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RESEARCH ARTICLE

EFFECT OF MICROENCAPSULATED *LACTOBACILLUS PLANTARUM* ON THE RHEOLOGICAL AND SENSORIAL PROPERTIES OF SYNBIOTIC ICE CREAM

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ABSTRACT

Synbiotic ice creams formulated with three probiotic variations (free *L. plantarum,* encapsulated *L. plantarum*, and a mixture of commercial *Bifido*. *Sp and L. plantarum*) were observed for their rheology and sensory properties. Microencapsulation of *L. plantarum* isolated from fermented cacao beans via freeze drying with 6% (w/v) lactose, 6% (w/v) skim milk and 3% solution of k-carrageenan coatings has shown the highest viscoelastic properties based on high fat content due to the addition of vegetable oil during the encapsulation process. Although there was no significant differences ($p<0.05$) in the overall acceptance attributes, the degree of sweetness for encapsulated *L. plantarum* ice cream was significantly higher ($p<0.05$) when compared to other samples. Henceforth, the encapsulation materials and freeze drying technique did improve the viscoelastic properties and overcome the sour taste of probiotics in ice cream formulation.

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INTRODUCTION

Nowadays, functional foods that could improve and confer benefit to human health are at the greatest demand (Gadhiya *et al*., 2015). One of the promising functional foods that is gaining its popularity and highly demanded is Probiotics. Probiotics are defined as living microorganisms which could bestow good health when consumed adequately (FAO, 2006). Referring to (Tien, 2009), the article (550) in the (Laws of Malaysia, 2006) have stated that lactic acid bacteria (LAB) is a legitimate food and the quantity must reach a minimum value of 10^6 CFU/g if the culture is to be labeled on food products. The success of probiotics in dairy products had display a positive image among consumers (Siro *et al*., 2008). Probiotics are sold as supplements and has reached more than US $28 billion worldwide in 2015, and is expected to increase up to US $96 billion by 2020 hence, showed an optimistic future (Trend Market Research, 2018). Some of the common probiotics are from lactic acid bacteria; Lactobacillus sp, and yeast such as *Saccharomyces boulardii* (Pochapin, 2000).

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The species of Lactobacillus and Bifidobacterium genera are commonly used as probiotics because they are known as GRAS (Generally Recognized As Safe) and is able to prove the effectiveness of the health of the human intestine and also mammals (Georgieva *et al*., 2009). Industrially, the consumption of probiotics foods is mainly related to functional food like fermented milk, yoghurt and fermented juice (Kalita *et al*., 2018). Lactic acid bacteria are more likely to grow and multiply at pH between 4.0 -4.5 (Bamforth, 2008). Part of sepsis lactic acid bacteria can survive at a cold room temperature of 4 ° C and as high as 45 ° C. The presence of lactic acid bacteria after 12 hours and over is as much as (10^3 CFU^{-1} WMg), and it is capable of producing lactic acid, acetic acid and carbon dioxide (Gálvez *et al*., 2007). The largest group of lactic acid bacteria is made up of genus Lactobacillus containing more than 50 different species (De Vries *et al*., 2006). The diversity of species lactic acid bacteria that dominate the fermentation of cocoa beans is limited, only species of *Lactobacillus plantarum* and *Lactobacillus fermentum* (Camu *et al*., 2007). According to (Ardhana *et al*., 2003), lactic acid bacteria *L. cellobiosus* was dominant at 36-48 hours, reaching a maximum population growth of 108-109 cfu / g after 36 hours, while *L. plantarum* was dominant at the beginning of 24 hours during the process cocoa beans fermentation in Indonesia.

In Malaysia, among the lactic acid bacteria found in fermented cocoa beans are *Lactobacillus collinoides, Lactobacillus plantarum* and some other species (Rose, 1982). The dairy industry have found a probiotic culture as a container to be formulated in a new functional food product (Champagne *et al*., 2005). Yogurt and fermented milk are the main medium of probiotic culture in addition to the introduction to new products being introduced into international markets such as ice cream, butter, mayonnaise and various types of cheese. The probiotic bacteria are capsule or powdered and included in cold minced products and fermented foods (Champagne *et al*., 2005). As such, there are more and more probiotic products that cause consumers to be more likely to buy the product. Various strains of *L. plantarum* were marketed as probiotics (Table 2.7). According to (Schillinger *et al*., 2005), lactic acid bacteria probiotic is often applied for fermented milk, where milk molecules can be protective against bacteria to counteract acidity of gastric juice. In (Shah *et al*., 2000), it was reported that the survival of probiotic bacteria in frozen dessert desserts can be increased by encapsulation. Hence, the technique of probiotic encapsulation is able to prolong the shelf life of frozen dairy products such as ice cream.

Synbiotic ice cream is one type of functional food with the combination of both probiotics and prebiotics. Prebiotics are also known as resistant, non-digestible fiber compounds named oligosaccharides which promote the growth of probiotics either bacteria or yeast in digestive tracts. Prebiotics performed as the food supply as well as the protective carrier for the probiotics survival in human digestive system (Chen *et al*., 2005). Many studies have proven that consuming probiotics could confer to various health benefits. In a study by (Zago *et al*., 2011), probiotic *L. plantarum* has shown its capability to treat many symptoms such as diarrhoea and constipation. Apart from that, it could reduce the risk of getting cancer, alleviate allergic symptom, and strengthening the immune system (Parvez *et al*., 2006). However, the direct addition of probiotics in food products is detrimental as the acidic environment in human gut might lead to the reduction number of their survival as they reach the intestinal tract. Moreover, their stability in the food products is extremely low and its survival in the human digestive tract is very limited. Although probiotic applications are widespread in the formulation of cheese and fermented milk, the application of this encapsulated microorganism in ice cream formulation as a medium of probiotic culture is something new (Homayouni *et al*., 2008). Owing to that reason, microencapsulation is the best protection method for bacterial cell upon cell release into the target site (Brinques, 2001). Microencapsulation technique is defined as a consolidation of food, bacterial cells, nutrients or nutraceutical substance forming small capsules which only release the contents at a particular target as a mean to protect the materials from damage and degradation due to environmental factors (Desai, 2005). As contended by (Nag *et al*., 2011), the purpose of probiotics bacteria microencapsulation process is to maintain the stability and survival during storage, to protect against environmental stress the digestive tract, and the control the release in the colon. Among the microencapsulation techniques, freeze drying is proven to be a very effective method for encapsulation of probiotics (Tee *et al*., 2014) since the encapsulation using high temperature could kill the good bacteria (Tripathi, 2014). Correspondingly, it was elucidated in some research that the survival rate for encapsulated probiotics was found significantly higher than the percentage of free cell, could offer greater protection (Thomas *et al*., 2014) and pleasant taste (Anand *et al*., 2018). As in (Foong *et al*., 2013), this technique could also prolong the shelf life of frozen dairy products like ice cream (Schillinger *et al*., 2005). Other than that, the application of microencapsulated probiotics in ice cream formulation is quite new (Homayouni *et al*., 2008). Hence, the aims of this study are to focus on the assessment of sensorial, physico-chemical and as well as rheological aspect.

MATERIALS AND METHODS

Isolation and microencapsulation of probiotic: Lactic acid bacteria were isolated from fermenting cocoa beans together with the pulp at day 4 of the fermentation located in Raub, Pahang, Malaysia. Then, 50g of the sample was mixed with 450ml of 0.1% peptone water. In this research, pour plate technique was applied. The sample was then diluted and spread on the deMan Rogosa and Sharpe (MRS, Oxoid, UK) agar plates for further anaerobic incubation at 37°C for 24- 48 h. The colonies that growth were found to have spherical, white and creamy colour which confirm to the morphology of lactic acid bacteria. Gram staining, microscopic observation and API 50 HCL (bioMérieux, Marcy-l'Étoile, France) system were carried out to verify the finding. Then, for single colony isolation, the strain from cultured *L. plantarum* from spreading technique was taken by loop wire and was subcultured in anaerobic jar similar to previous technique mention above. Later, the cultures were stored in MRS broth composed of 20% (w/v) glycerol at –20°C for further analysis (Otero *et al*., 2007).

In this research, the encapsulation technique was done by referring to (Johansson *et al*., 1998), Subculturing in the MRS broth was done twice, inoculated in 2% (v/v) 800ml MRS broth and incubated at 37°C for 24-48 h. Culture was then centrifuged at (6000 x g for 10 min at 4°C) washed twice with 0.1/100 g sterile pepton water. Then, suspended bacteria was then transferred into a solution containing 6% (w/v) lactose and 6% (w/v) skimmed milk as cryoprotective agent which were believed to protect and enhance the viability of *L. plantarum* through the process of freeze drying. Twenty-five ml of the bacterial suspension was mixed with 60 ml of 3% κ-carrageenan. A mixture of 0.1% Tween 80 and 100 ml of vegetable oil with was heated at 45°C and stirred homogeneously for 3 min. Then, both mixtures were mixed and stirred slowly for 10 min. An amount of 150 ml of 0.3M KCl was poured slowly at the side of the beaker and rested for 30 min at room temperature in order to break the emulsion. Separation of oil was done by sucking and filtering microencapsulated cells. The cells were stored overnight at –20°C and freeze dried at –40°C and 0.3 mPa for 24 h using a freeze drier (Freezone Plus 6, Labconco, USA).

Preparation of synbiotic ice cream: For ice cream preparation, the materials were purchased from Giant Hypermarket, Bandar Baru Nilai, Negeri Sembilan. The synbiotic ice cream was formulated with the addition of prebiotic and probiotics following the work of (Schillinger *et al*., 2005) with some modifications. The formula was based on the calculation of 38-39% total solids for one liter ice cream production. About 40 percent of milk and were added before elevating the temperature up to 50°C. Then, milk powder, prebiotic oligosaccharide, stabilizers, vanilin and sugar were added together into the mixture. It was homogenized at 250 bar using a homogenizer (Company Niro Soavi Italy) just before it is being pasteurized at temperature of 80°C for 20 seconds.

The mixture was then cooled to 4°C and kept for 12 h to promote the ripening process of ice cream. Later, it was poured into the ice cream maker (Taylor Company, Italy) to obtain a smooth distribution of small, disc-shaped crystals. The next stage of synbiotic ice cream formulation was the incorporation of probiotic bacteria in which one percent (1%) of *L. plantarum* which has been encapsulated was added into a 100 ml plastic cup with lid of the prepared ice cream and labeled as A. Then, one percent (1%) of free, non-capsulated *L. plantarum* was introduced in the ice cream as B. Also, an amount of one percent (1%) free *L. plantarum* and 4% of Bifidobacteria starter culture were added together in the ice cream and labeled as C, while ice cream without any addition of microorganism was assigned to be the control sample and labeled as D. Semi-frozen mixture was cooled freeze at -18° C. During the process of freezing, frozen product temperature must be controlled (Cruz *et al*., 2009). Samples were stored in the freezer room for a month and analyzed each week. Experiments were conducted in triplicate. After 48 h of fermentation at 37°C, 1 ml of each bacterial culture was transferred to 1.5 ml microcentrifuge tube and centrifuged at 2,000 x *g* for 5 min at room temperature (Microcentrifuge; Eppendorf, USA). The pellet cells which are intracellular proteins were then separated from fermentation medium and washed with phosphate-buffered saline (Cambrex Bioscience, Belgium) twice, before being dissolved in sterile distilled water. The protein suspension was then mixed with Laemmli buffer (Bio-Rad, USA) and β-mercaptoethanol (Sigma-Aldrich, USA) in 1:1 ratio before being heated at 95°C for 10 min and cooled on ice. The protein samples were further analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Rheological analysis of ice cream: Fermentation process for production of extracellular proteins was conducted based on the method proposed by (4). In this process, a single colony of *B. subtilis* ATCC21332 was inoculated with 50 ml of MHB and incubated at 30°C with gentle agitation. *C. nardus* essential oil at concentration of 0.02 % was then added to bacterial culture after 8 h of cultivation and the culture was further shaken vigorously at 30°C for 48 h and 72 h. A culture to which essential oil was not added serve as a control. Ice cream samples were fit into a rheometer (oscillatory rheometry, Physica MCR301, Anton Parr GmbH, Graz, Austria) according to the manual. Amplitude sweep test was conducted according to (Waterhouse *et al*., 2011) to determine the linearity of the viscosity-elastic region of ice cream at a fixed frequency of 10 rad / s with strain between 0.1 to 100%. Frequency sweep test was also carried out between 0.1 and 100 rad / s at a temperature of 0 and 20 °C with a gap setting, 0 mm. Data obtained in 10 points value was explained by Rheoplus V2.66 (Anton Paar GmbH, Graz, Austria).

Sensory analysis of ice cream: The organoleptic sensory for the ice cream samples were evaluated by referring to the affective test recommended by (Aminah, 2000). The idea is to test the level of consumer acceptance and preference towards the food samples. A total of 30 untrained consumer panels were selected randomly among the undergraduate students and graduate of Food Biotechnology from Faculty of Science and Technology. Assessment was carried out under fluorescent light which took place in a sensory laboratory, Faculty of Science and Technology, USIM. A total of four attributes evaluated in this assessment were aroma, taste, texture and overall acceptability.

The 7-point hedonic scales range from “most disliked” to “most prefer” were given to the panelists. Samples were stored and frozen at -18 ^{o}C to maintain the integrity of the ice cream samples.

Statistical analysis: Statistical analysis was conducted using SAS (version 18.00) to determine the means and standard deviation for the physico-chemical and sensory data. The ANOVA Duncan’s test was conducted to determine differences between means.

RESULTS AND DISCUSSION

Statistical analysis Biochemical API 50 CHL L. plantarum identification: The isolated lactic acid bacteria from Theobroma cacao was morphologically confirmed by gram staining, microscope and Biochemical API 50 CHL *L. plantarum* identification. It was observed that the colony showed a spherical, shiny and creamy as explained by (Johansson *et al*., 1998). Subsequently, they were found out to be gram-positive bacteria, with rod-shape when observed under microscope which further narrow down the taxonomy of LAB. According to (Hutkins, 2006), Lactobacillus species has rod shaped looks which could support this research findings. Interestingly, the API 50 CHL system also shows 99.9% probability of the colonies were from L. plantarum species (Figure 1). The analysis of the strips after 48 hours fermentation showed some positive tests that changed the color of the bromocresole indicator purple to the yellow color. Yellow means positive while blue means negative. The black color for tubes numbered 25 is positive. Color conversion indicates that the lactobacillus is able to carry out the fermentation process and use its carbohydrates and acne (heteroside, polyalcohol and uric acid) for metabolic purposes and produce acids with acidic pH of 5.2 (Barbu, 2006). Table 1 shows that there are 24 types of carbohydrates that show a positive reaction of D-Galactose, D-Glucose, D-Fructose, D-Mannose, L-Rhamnose, D-Mannitol, Methyl-α D-Mannopyranoside, N-Acetyl Glucosamine, Amygdalin, Arbutin, Eskulin citric iron, Salicin, D-Celiobiose, D-Maltose, D-Laktose, D-Mellibiose, D-Sacarose, D-Trehalose, D-Melezitose, D-Raffinose, D-Arabitol, Gentiobiose, D-Turanose and Potassium Gluconate. For the 25th tube, the color exchange is from purple to black. This is due to iron reactions with eskulin produced in the process of hydrolysis of the eskulin (Seldin, 1986).

Rheological properties of synbiotic ice cream: Ice cream stability is influenced by rheological properties as it determines the behavior of the flow and also the taste of a product. In the Amplitude Sweep Test, the viscoelasticity of each sample of ice cream has a linear response for the strain range (0.1-1%) as shown in Figure 2. In general, G 'modulus of shear storage of a substance is dependent on time, which indicates the elastic properties as well as the energy saving. In this study, there is a linear state in a certain strain range because G 'is independent of time, the amplitude of the swell or the swelling pressure, and also the Applied Frequency Swings. The modulation of the G 'modulus pattern for all ice cream samples (control, *L. plantarum* free, *L. plantarum* encapsulated and mixtures of *L. plantarum* and Bifido) are the same but have different amplitude when the strain or strain value is higher. G 'position is consistent with the strain range (0.1-1%). The highest reading of G 'value is for *L. plantarum* capsule ice cream

Table 1. Spectrum of antimicrob biochemical carbohydrate metabolism profile by *L. plantarum*

Tube	Carbohydrate	Result	Tube	Carbohydrate	Result
0	Control	-	26	Salicin	+
1	Glycerol	-	27	D-Celiobiose	+
2	Erythritol	-	28	D-Maltose	+
3	D-Arabinose	-	29	D-Lactose	+
4	L-Arabinose	-	30	D-Mellibiose	+
5	D-Rybose	-	31	D-Sacarose	+
6	D-Xylose	-	32	D-Trehalose	+
7	L-xylose	-	33	Inulin	-
8	D-Adonitol	-	34	D-Melezitose	+
9	Metil-β-D xylopyranoside	-	35	D-Raffinose	+
	D-Galactose		36	Amidon(Starch)	-
10	D-Glucose	+	37	Glycogen	-
11	D-Fructose	+	38	Xylitol	-
12	D-Mannose	+	39	Gentiobiose	+
13	L-Sorbose	+	40	D-Turanose	+
14	L-Rhamnose	-	41	D-Lyxose	-
15	Dulcitol	+	42	D-Tagatose	-
16	Innositol	-	43	D-Fucose	-
17	D-Mannitol	-	44	L-Fucose	-
18	D-Sorbitol	+	45	D-Arabitol	+
19	Metyl-α-D-	-	46	L-Arabitol	-
20	Mannopiranoside	+	47	Potassium Gluconate	+
	Methyl-α D-			Potassium 2-ketogluconate	
21	Glucopyranoside	-	48	Potassium 5-ketogluconate	-
	N-Acetyl Glucosamine				
22	Amygdalin	+	49	Isolate Identity	-
	Arbutin				
23	Eskulin iron citrate	+			
24		+			99.9% ID *Lactobacillus*
25		+			*plantarum* 1

Table 2. Mean scores or sensory analysis of ice cream samples

	4 weeks of ice cream storage at-18°C			
Atrribute Score	Control	*L. plantarum* (free)	*L. plantarum* (encapsulated)	*L. plantarum*+ *Bifido* mix
Aroma (n=30)	4.87±1.04[a]	4.83±1.23[a]	4.80±1.35[a]	4.43±1.38[a]
Texture (n=30)	4.97±1.25[a]	4.40±1.93[a]	5.0±1.51[a]	4.63±1.52[a]
Sweetness (n=30)	4.73±1.26[ab]	4.43±1.38[a]	4.77±1.57[a]	4.4±1.35[ab]
Sourness (n=30)	5.03±1.38[a]	4.37±1.38[a]	5.33±1.27[a]	5.17±1.18[a]
Overall acceptance (n=30)	5.13±0.82[a]	4.50±1.61[a]	5.17±1.42[a]	4.8±1.42[a]

[a-b]Different alphabet at the same column showed a significant different at ($p<0.05$).

Fig. 1Color change of fermentation reaction on API 50 CHL medium tube

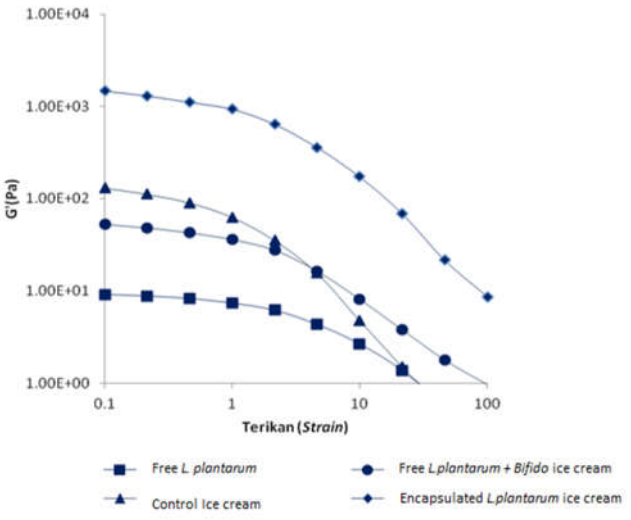

Fig. 2 Rheological analysis of the amplitude sweep point for *encapsulated L. plantarum* ice cream, free *L. plantarum* ice cream, ice cream mix of *L. plantarum* and *Bifido* and also control ice cream.

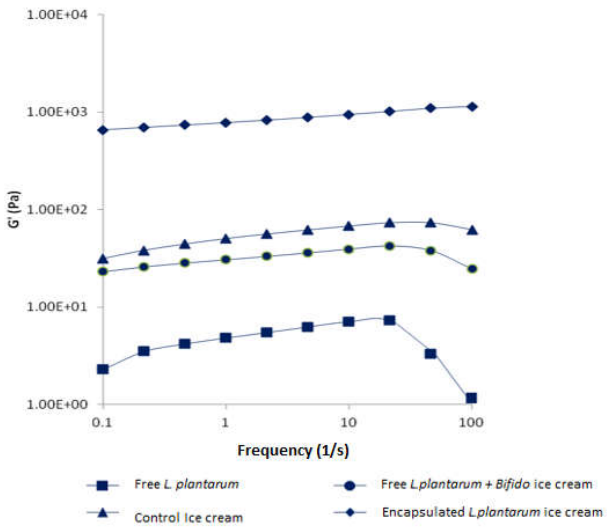

Fig. 3. G'storage Modulus Analysis as a function of frequency for ice creams; (encapsulated *L. plantarum*), (free *L. plantarum*), (*L. plantarum* and *Bifido*) and (control)

showing a high elastic viscosity followed by control ice cream, *L. plantarum* ice cream and *Bifido* and free *L. plantarum* ice cream. For the Frequency Sweep Test, the relationship between G' and frequency in the range 0.1 to 100 s-1 were not completely linear (Figure 3). The reading of G' modulus increased with frequency in the range 0.1-100 s-1. Stability of the ice cream was influenced by the rheological properties. In general, G ' or the shear storage modulus of a material was time dependent, which showed the elastic and the internal, core, energy. In this study, there was a linear behavior in the range of the particular strain because G' was not depended on time, amplitude of the oscillating strain or pressure, and also the applied frequency. The value of G' was the highest for sample A, which showed that the sample has a high viscosity elastic component. In this study, the G' modulus of all ice cream samples exhibited were depended on the frequency range tested. In addition, the high value of the elastic properties of ice cream samples also affected the slow melting rate. According to (Vélez-Ruiz *et al*., 1997), viscosity, elastic characteristics of ice cream mix was affected by fat globule size, the distribution of air cells, the physical properties of emulsifier, protein layer, the droplets of fat, and processing procedures such as the homogenization and storage period. The instability level of both fat and protein on the surface of the micelles influenced the elastic properties of ice cream.

In general, encapsulated probiotics greatly enhanced the elastic and rheological properties of ice cream. Table 2 shows the mean scores of ice cream perception recorded by the 30 panelists. However, in this study, the level of ice cream synbiotic acceptance was not significant at $p<0.05$ except for the sweetness attribute. Results found that sample A (encapsulated probiotics) showed good acceptance by sensory panels. Similar findings was reported by (Nousia *et al*., 2011) on the acceptance of probiotics ice-cream samples incorporated with encapsulated *L. acidophilus.* Panelists thought that the sweetness is an important characteristic for an ice cream product to be acceptable. The sweet taste of ice cream mixed with free *L. plantarum* was less favored due to higher acidity. For the encapsulated probiotics ice cream, the level of sweetness was stable and well-received by the sensory panel.

According to the sensory panel, sourness was a sense of unfriendliness ice cream attribute with free probiotics. However, free *L. plantarum* ice cream was more aromatic than encapsulated *L. plantarum*. In general, the results indicate that the sample of ice cream with the assimilation of encapsulated *L. plantarum* showed higher level of optimistic acceptance among the sensory panels.

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Conclusions

Therefore, other related factors such as pH of the medium, incubation period, temperature and cell density in which could affect the enhancement of antimicrobial protein production should be considered for further research. Encapsulated probiotics *L. plantarum* may greatly influence the rheological properties of ice cream in terms of elasticity and melting rate also could be improved by assimilating the encapsulated probiotics in the ice cream product. Ice cream prepared with freeze dried encapsulation technique may have higher viscoelasticity and slower melting rate. Our sensory results also indicated that the ice cream made with encapsulated probiotics have higher mean scores for overall acceptability among panels which thus suitable for commercialization although it is not significant. Further shelf life study for the synbiotic ice cream and the therapeutic effects need to be conducted in the future.

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