

Synbiotic Cocoa Cream Produced via Incorporation of Microencapsulated *Bifidobacterium animalis* subsp. Lactis and Inulin: Physicochemical, Rheological, and Sensory Properties

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ABSTRACT

The use of microencapsulation technique and prebiotic compounds are among the methods used to increase the probiotics survival. This study was done to investigate the effect of combination of inulin as a prebiotic agent and inoculation of *Bifidobacterium animalis* subsp. lactis as a probiotic bacterium microencapsulated with sodium alginate, to produce a synthetic biochemical product with a health characteristic in cocoa cream. The study included inoculated samples that were prepared by microencapsulated *B. animalis* containing 2 and 3% of inulin and the control sample without it. The samples were kept at 4°C for 6 weeks and acidity, viscosity, *B. animalis* survival, and sensory properties were studied on days 1, 7, 14, 21, 28, 35, and 42. It was found that in cocoa cream samples, acidity and viscosity were significantly increased and reduced, respectively, during the storage period. In samples with 2 and 3% inulin, reduced *B. animalis* was not significantly different, but this reduction was significantly lower than samples without inulin. After 42 days of storage at 4°C, there was no significant difference among sensory properties of samples including taste, texture, and total acceptability. The product had an appropriate physicochemical, sensory, and probiotic survival properties for industrial production.

Keywords: Digestive microorganisms, Health index, Probiotics survival.

INTRODUCTION

The microbial flora of the human digestive tract is the group of microorganisms that are in symbiotic relationship with digestive microorganisms and each other. Among these, *Lactobacillus* spp. and *Bifidobacterium* have probiotic properties relative to pathogenic bacteria and promote the health index in individual (Niazmand *et al.*, 2010; Sekhon and Jairath, 2010). Probiotics should be non-pathogenic and non-toxic; they should also survive in the food matrix during storage and in the gastrointestinal tract until proceeding to the colon (Wallace *et al.*, 2011; Farnworth, 2016). The role of probiotics is to establish a balanced bacterial media in which the

immune system function, some kinds of gastrointestinal diseases such as, diarrhea, constipation, irritable bowel syndrome, and infectious intoxication can be improved. In addition, food allergies and liver disease can be treated (Wallace *et al.*, 2011; Gbassi and Vandamme, 2012). In addition, Probiotic products play an efficacy role in reducing the risk of heart attacks and the level of serum cholesterol (Niazmand *et al.*, 2010).

Prebiotics are fermentable substances such as lactulose and inulin that have been proposed for particular changes in the composition and activity of these microorganisms in the host gastrointestinal tract (Gibson *et al.*, 2010; Emami *et al.*, 2018). Using these compounds, a protective layer for probiotics was able to increase their

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survival in the digestive tract (Gibson *et al.*, 2010). Inulin belongs to a class of dietary fibers known as fructans with bonds B (6 \rightarrow 1) that stimulates the growth of gastrointestinal tract of bifidobacteria and enhances calcium absorption and keeps bones and teeth strong and healthy (Sekhon and Jairath, 2010; Emami *et al.*, 2018). The structure of inulin gives it different applications such as fat replacement. Through small crystallites, inulin shapes into gels (Beikzadeh *et al.*, 2018).

For the development of health benefits of probiotics, it is necessary to have CFU 10^6 to 10^7 of these bacteria per gram of probiotic food products (Farnworth, 2016). In order to survive, these microorganisms are used in the form of microencapsulation of materials with enveloping properties. Calcium alginate is a water-insoluble, hydrocolloid that is used to form coatings for probiotics' microencapsulation due to low price and non-toxic properties (Gbassi and Vandamme, 2012). Studies have been reported on microencapsulated probiotics inoculated into products such as fermented sausages (Muthukumarasamy and Holley, 2006), ice cream (Homayouni *et al.*, 2008), chocolate (Possemiers *et al.*, 2010), infant formula powder (Weinbreck *et al.*, 2010), bread (Altamirano-Fortoul *et al.*, 2012), mayonnaise (Mohammadi *et al.*, 2012), cream-filled cake (Zanjani *et al.*, 2012), yogurt (Niazmand *et al.*, 2010; Ladjevardi *et al.*, 2016) and cake (Beikzadeh *et al.*, 2018).

There is no study on the inoculation of *Bifidobacterium animalis* in cocoa cream. Also, it is seen that the presence of significant amounts of free fat and free water constraints makes probiotics being not alive in mayonnaise (Mohammadi *et al.*, 2012) and cream-filled cake (Zanjani *et al.*, 2012). In addition, their growth and activity lead to changes in the texture benefits and taste of the product (McClements, 2015). Therefore, microencapsulation technique with sodium alginate has been used to maintain bacterium survival and improve intestinal microorganism activity in order to obtain pleasant effect on consumer health (Zanjani

et al., 2012). Besides, this technique can contribute to prevention of undesired tissue changes. As it was shown in previous studies, prebiotics have the ability of improving the flavor and aroma in different foods (Beikzadeh *et al.*, 2018).

Here, we aimed to produce synbiotic cocoa cream by using inulin to help the *B. animalis* microbial growth with sodium alginate and investigate its physicochemical properties such as acidity, viscosity and particle size of microcapsules and sensory characteristic including taste, texture, and general acceptability.

MATERIALS AND METHODS

Materials

Cocoa powder and sugar were purchased from the local market. Analytical grade chemicals containing calcium alginate, tween 80, calcium chloride, glycerol, phosphate buffered saline, lecithin, skim milk powder, and gelatin powder were obtained from the Merck Company (Darmstadt, Germany). Inulin was provided from Pyson Company (China). The media for microbial culture, including Man, Rogosa and Sharpe (MRS) broth and agar were purchased from the Merck Company (Darmstadt, Germany).

Preparation of Cocoa Cream

Cocoa cream was prepared by mixing all the ingredients including 20 g of cocoa cream oil, 50 g of cocoa powder, 14 g of sugar, 4 g of gelatin powder, 8 g of skim milk powder. The mixture was then heated to 80-85°C in the water bath. In samples containing inulin, two different treatments with 2 and 3% inulin ratios, i.e. 2 and 3 grams of inulin were added with sugar. After reaching 80-85°C, using an ice bath equipped with a stirrer, the temperature of the mixture was lowered quickly to 45°C, and 4 g of lecithin was added and further

stirred for 2 minutes. At this stage, microencapsulated microbial strain was added at the rate of 2×10^{10} CFU g^{-1} to prepare synbiotic cocoa cream. The temperature of cocoa cream was brought to 4°C. All the samples were then stored in dark air tight plastic containers away from light for a period of 42 days at 4°C.

Preparation of Cell Suspension

Using a sterile loop, a portion of lyophilizing *B.animalis* subsp. lactis (ATCC 27673) was removed from sterile ampoules and entered into 10 mL MRS broth (Merck, Germany). This pure strain was provided from the University of Tehran's microbial collections. This anaerobic tube containing bacterial strain was incubated for 24 hours at 37°C. Then, it was cultured on MRS agar (Merck, Germany) using a sterilized loop and the plate was incubated at 37°C for 24 hours. The result of biomass was inoculated into 50 mL of MRS medium and multiplied under these conditions. The medium was centrifuged (Sigma 3-k, Germany) at about 25°C for 15 minutes at 4,000 rpm and the resulting biomass was isolated and washed twice with 0.1% sterile pure peptone water solution.

Microencapsulation and Inoculum *B.animalis* into the Cocoa Cream

Microencapsulation was done by emulsion method (Sultana *et al.*, 2000). Then, 2.5 g of calcium alginate (Merck, Germany) was dissolved in 100 mL of distilled water, and after autoclaving of isothermal solution at room temperature, microbial suspension was added. To make an emulsion, 50 mL of maize oil was added to the solution containing 0.3% tween 80 (Merck, Germany) and mixed by a magnetic stirrer for 20 minutes at 400 rpm. Afterwards, 0.1 molar of calcium chloride (Merck, Germany) was added to the solution to make the capsule, along with mixing operation for

30 minutes. After the formation of the sediment caused by emulsion breakdown in a decanter funnel, in order to complete the separation process, the samples were centrifuged at 350 rpm. The resulting capsules were washed several times with sterilized distilled water and 10% glycerol (Merck, Germany), and stored at 4°C until they were inoculated. Cocoa cream with less viscosity was inoculated for 1 gram of 2×10^{10} CFU g^{-1} microbial suspension at 45°C.

Changes in Acidity

Extraction of cocoa cream fat samples was performed using Soxhlet method according to the 920.39C method of AOAC (2005). The acidity was measured using AOAC (1984) with three replications, and the figures were reported as gram of oleic acid per 100 g of the sample.

Viscosity Measurement

The viscosity was measured by Spindle (No. 60) viscometer (Brookfield DV-II+Pro, USA) and 100 rpm. The samples were measured in 600 mL petri dish at the temperature of 23°C by 75% torque with three replications (Ladjevardi *et al.*, 2016).

Particle Size of Microcapsules

The size of *B.animalis* microencapsulation particle was measured by the particle size analyzer (Mastersizer 2000, UK).

Survival of Encapsulated *B.animalis* in the Cocoa Cream

Five g of Cocoa cream sample was stirred in 45 mL of 0.1 M phosphate buffered saline (Merck, Germany) for 110 minutes by stomacher (Funk Gerber, Germany) and then cultured by pour plate method after making appropriate dilution in MRS culture



medium. After 48 hours, the incubation was performed at the temperature of 37°C (Krasaekoopt *et al.*, 2006; Sultana *et al.*, 2000).

Sensory Evaluation

This test was performed using a five-point hedonic test 1 (dislike extremely) to 5 (like extremely) and 10 semi-trained panelists (five women and five men, ages: 25-35 years) (Beikzadeh *et al.*, 2018). Even though the 9-point scale is used in many food products, in some studies, the 5-point is more preferable to reduce the potential problem of the consumers avoiding expressing extreme reactions (“end use avoidance”) (Lawless and Heymann, 2010). In order to evaluate sensory properties, 20 g of the sample in each container was provided for the panelists. For each sample, the panelists scored the texture, flavor, and total acceptability. The higher mean of scores indicated more panelists’ interest.

Statistical Analysis

The experiments were carried out using a factorial experiment in a completely randomized design with three replications. The SPSS software (Version 20; SPSS Inc., USA) was used to analyze the results, and Duncan multi-domain test was used at the statistical significance level 5% in order to compare the treatments.

RESULTS AND DISCUSSION

Acidity

Table 1 shows the mean acidity in terms of oleic acid in cocoa cream samples after 42 days. The acidity of the samples containing 2 and 3% inulin were compared to the control sample. The discussed factor increased significantly during the storage period until the 21st day ($P < 0.05$), but finally stopped and

reached a constant level. In all *B.animalis* samples with or without inulin, a significant increase was observed in acidity at all days of the test ($P < 0.05$). Due to high fat content in cocoa cream, hydrolysis of its fat produced free fatty acids and increased the acidity feature of the product. The major products derived from the carbohydrates metabolism by Bifidobacteria were lactic acid and acetic acid; they caused increase in the acidity during storage as indicated by Farnworth (2016) and Ladjevardi *et al.* (2016). Patel *et al.* (2008) have reported the same results related to the acidity of liquid chocolate, inoculated with *Lactobacillus paracasei* and inulin as a prebiotic compound. During the 28 days of storage, the addition of inulin alone did not cause any changes, but in samples with *Lactobacillus paracasei*, acidity increased significantly compared to the other treatments ($P < 0.05$).

Viscosity

The mean viscosity of cocoa cream samples after 42 days is show in Table 2. At the beginning, the viscosity measurements and viscosity process during 15 minutes study showed that cocoa cream viscosity decreased like a thixotropic fluid (Chevalley, 1994; Servais *et al.*, 2003). At the first day of the test, viscosity of all samples was close to each other, but in all samples, the mean viscosity of the samples showed a significant reduction over time ($P < 0.05$). In the chocolate colloidal system, the existence of oil, sugar, cocoa powder, milk, and lecithin causes the resistance to stress and, in fact, resistance to the viscometer spindles motion during viscosity measurements (Chevalley, 1994; Rad *et al.*, 2012). Cocoa cream produced with a certain percentages of compounds can provide a good texture and viscosity. When inulin was added to treatments, the effect on these factors reduced viscosity. The samples that contained inulin had a higher viscosity than microencapsulated samples. In addition, viscosity of the samples that contained

Table 1. Acidity (g oleic acid 100 g⁻¹) of cocoa cream samples during 42 days (means±standard deviation).

Sample	Time (Day)						
	0	7	14	21	28	35	42
Control ^a	0.39±0.03 ^{cd}	0.39±0.16 ^{cd}	0.39±0.07 ^{cd}	0.37±0.04 ^d	0.35±0.06 ^{de}	0.34±0.23 ^e	0.34±0.03 ^e
2% Inulin	0.4±0.07 ^{cd}	0.39±0.07 ^{cd}	0.39±0.01 ^{cd}	0.37±0.27 ^d	0.36±0.03 ^{de}	0.35±0.11 ^{de}	0.33±0.62 ^e
3% Inulin	0.4±0.21 ^{cd}	0.39±0.01 ^{cd}	0.4±0.09 ^{cd}	0.37±0.10 ^d	0.37±0.02 ^d	0.35±0.04 ^{de}	0.34±0.32 ^e
<i>B. animalis</i>	0.49±0.11 ^{ab}	0.45±0.26 ^{bc}	0.42±0.15 ^c	0.39±0.05 ^{cd}	0.39±0.18 ^{cd}	0.35±0.07 ^{de}	0.34±0.02 ^e
<i>B. animalis</i> +2%Inulin	0.5±0.19 ^a	0.46±0.03 ^b	0.44±0.18 ^{bc}	0.4±0.03 ^d	0.39±0.08 ^{cd}	0.35±0.21 ^{de}	0.33±0.18 ^e
<i>B. animalis</i> +3%Inulin	0.51±0.14 ^a	0.47±0.04 ^b	0.44±0.06 ^{bc}	0.4±0.16 ^{cd}	0.4±0.06 ^{cd}	0.36±0.05 ^{de}	0.34±0.01 ^e

^a Sample cocoa cream without inulin and *B. animalis*. ^{a-e} Means with different letters within a column indicate significant differences (P≤0.05).

Table 2. Viscosity (centipoise) of cocoa cream samples during 42 days (means±standard deviation).

Sample	Time (Day)						
	0	7	14	21	28	35	42
Control ^a	30298±381 ^a	29998±489 ^{ab}	30002±221 ^b	29586±560 ^c	29539±122 ^c	29535±25 ^c	29530±576 ^c
2% Inulin	30287±485 ^{ab}	29892±658 ^{bc}	29811±115 ^{bc}	29628±557 ^d	29681±113 ^d	29634±637 ^d	29631±572 ^d
3% Inulin	30283±386 ^{ab}	29885±896 ^{bc}	29701±683 ^{cd}	29521±344 ^e	29498±75 ^{ef}	29477±97 ^f	29424±280 ^f
<i>B. animalis</i>	30297±64 ^a	29994±556 ^{ab}	29990±960 ^{ab}	29593±371 ^{de}	29518±371 ^e	29512±580 ^e	29515±706 ^e
<i>B. animalis</i> +2%Inulin	30293±548 ^a	29785±595 ^c	29684±619 ^d	29321±476 ^g	29218±456 ^h	29211±623 ^h	29098±480 ^{hi}
<i>B. animalis</i> +3%Inulin	30288±75 ^{ab}	29685±553 ^d	29621±864 ^d	29021±595 ⁱ	29017±620 ⁱ	29011±120 ⁱ	29014±342 ⁱ

^a Sample cocoa cream without inulin and *B. animalis*. ^{a-i} Means with different letters within a column indicate significant differences (P≤0.05).



B. animalis with 3% inulin was significantly lower than 2% ($P < 0.05$). As considered in current study, the replacement of sugar with inulin and stevia reduces viscosity and increases consistency of chocolate milk (Rad et al., 2012). In contrast to these results, Akalın and Erişir (2008) reported a significant increase in viscosity on probiotic ice cream treated with inulin and oligofructose ($P < 0.05$). The reaction between water in ice cream matrix and dietary fiber increased gelatin state and viscosity of the product.

Particle Size

The mean diameter of microencapsulated *B. animalis* measured in the treatment without inulin and in presence of 2 and 3% inulin was 123 ± 1.1 , 123.2 ± 1.4 and 123.4 ± 0.9 μm , respectively. The results showed that addition of inulin had no effect on the size of the particles, which were formed by *B. animalis* microencapsulation. The smaller particles, compared to the reported numbers in other studies, produced a softer texture and prevented the formation of sandy texture (Mohammadi et al., 2012; Sultana et al., 2000). In a study by Hansen et al. (2002), this finding was validated. They

concluded that if the microencapsulation layer was thin and the diameters of the particles were less than 100 micrometers, it cannot protect the probiotic bacteria well, reducing any positive effect on the bacterial survival.

Viability of Probiotic Bacteria

Figure 1 shows the mean viability of microencapsulated *B. animalis* in the cocoa cream after 42 days. The number of the bacteria decreased in all samples during storage. Since microencapsulation is not fully effective, the faster reduction in bacteria in the first 14 days indicates the loss of a part of the bacteria that was not either fully or partially microencapsulated, as stated by the Gbassi and Vandamme (2012). The reduction in microencapsulated samples with 2 and 3% inulin was approximately the same, but in samples that did not have inulin and only microencapsulated bacteria were inoculated, the reduction number was significantly higher ($P < 0.05$). As a result, the prebiotic agent increased viability of probiotic bacteria, which agreed well with the previous studies (Akalın and Erişir, 2008; Patel et al., 2008; Sekhon and Jairath, 2010; Ladjevardi et al., 2016) (Figure 1).

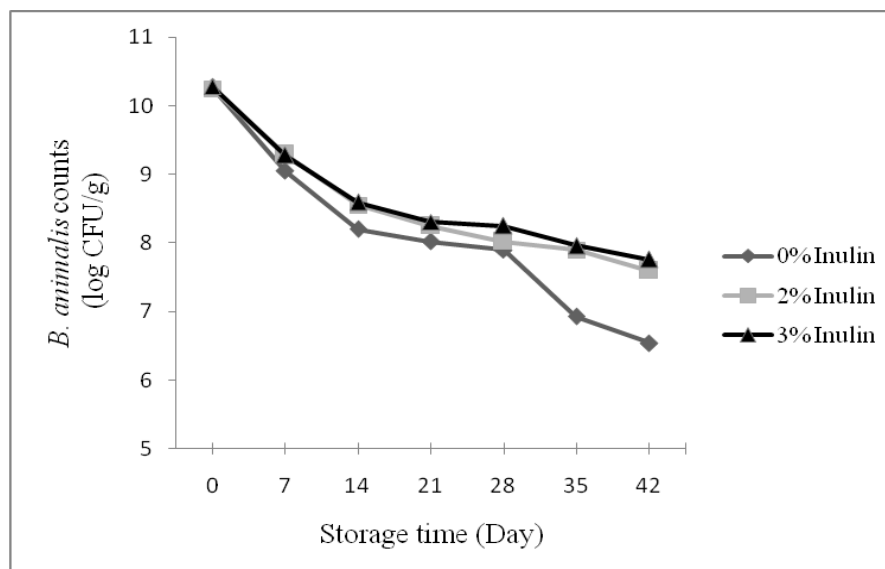


Figure 1. Viability of microencapsulated *B. animalis* in the cocoa cream during 42 days.

Sensory Evaluation

The sensory characteristic of the cocoa cream samples are represented in Figure 2. No significant difference was observed in terms of taste, texture, and general acceptability of different samples ($P > 0.05$). This suggests that the addition of microencapsulated *B. animalis* and inulin as a prebiotic compound had no effect on the sensory acceptance of cocoa cream samples. Similar observations of sensory properties have been widely reported by previous literature with regard to other kinds of food such as ice cream (Homayouni *et al.*, 2008), cheese (Mirzaei *et al.*, 2012), cream-filled cake (Zanjani *et al.*, 2012), chocolate (Possemiers *et al.*, 2010), and fermentation sausages (Muthukumarasamy and Holley, 2006), in which probiotic bacteria were microencapsulated by different materials. In these studies, the reason for not changing the sensory properties is related to the small size of the capsules made by the emulsion method (Muthukumarasamy and Holley, 2006; Zanjani *et al.*, 2012) (Figure 2).

CONCLUSIONS

In this study, microencapsulation technique with inulin was effectively used to maintain and decrease the *B. animalis* survival (as per the graph, it decreased). The

results showed that probiotic cocoa cream production is possible through microencapsulation of *B. animalis* and inulin. While this research-based study contributes in this area and its findings can be appropriate for bringing the products to industrial level, further studies are needed to open gateway for pilot studies and evaluation of novel synbiotic products.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the facilities support and technical assistance provided by Islamic Azad University, Science and Research Branch of Iran.

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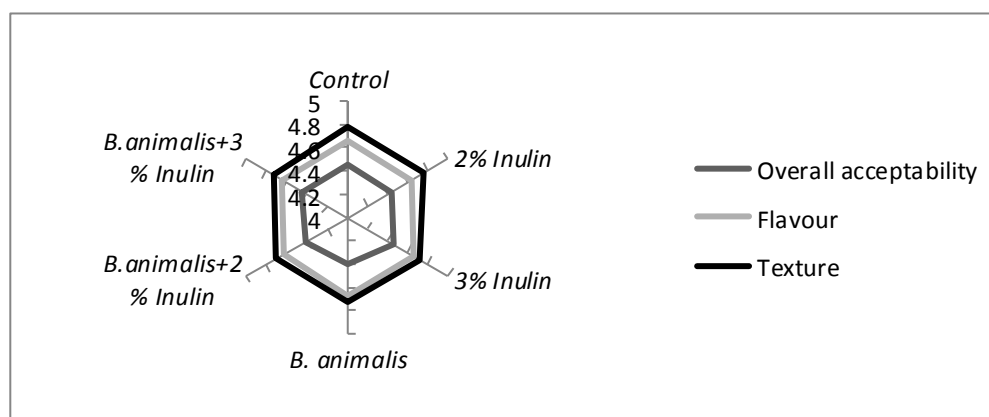


Figure 2. Sensory evaluation of the cocoa cream.



- ISBN: 0-925584-24-2, Association of Official Analytical Chemists, Washington, DC, USA.
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کرم کاکائو سین بیوتیک تولیدی بوسیله افزودن بیفیدوباکتر انیمالیس زیرگونه لاکتیس ریزپوشانی شده و اینولین: خصوصیات فیزیکیوشیمیایی، رئولوژیکی و حسی

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چکیده

استفاده از روش ریزپوشانی و ترکیبات پروبیوتیک از جمله روشهایی هستند که برای افزایش زندهمانی پروبیوتیکها استفاده می شود. این مطالعه به منظور بررسی اثر اینولینبه همراه ترکیب پروبیوتیک و تلقیح بیفیدوباکتر انیمالیس زیرگونه لاکتیس به عنوان یک باکتری پروبیوتیک ریزپوشانی شده با آلژینات سدیم انجام شده است. همچنین یک محصول بیوشیمیایی سنتزی دارای ویژگی سلامتی بخش در کرم کاکائو تولید گردیده است. نمونه ها شامل ۲ و ۳٪ اینولین همچنین، نمونه های تلقیح شده با بیفیدوباکتر انیمالیس ریزپوشانی شده با و بدون ۲ و ۳٪ اینولین و نمونه شاهد نیز بدون آن تهیه شده اند. نمونه ها به مدت ۶ هفته در دمای ۴°C نگهداری شدند و اسیدیته، ویسکوزیته، زندهمانی بیفیدوباکتر انیمالیس و خواص حسی آن ها در روزهای ۱، ۷، ۱۴، ۲۱، ۲۸، ۳۵ و ۴۲ مورد بررسی قرار گرفته است. در نمونه های کرم کاکائو اسیدیته و ویسکوزیته در طی زمان نگهداری به طور معنی داری به ترتیب افزایش و کاهش داشته اند. کاهش تعداد بیفیدوباکتر انیمالیس در نمونه های حاوی ۲ و ۳٪ اینولین با هم تفاوت معنی داری نشان نداده اند اما این کاهش در مورد نمونه های بدون اینولین به طور معنی داری کمتر بوده است. بعد از طی ۴۲ روز نگهداری در دمای ۴°C، ویژگی های حسی نمونه ها شامل مزه، بافت و پذیرش کلی تفاوت معنی داری نشان نداده اند. توانایی تولید کرم کاکائو سین بیوتیک بر مبنای روش ریزپوشانی بیفیدوباکتر



انیماليس و افزودن اينولين انجام شده است. محصول توليدي داراي ويژگي هاي مطلوب فيزيكوشيميايي، حسي و زنده ماني پروبيوتيك براي توليد صنعتي مي باشد.