formulations in spite of influencing the rheological properties had no effect on the mathematical model fitting and the same model was used to determine the flow behavior of all the samples.

$$RMSE = \left[\frac{1}{n} \sum_1^n (x_{\exp} - x_{pred})^2 \right]^{0.5} \qquad \text{Eq. 5}$$

Where, n is the number of experimental data, x_{exp}, is the value obtained from experiment, x_{pred} is the predicted value by the corresponding model.

Casson model is widely used and offered by International Office of Cocoa, Chocolate and Confectionary to describe flow behavior and rheological characteristics of chocolate [42,43]. The Casson viscosity and Casson yield stress were assessed using $\sigma^{0.5}$ and $\gamma^{0.5}$ curves, where square of the slope and the intercept belong to Casson viscosity and Casson yield value, respectively.

3.2.1. Casson plastic viscosity

The results illustrated that there were significant differences (p≤0.05) between the Casson viscosity of prebiotic chocolates and controls (Table 2). In the present study, chocolate with GOS exhibited higher viscosity than the control. But the plastic viscosity of chocolate containing high concentrations of tagatose was lower than the control (Table 2). Casson viscosity values ranged between 2 and 4.51 Pa.S (Table 2), which is in agreement with the data reported by Aeschlimann and Beckett for milk chocolate (2.2-5.5 Pa.S) [44].

Table 3. Effects of prebiotics on fitting of experimental data with mathematical models based on coefficient of determination and Root Mean Square Error parameters

Sample no	Mathematical model	R^2	RMSE	Sample no.	Mathematical model	r^2	RMSE
1	Power law	0.992	0.572	6	Power law	0.989	0.478
	Bingham	0.995	4.587		Bingham	0.995	9.127
	Herschel-Bulkley	0.987	1.207		Herschel-Bulkley	0.987	0.407
	Casson	0.998	0.245		Casson	0.998	0.266
2	Power law	0.989	0.867	7	Power law	0.992	0.999
	Bingham	0.995	5.648		Bingham	0.998	7.659
	Herschel-Bulkley	0.985	1.448		Herschel-Bulkley	0.979	0.786
	Casson	0.999	0.189		Casson	0.999	0.280
3	Power law	0.990	0.657	8	Power law	0.995	0.875
	Bingham	0.992	7.325		Bingham	0.998	8.248
	Herschel-Bulkley	0.991	0.872		Herschel-Bulkley	0.982	0.852
	Casson	0.998	0.301		Casson	0.998	0.268
4	Power law	0.993	0.966	9	Power law	0.997	0.292
	Bingham	0.997	6.982		Bingham	0.995	4.237
	Herschel-Bulkley	0.992	0.359		Herschel-Bulkley	0.987	0.357
	Casson	0.999	0.274		Casson	0.997	0.253
5	Power law	0.995	0.555	10	Power law	0.990	0.521
	Bingham	0.997	4.273		Bingham	0.997	8.654
	Herschel-Bulkley	0.994	0.465		Herschel-Bulkley	0.991	0.616
	Casson	0.997	0.213		Casson	0.998	0.299

r^2= Coefficient of determination, RMSE: Root Mean Square Error

It means that these prebiotic compounds can be easily used for milk chocolate production. There was a significant increase in the Casson viscosity upon addition of GOS to chocolate formulation. Higher plastic viscosity caused by GOS may be associated with its molecular structure and physical characteristics such as hygroscopicity and crystallinity. Chocolate containing 7.5% GOS exhibited the highest moisture content and had the highest Casson viscosity. Conversely, 7.5% tagatose with relatively low moisture content exhibited the lowest Casson viscosity. GOS is very hygroscopic, which can absorb moisture from the environment (moisture released from milk powder) [45]. Saputro et al. reported that free moisture within the matrix of chocolate leads to the sugar particles dissolving and sticking together, accordingly increasing the viscosity. Moreover, the differences in the structures of GOS (oligosaccharide) with D-tagatose (monosaccharide) are one of the factors for the interaction between the particles in chocolate making and their flow resistance [46].

Shourideh et al. reported that plastic viscosity values of dark chocolate samples containing 25% inulin-75% D-tagatose and 100% D-tagatose had no difference with a control (p≤0.05). The Casson viscosity of the control sample was 1.32 Pa.S [28].

3.2.2. Casson yield stress

Casson yield values ranged between 6.13 and 8.66 Pa.S (Table 2). Casson yield stress for milk chocolate has been reported to be between 2-18 Pa [44]. The highest Casson yield was achieved using 7.5% GOS. In contrast, milk chocolate formulations with 5% tagatose resulted in the lowest Casson yield. Samples containing the highest tagatose were found to be close to the controls in tested Casson yield value (Table 2).

Particle-particle interaction, the amount of specific surface area, emulsifiers and moisture are the factors that determine the yield stress value. Low yield values in the formulations containing high concentration of tagatose illustrates that interaction forces between tagatose particles were weak, thus, less force is needed for the flow of the formulated chocolates.

On the other hand, the high yield value for samples containing 7.5% GOS can be attributed to high molecular mass of the ingredient [45]. High molecular weight of GOS increases the intermolecular (non-polar) interactions in chocolate mass. Therefore, mass becomes rigid and agglomerates and as a result more energy is required to start the flow. Therefore, higher yield value of chocolate with GOS could be associated with the high molecular mass of GOS (504.438) vs. tagatose (180.16). In a similar research, Suter reported that there was a significant increase in the yield stress upon addition of GOS to the chocolate system [27].

3.3. Hardness

According to the statistical analysis, no significant differences were generally observed between hardness of the samples (p>0.05). The hardness values were found to be between 27 N to 29 N (Table 2). Coarseness and textural properties of solid tempered chocolate correlated with the largest particle size [47]. Afoakwa et al., reported that the hardness of chocolate is dependent on the type of

fat and its content, particle size distribution, sugar type and also the tempering process [36].

Although there have not been any particle size measurements in this study, such similarity can be attributed to the same particle size of prebiotics in comparison to the sugar used in the control formulation. Shourideh et al. [28] reported that the hardness value of formulation with 100% D- tagatose was similar to the control (100% sucrose). Gaio also stated that the hardness of the chocolate samples containing sucrose or tagatose was the same [40].

3.4. Sensory acceptability of formulated chocolates

The odor and sweetness scores of all samples in this study ranged from 7.1 to 7.8 and 7.2 to 7.8 respectively with no significant ($p>0.05$) differences between samples. The use of prebiotics had no significant effects ($p>0.05$) on chocolate flavor (Table 4). Among the chocolates produced in this research, the control chocolate was scored the best in terms of total acceptance (Table 4).

Mouthfeel is another sensory property and appears as one of the most important characteristics. The control sample perceived as the most liked (7.9) followed by samples containing 5% tagatose (7.7). Mouth-feel properties of samples exhibited more significant ($p\leq0.05$) differences depending on concentration of GOS used. However, there were no statistical difference for overall acceptance between the control sample and low concentrations of GOS samples. Melting in mouth of chocolate made of 2.5% tagatose was the most similar to the control chocolate.

Jackson stated that different component proportions, cocoa types, differences in processing techniques and particle size distribution will result in different sensory properties [48]. Also the rheology of chocolate has been shown to impact sensory characteristics [49]. Suter reported that there were no remarkable differences in overall acceptance between the control sample and the 3.75% GOS sample [27]. Shourideh et al, claimed that by

increasing the tagatose concentrations, the overall acceptability of the chocolate treatments increased [28]. In the study of Gaio the use of D-tagatose with a 0.2-1%, dosage, improved the flavor profile of soft drinks [40].

Generally, it can be observed that conventional chocolate was preferred by the panelists over all chocolate samples regarding their total acceptance. Chocolate containing 5% tagatose and control were identified as having the highest overall acceptance, and chocolate made of 7.5% GOS, exhibited the lowest acceptability.

When tagatose and a low concentration of GOS are added to chocolate, the sensory quality of the product will not be affected strongly due to their limited effects on viscosity. The results of this study indicate the potential use of prebiotics to partially replace sucrose and achieve the desired sensory properties.

3.5. Selecting the optimum formulation

Taking all quality properties into account (Casson viscosity, Casson yield, moisture content and prebiotic properties), formulation one, consisting of 2.5% GOS and 2.5% tagatose was selected as having the maximum desirability. The simultaneous use of several prebiotics leads to high health benefits as well as stimulating the growth of different indigenous gut bacteria. High potential is attributed to synergistic effects of prebiotics. On the other hand, GOS has been broadly used to stimulate the growth of useful bacteria in human intestine. These findings indicate that the formulations containing high concentrations of GOS did not demonstrate desirable rheological properties due to their high moisture content. It can be finalized that GOS and tagatose can be used in the recommended range for improving the flow and sensory properties. Therefore, the milk chocolate consisting of tagatose: galactooligosaccharide ratios of 2.5%:2.5% were chosen as the optimum formulation. The overall acceptability and viscosity properties of the mentioned formulation were in the acceptable limit.

Table 4. Sensory evaluation of milk chocolate samples prepared using prebiotics

Samples	Mouthfeel	Sweetness	Melting in mouth	Odor	Flavor	Total acceptance
1	6.9[b]	7.4[a]	7.0[b]	7.1[a]	7.6[a]	7.1[b]
2	6[c]	7.3[a]	6.1[c]	7.2[a]	7.4[a]	6.2[c]
3	4.9[d]	7.2[a]	5.3[d]	7.5[a]	7.3[a]	5.5[d]
4	7.6[a]	7.4[a]	7.8[a]	7.6[a]	7.7[a]	7.6[a]
5	7.7[a]	7.6[a]	7.9[a]	7.7[a]	7.8[a]	7.7[a]
6	7.5[a]	7.8[a]	7.7[a]	7.8[a]	7.4[a]	7.6[a]
7	7.4[a]	7.3[a]	7.6[a]	7.5[a]	7.5[a]	7.5[a]
8	6.8[b]	7.5[a]	6.8[b]	7.5[a]	7.3[a]	7.5[a]
9	6.1[c]	7.6[a]	6.2[c]	7.7[a]	7.8[a]	6.2[c]
10	7.9[a]	7.5[a]	7.8[a]	7.6[a]	7.9[a]	7.8[a]

Means in same columns shown with different letters are significantly different ($p\leq0.05$).
1: sample containing 2.5% GOS, 2: sample containing 5% GOS, 3: sample containing 7.5% GOS, 4: sample containing 2.5% tagatose, 5: sample containing 5% tagatose, 6: sample containing 7.5% tagatose, 7: sample containing 1.25% GOS and 1.25% tagatose, 8: sample containing 2.5% GOS and 2.5% tagatose, 9: sample containing 3.75% GOS and 3.75% tagatose, 10: control

3.6. Viability of probiotic bacteria

The obtained results in this study indicated that the survival of probiotic strain *L. paracasei* in milk chocolate was generally very good. Results of statistical analysis using the Tukey's test (Table 5) showed that during storage at 22°C, there was no statistically significant difference in the number of viable cells of probiotic in milk chocolate. Total number of live cells was maintained at the functional level. This means that the probiotic used exhibited high viability at 22°C which indicates that chocolate can preserve its functionality during storage at the mentioned temperature. In fact, the probiotic after an early decrease of about 1.0 log CFU g^{-1}, showed a substantially constant trend during the remaining period of storage, with final loads of about 8 log CFU g^{-1} (Table 5).

Table 5. Survival of *L. paracasei* in prebiotic chocolate at 22°C during 6 months storage.

Sample	Storage time (day)									
	1	7	14	21	28	60	90	120	150	180
L. paracasei	9.33±0.58^A	8.67±0.58^A	8.33±0.58^A	8.33±0.58^A	8.33±0.58^A	8.00±1.00^A	8.33±0.58^A	8.00±1.00^A	8.00±1.00^A	9.00±1.00^A

*Different capital letters in each row indicate significant differences at α≤0.05.

It can be concluded that tagatose and GOS as prebiotic ingredients promoted the survival of probiotics. Gibson et al, reported that using inulin in human diet, increased the number of probiotic bifidobacteria [50].

Different stresses such as oxygen exposure, sugar concentration, osmotic effects and mechanical shearing all influence the number of probiotic live cells and can cause a decrease in their viability, both during the production and storage period [9,51-52]. These factors absolutely contribute to the gradual reduction of live cells used in the current study.

The chocolate production technology involves some procedures that can stimulate the damages to probiotic bacteria [53,54]. Therefore, the incorporation of probiotics into milk chocolate was performed before the tempering process to avoid any deleterious effect of temperature on bacterial cells. Low water activity and high concentration of sugar and fat in chocolate ensures the maintenance of probiotic bacteria in an inactive state. Also, the packaging with aluminum foil limits penetration of oxygen and protects the chocolates from humidity and other damages during storage period [39,55].

Żyżelewicz et al. produced probiotic (*L. casei* and *L. paracasei*) chocolate. The number of two strains remained at the functional level of 10^6-10^8 CFU g^{-1} during 12 month storage period. Aragon-Alegro et al incorporated a *L. paracasei* and inulin into chocolate mousses and observed

that the population maintained above 6 log CFU g^{-1} during 28 days of storage at 5°C [55].

The use of the potentially probiotic strain of *L. paracasei* and prebiotics for the production of milk chocolate was shown to be advantageous. The population of the mentioned strain after 180 days storage in optimal formulation was satisfactory, which remained above 8 log CFU g^{-1}. It can be concluded that tagatose and GOS based chocolates may be an ideal vehicle for probiotic strains.

4. Conclusion

Overall, the result of this research has shown a positive outcome regarding the incorporation of *L. paracasei*, GOS and tagatose in the chocolate formulation. GOS and tagatose did not interfere with the viability of *L. paracasei*. Considering a threshold of about 8 log CFU g^{-1} of viable probiotic bacteria, our results indicated an acceptable amount of probiotic strains assayed in milk chocolates during a storage period of 180 days. It can be said that the milk chocolate can be an excellent food matrix for adding probiotic microorganisms especially *L. paracasei*, since high populations were observed in the product during storage. Although increases in yield stress and Casson viscosity were observed upon GOS addition, it was noted that the low dosages did not contribute to the noticeable quality changes. Chocolate containing tagatose and low concentrations of GOS received desirable sensory acceptance. It can be concluded that chocolate samples with GOS: tagatose ratio of 2.5%:2.5% can act as a proper partial of sucrose substitute. Since GOS and D-tagatose both have prebiotic properties, chocolate samples prepared with these substances are also desirable from a nutritional point of view and can thus be considered as functional foods.

5. Acknowledgements

6. Conflict of Interest

The authors declare no conflict of interest.

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تولید شکلات شیری سین‌بیوتیک غنی شده با لاکتوباسیلوس پاراکازئی، D- تاگاتوز و گالاکتوالیگوساکارید

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چکیده

سابقه و هدف: پری‌بیوتیک‌ها مواد متشکله غذایی هستند که باعث تحریک رشد یا افزایش فعالیت باکتری‌های مفید (بیفیدوباکتری‌ها و لاکتوباسیل‌ها) می‌شوند. گالاکتوالیگوساکارید و تاگاتوز از جمله ترکیبات پری‌بیوتیک عمده مورد استفاده در صنعت غذا می‌باشند. شکلات در سراسر جهان مصرف زیادی دارد و می‌تواند به‌عنوان حامل مواد پری‌بیوتیک مورد استفاده قرار گیرد. به‌علاوه، استفاده از پری‌بیوتیک‌ها در شکلات، امکان ادعای اثرات سلامت‌بخشی آن را افزایش می‌دهد. هدف از مطالعه حاضر، بررسی اثر استفاده از تاگاتوز و گالاکتوالیگوساکارید بر ویژگی‌های فیزیکوشیمیایی و حسی شکلات شیری و زنده‌مانی لاکتوباسیلوس پاراکازئی در فرمولاسیون بهینه بود.

مواد و روش‌ها: شکلات شیری پروبیوتیک حاوی لاکتوباسیلوس پاراکازئی با جایگزینی بخشی از ساکارز توسط پودر گالاکتوالیگوساکارید و تاگاتوز فرموله شد. بدین منظور، غلظت‌های گوناگونی از گالاکتوالیگوساکارید و تاگاتوز (۲/۵، ۵ و ۷/۵٪ وزنی/ وزنی) به‌همراه استویا در فرمولاسیون شکلات شیری استفاده شد. برای یافتن غلظت بهینه این ترکیبات، ویژگی‌های فیزیکوشیمیایی، مکانیکی و حسی ۹ تیمار شکلات فرموله شده تعیین شد. لاکتوباسیلوس پاراکازئی لیوفیلیزه (خشک شده به روش انجمادی) به فرمولاسیون بهینه شکلات شیری تلقیح شد. زنده‌مانی باکتری های پروبیوتیک طی ۶ ماه نگهداری در دمای ۲۲°C مورد بررسی قرار گرفت.

یافته‌ها و نتیجه‌گیری: به طور کلی، در فرمول‌های شکلات حاوی مقادیر بالای گالاکتوالیگوساکارید، بیشترین گرانروی پلاستیک- کیسون و تنش تسلیم به‌دست آمد. کمترین گرانروی و تنش تسلیم برای نمونه‌های حاوی درصد بالای تاگاتوز و شاهد مشاهده گردید. به‌علاوه، نسبت بالاتر گالاکتوالیگوساکارید کمترین اثرات مطلوبیت حسی را به‌همراه داشت در حالی‌که تاگاتوز موجب بهبود قابلیت پذیرش کلی شد. می‌توان نتیجه‌گیری کرد که امتیاز قابلیت پذیرش کلی (۷/۵) برای نمونه‌های شکلات شیری حاوی ۲/۵٪ تاگاتوز و ۲/۵٪ گالاکتوالیگوساکارید، نشان می‌دهد که این محدوده، بهینه مطلوب به‌عنوان پری‌بیوتیک می‌باشد. باکتری‌های لاکتوباسیلوس پاراکازئی به میزان بیشتر از ۱۰⁸ log CFU g⁻¹ تا ۶ ماه در شرایط محیط زنده باقی ماندند. بدین ترتیب، شکلات شیری می‌تواند حامل بسیار مناسب لاکتوباسیلوس پاراکازئی و ترکیبات پری‌بیوتیک تاگاتوز و گالاکتوالیگوساکارید بدون تاثیر نامطلوب بر زنده‌مانی پروبیوتیک باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.