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The Viability of Free and Encapsulated *Lactobacillus casei* and *Bifidobacterium animalis* in Chocolate Milk, and Evaluation of Its pH Changes and Sensory Properties during Storage

Reza Ghasemnezhad[1], Vadood Razavilar[1], Hadi Pourjafar[2*], Kianoush Khosravi-Darani[3] and Khadijeh Ala[4]

[1]Department of Food Hygiene and Quality Control, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Public Health, Maragheh University of Medical Sciences, Maragheh, Iran.
[3]Department of Food Technology, National Nutrition and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[4]Tabriz Branch, Islamic Azad University, Tabriz, Iran.

Authors' contributions

This work was carried out in collaboration between all authors. Authors RG, VR and HP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KKD and KA managed the analyses of the study. Author HP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The viability of encapsulated and free *Lactobacillus casei* and *Bifidobacterium animalis* subsp. lactis bacteria in chocolate milk for the first time as well as the influence of the bacteria on acidification and sensory acceptability of the product at 5℃ for 21 days.
Study Design: Research study
Place and Duration of Study: Department of Public Health, Maragheh University of Medical Sciences, between October 2016 and April 2017.

**Corresponding author: E-mail: pourjafarhadi59@ut.ac.ir;*

Methodology: The *Lactobacillus casei* and *Bifidobacterium animalis* subsp. Lactis were injected into chocolate milk in free and microencapsulated forms. Sodium alginate plus resistant starch and sodium alginate plus chitosan were used via extrusion method for microencapsulation. The changes in probiotic bacteria count and their sensory acceptability were evaluated at 5℃ for 21 days.
Results: The chocolate milk containing microencapsulated bacteria had a slight decrease in pH compared with the products with free bacteria. Further, the rate of microencapsulated bacteria viability was high in comparison with free bacteria in all conditions ($p<0.05$). Also, a product with microencapsulated bacteria has a more desirable sensory properties compared with a product with free bacteria at 5℃ for 21 days; hence, it has a high er acceptability.
Conclusion: Probiotic bacteria microencapsulation in the chocolate milk, as well as low temperature storage, can increase the viability of probiotics into product and postpone the fermentation process in chocolate milk. Based on the sensory evaluations and total acceptability scores, the chocolate milk containing microencapsulated *Lactobacillus casei* with an expiration of 6 days and containing *Bifidobacterium animalis* with an expiration of 10 days can be produced.

Keywords: Chocolate milk; Microencapsulation; Lactobacillus casei; Bifidobacterium animalis.

1. INTRODUCTION

Probiotics are live microorganisms which exert health beneficial impacts on human body including improving lactose and food digestion, stimulating the immune system, decreasing the blood cholesterol, as well as having anti-mutation and anticancer properties. Probiotics foods should contain at least 10^7 cfu ml^{-1} live bacteria and be consumed more than 100 ml per day to have helpful effects on health [1-3].

Incorporation of probiotics into several foods, e.g. cornflakes, pomegranate juice, cheese, yogurt, fermented milk, etc, has been reported [4,5]. Probiotics have many health-beneficial intrinsic properties for their host. Also, they can produce many useful metabolites during their growth and metabolism such as production of bioactive compounds, conjugated linolenic acid, propionic acid and etc [5,6]. Recently, it has been reported that these amazing microorganisms remove toxins and heavy metals and thereby reduce oxidative stress and inflammatory factors [1].

Chocolate consumption offers an encouraging role to human nourishment throughout the provision of antioxidants, and polyphenols including flavonoids for instance catechin, epicatechin, and procyanidins. Furthermore, chocolate has got some essential minerals, particularly iron, potassium, copper, and magnesium. Chocolate milk with its healthy properties is one of the most promising dairy products which may improve societies, especially children's health. The integration of probiotic microorganisms into chocolate could present an excellent alternative to regular dairy products and permit to expand the wellbeing claims of chocolate based food produces [7-9]. Due to high carbohydrate in chocolate milk, free probiotic bacteria have a fast growth; hence, they cause a sudden drop in pH [10].

The difficulties associated with the integration of probiotic microorganisms into different foods are their viability and stability at appropriate level throughout the processing, preservation, storage, and gastrointestinal (GI) passage. Several methods have been expanded to guard the probiotics from environmental stresses in food matrices, processing, storage, and GI tract transit. Amongst these, microencapsulation has been found to be, the most proper technique to guard the living probiotic microorganisms. Microencapsulation of probiotics decreases the accessibility of probiotic bacteria into the product and thereby leads to greater stability over storage period and transition through human GI tract [7,11-13].

"Extrusion technique" employed to encapsulate microorganism, is a mild method which doesn't have harmful solvents and can be conducted in aerobic and anaerobic conditions. In this method, the probiotic bacteria are surrounded by wall substances. The process consists of directing the dispersed nuclear substances in a mass of melted carbohydrates, to a water-absorbent liquid bath. When they contact with the liquid, the carbohydrate-capsule hardens and the probiotic bacteria are trapped. Capsulated beads prevent cell release, allow sufficient mass transfer and increase the chemical and physical stability of the cells [14-16].

The chocolate milk is a highly valuable drink containing high energy and fiber compared with white milk. In a study conducted in the USA, it was revealed that 91% of 8-12 years-old children and 57% of children preferred chocolate milk to white milk. Considering that childhood period is the time of receiving sufficient calcium to reach maximum bone density, the milk consumption by children has a special importance to prevent osteoporosis at old ages. In fact all milk, including flavored milk such as chocolate milk, includes a unique mixture of nutrients significant for growth and progress in children, and for most favorable healthiness and disease hindrance in adults. As chocolate milk has a favorable taste for children, adding probiotics can increase its valuable properties [17-21]. The present study aimed to evaluate both the viability of encapsulated and free *Lactobacillus casei* and *Bifidobacterium animalis* subsp. lactis bacteria in chocolate milk for the first time as well as the influence of the bacteria on acidification and sensory acceptability of the product at 5℃ for 21 days.

2. MATERIALS AND METHODS

2.1 Obtaining and Preparing the Starter Culture

The *Lactobacillus casei* and *Bifidobacterium animalis* subsp. lactis were obtained from Scientific-Industrial research Organization of Iran, the branch of the microbial collection. One lyophilized vial of each bacterium was inoculated into 5 ml MRS broth (de Man-Rogasa-Sharpe) medium and incubated at 37℃ for 24 h. Then, the samples were inoculated to 95 ml of MRS broth medium and incubated again at 37℃. Finally, the obtained biomass was centrifuged at 5000 g for 10 minutes. In order to completely rinse the bacteria sediment in the microtubes, it was centrifuged again with a physiologic serum. Eventually, this bacterial emulsion was used for direct inoculation of chocolate milk [22].

2.2 Microencapsulation of the Bacteria

In the present study, the microencapsulation process was conducted via sodium alginate (Sigma, USA) + resistant starch (Merk, Darmstadt, Germany), and sodium alginate + chitosan, by extrusion method [22,23]. At first, 4 g of sodium alginate was added to 100 ml distilled water. Then, the solution was sterilized and kept in refrigerator over a night in order that alginate particles absorb water. Then, 10 ml of the prepared bacterial emulsion was added to alginate and the obtained suspension was injected into the 0.1 mol calcium chloride solution by insulin syringe. After the formations of beads into the calcium chloride solution, they were drained and rinsed. The beads were passed through two separate paths in order to get encapsulated: i. 10 g of the beads was added into 1% starch solution and was stirred for 10 minutes; ii. 10 g of the beads was added into 100 ml of 1% chitosan solution and was stirred for 10 minutes. The microencapsulated beads with chitosan and resistant starch were kept in sterile peptone (0.1 g 100 g^{-1}) at 4℃ to conduct the related tests [14,22-24].

2.3 Inoculation of Free and Microencapsulated Probiotic Bacteria into the Chocolate Milk

The mentioned probiotic bacteria were inoculated in two forms, free and microencapsulated, into the milk in a sterile condition. The production was conducted in two replications for each sample.

2.4 Evaluating the Physicochemical and Sensory Properties of Chocolate Milk

PH, acidification, and fat (physicochemical properties) were measured from the first day (produced day) through the 21st with an interval of 7 days. pH meter, Dornic method, and Gerber method were used to measure the mentioned variables. The evaluation of sensory properties, as well as bacterial count, was conducted on the same day by a 32-person panel under equal conditions of place, light, and dish. In this method, a questionnaire consisting of three items including color and appearance (1-5 scores), texture (1-5 scores), and taste and flavor (1-10 scores) was used and a general acceptability (1-20 scores) was recorded for the product. The total acceptability scores were; Like very much (20), like moderately (17-20), neither like nor dislike (14-17), Dislike moderately (10-14), Dislike very much (<10). Mean scores for each characteristic were calculated for the comparison of the samples.

2.5 Evaluating the Viability of Probiotic Bacteria in Chocolate Milk

The number of the survived probiotic bacteria in each sample was measured immediately after the preparation of chocolate milk, and during 21 days of storage at 5℃ with 7 day intervals. The counting of free bacterial samples was as follows:

10 ml of chocolate milk containing free bacteria was added to 90 ml of peptone water and was diluted to 10-12. Then, 1 ml of any dilution was cultured in plates containing MRS-Salicin-agar in two replications. The plates containing *Bifidobacterium animalis* as well as the plates containing *Lactobacillus casei* were kept in anaerobic and aerobic conditions and were incubated at 37℃ and 30℃ for 72 h, respectively. Then, the number of colonies was determined in two replications. The bacteria needed to be released from the beads in order to count the microencapsulated probiotic; thus, 9 ml of the prepared sodium citrate (16 g sodium citrate was dissolved in 1 liter water and was sterilized in autoclave to prepare the solution) and 1 g of the beads were stirred on the shaker for 30 min. to release the bacteria from the beads, and then the above, mentioned stages were used to count the bacteria [4,11,25,26].

2.6 Statistical Analyses

All statistical analysis was performed by SPSS (version 8.5) software. Data were presented as mean (±SD) or median (Quartile1–Quartile3). The regularity of the overall acceptability score was tested and confirmed by Kolmogorov-Smirnov test. These comparisons were carried out for appearance, texture, and taste by Mann–Whitney and Sign test, respectively. P< 0.05 considered to be significant.

3. RESULTS AND DISCUSSION

3.1 The Effect of Microencapsulation on Probiotic Bacteria Viability

The main value of a product containing probiotic bacteria is the number of survived probiotic cells in each ml of product at the time of consumption. So, considering the necessity of the presence of determined probiotic bacteria at the time of consumption of a product, the effect of microencapsulation on *Lactobacillus casei* and *Bifidobacterium animalis* subsp. lactis viability was studied. Table 1 shows the changes of the survived cells' count of the two bacteria in eight samples during the storage period. This table shows a slight increase and decrease in the viability of microencapsulated bacteria, which confirms the effect of coating the beads on protecting the probiotic bacteria during storage of the product. Furthermore, it was observed that using the sodium alginate + resistant starch to coat the beads cause greater viability of probiotic bacteria in comparison with sodium alginate + chitosan. Calcium alginate and starch are safe for acid lactic bacteria nevertheless chitosan may decrease survival rate of them. This can be attributed to the antimicrobial characteristic of chitosan even as the second layers. The antimicrobial activity of chitosan against a wide range of microorganisms mainly bacteria has been revealed in some studies [27,28].

Microencapsulated probiotic bacteria, at 5℃ storage temperature, need a longer time to decrease logarithmic cycle compared with the free bacteria, which is evident in Table 1. The functional foods should have at least 10^7 cfu g or ml^{-1} probiotic bacteria to affect consumers beneficially [29,30]. In the present study, the count of microencapsulated probiotic bacteria was higher than its rate (10^7 cfu ml^{-1}) during the storage period of the product in refrigerator.

In a similar study, Mandal et al. [7] investigated integration of encapsulated *Lactobacillus casei* NCDC298 and inuline (as a prebiotic) into chocolate milk. After 1 month of storage at room temperature, the mentioned probiotic counts diminished at about 3 and 2 log cycles from the first level of 8 log cfu g^{-1} in chocolate milk with free and microencapsulated lactobacilli, respectively; nevertheless, at the refrigeration temperature, the survival rate of the free and microencapsulated lactobacilli was unchanged up to 2 month. Also, Possemiers et al. [19] proved chocolate as a possible carrier for oral delivery of a microencapsulated combination of *Lactobacillus helveticus* CVCM I-1722 and *Bifidobacterium longum* CNCM I-3470.

3.2 The Effect of Microencapsulation on pH Changes in Chocolate Milk

Based on the obtained results, free bacteria caused more pH decrease and acidity increase during the chocolate milk storage than microencapsulated bacteria. The reason can be attributed to the decreased activity of microencapsulated bacteria; therefore, the pH of samples containing microencapsulated bacteria was higher than the free bacteria. One of the factors influencing the metabolic activity of microencapsulated bacteria in the products is the size of alginate layer; the more encapsulation layers, the lower the acidity trend. In the present study, two layers were used in microencapsulation. As it is observed in Table 2, the acidity trends were decreased in samples containing microencapsulated bacteria.

Also, based on Table 2, the samples containing free *Lactobacillus casei* showed the earliest pH changes at 5℃ of storage while the samples containing *Bifidobacterium animalis* subsp. lactis microencapsulated with sodium alginate + chitosan showed the latest pH changes. Moreover, the formed coating around the bacteria made the nutrition absorption slow and thereby decreased the pace of organic acids release. Furthermore, due to its need for longer time to grow, *Bifidobacterium animalis* subsp. lactis caused less pH decrease compared with *Lactobacillus casei*.

3.3 The Effect of Microencapsulation on Sensory Properties of Chocolate Milk

Suitable sensory properties are the main factors of the acceptability of products. The microencapsulation had not any influence on the samples' color suggesting that the sodium alginate, resistant starch, and chitosan advantages were colorless compounds thus made no change in the product's appearance.

One of the important factors in samples' texture acceptability is their uniformity. In the present study, the size of capsules was 300-500 µm and influenced the texture of the samples at a low rate; so that, the chocolate milk containing microencapsulated bacteria had lower scores in terms of texture compared with the samples containing free bacteria. This miller size of capsules made fewer changes in the product texture and prevented the sandy appearance of the product. In extrusion microencapsulation, the size of the capsules is larger so, that when consuming the product, they feel sandy [2,22, 31-33]. In terms of flavor and taste, the microencapsulation had not any influence on the chocolate milk samples. Also, considering Fig. 1, the total acceptability of chocolate milk containing free bacteria was lower at refrigerator temperature during 21 days of storage compared with the chocolate milk containing microencapsulated bacteria. The lowest acceptability was associated with the chocolate milk containing free *Lactobacillus casei*, which obtained the total acceptability score about 6 during 3 days of storage due to pH of 5.3 and presence of chocolate milk clots. On the other hand, the highest total acceptability was associated with the chocolate milk containing *Bifidobacterium animalis* microencapsulated with sodium alginate + chitosan that the total acceptability score reached 9 on the day 21 of storage.

Table 1. Evaluating the viability of probiotic bacteria (cfu ml-1) in chocolate milk

Group	Bacterium	Day 1	Day 7	Day 14	Day 21
Control	***No bacterium***	**0**	**0**	**0**	**0**
Free	*Lactobacillus casei*	$1.6±0.1×10^9$	$2.6±0.1×10^9$	$2.7±0.1×10^8$	$7.0±0.4×10^7$
	Bifidobacterium animalis	$4.1±0.6×10^9$	$8.6±0.7×10^9$	$5.2±0.6×10^8$	$5.6±0.4×10^7$
Microencapsulated with starch	*Lactobacillus casei*	$2.3±0.9×10^9$	$2.4±0.1×10^9$	$1.8±0.2×10^9$	$1.6±0.7×10^9$
	Bifidobacterium animalis	$3.6±0.6×10^9$	$5.4±0.3×10^9$	$2.3±0.1×10^9$	$1.0±0.1×10^9$
Microencapsulated with chitosan	*Lactobacillus casei*	$1.7±0.2×10^9$	$3.1±0.4×10^9$	$6.7±0.9×10^8$	$2.1±0.2×10^9$
	Bifidobacterium animalis	$3.1±0.6×10^9$	$2.8±0.1×10^9$	$1.4±0.3×10^9$	$6.3±0.4×10^8$

Table 2. pH changes of chocolate milk prepared with free and microencapsulated probiotic bacteria

Bacterium	Product type	Day 0	Day 7	Day 14	Day 21
Lactobacillus casei	Control	6.70	6.70	6.70	6.70
	Free bacterium	6.62	4.75	3.94	6.70
	Microencapsulated with alginate/starch	6.62	6.26	5.33	4.25
	Microencapsulated with alginate/chitosan	6.64	6.33	5.40	4.31
Bifidobacterium animalis	Free bacterium	6.67	6.0	5.6	5.10
	Microencapsulated with alginate/starch	6.67	6.40	5.92	5.35
	Microencapsulated with alginate/chitosan	6.67	6.52	6.0	5.37

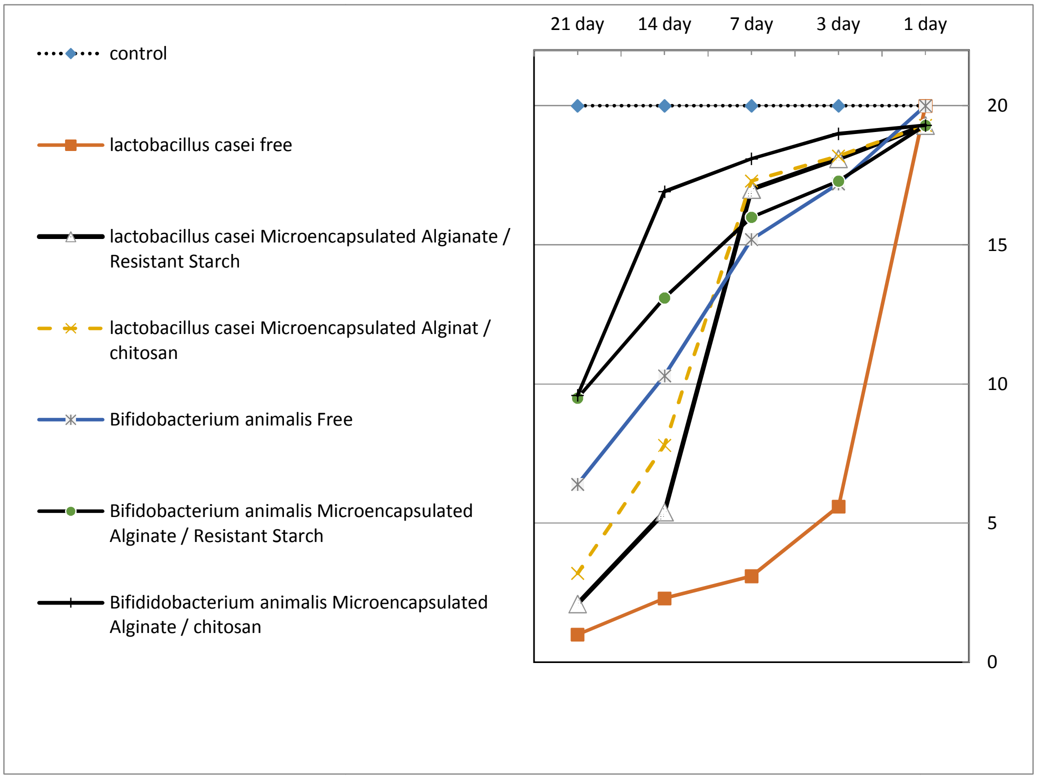

Fig. 1. Comparing the total acceptability of chocolate milk containing free and microencapsulated bacteria during storage time

Table 3. Maximum storage time of chocolate milk containing microencapsulated probiotic bacteria and score of total acceptability of the product at the end of the maximum storage time

Bacterium	The used wall	The maximum storage time	The score of total acceptability (1-20 scores)
Lactobacillus casei	Sodium alginate + resistant starch	6 days	17
Lactobacillus casei	Sodium alginate + chitosan	8 days	17.2
Bifidobacterium animalis	Sodium alginate + resistant starch	9 days	17
Bifidobacterium animalis	Sodium alginate + chitosan	10 days	17.5

Also, maximum storage time of chocolate milk containing microencapsulated probiotic bacteria and the score of total acceptability of the product at the end of maximum storage time are shown in Table 3 above.

4. CONCLUSION

Based on the results of the present study the acidity trend in chocolate milk can be postponed with microencapsulation of *Lactobacillus casei* and *Bifidobacterium animalis* subsp. lactis. Furthermore, it is possible to produce chocolate milk containing microencapsulated probiotic bacteria with a determined storage time in refrigerator. In addition, considering the tested sensory properties and the obtained total acceptability score, according to Table 3, chocolate milk containing probiotic bacteria can be produced and released to market.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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