IJBPAS, April, 2016, 5(4): 837-849

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

SURVIVAL OF PROBIOTIC BACTERIA MICROENCAPSULATED WITH CALCIUM ALGINATE AND RESISTANT STARCH UNDER SIMULATED GASTROINTESTINAL CONDITIONS AND DURING STORAGE INTO CHOCOLATE MILK, AND EVALUATION OF SENSORY PROPERTISES OF PRODUCT

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ABSTRACT

The aim of this study was to evaluate the effect of microencapsulation with calcium alginate and resistant starch on viability of Lactobacillus casei and Bifidobactrium animalis subsp. lactis in chocolate milk and the survival rate in simulated gastrointestinal juice conditions. Two types of probiotic chocolate milk, with free and microencapsulated probiotic bacteria, were manufactured in two repeat under the same conditions. The number of viable cells during 21 days of storage in refrigerated conditions and in 0.6% bile salt solution and simulated gastrointestinal condition was evaluated. Results showed that microencapsulation affected PH, acidity, sensory properties and viability of probiotic bacteria in chocolate milk samples .The number of viable cells of probiotic bacteria was altered significantly during storage period in both types of chocolate milk , but alteration in the chocolate milk samples containing free cells was significantly ($p < 0.05$) higher than the chocolate milk containing microencapsulated cells. The results indicate that, microencapsulation in calcium alginate and resistant starch was able to increase the survival rate of *L.casei* and *B.animalis* in chocolate milk after 21 days of storage. Survivability of microencapsulated cells in a simulated gastrointestinal condition was significantly more than free cells ($p<0/05$). Total acceptability of chocolate milk samples containing microencapsulated was better than samples with free one.

Key words: microencapsulation- calcium alginate- resistant starch-gastrointestinal juice – chocolate milk

INTRODUCTION

Lactobacillus casei and *Bifidobacterium animalis subsp. lactis* are considered to be probiotic and have been incorporated into various dairy products [38]. These microorganisms benefit person health by improving the balance of intestinal microbiota and by strengthening mucosal defenses against pathogens [5]. However, for probiotics to be therapeutically effective, it has been suggested that products should contain at least10^{7}cfu/g probiotic bacteria and consumed at levels higher than 100 g/day to have helpful effects on health [33]. Microencapsulation of probiotic bacteria is currently drawing more and more attention for being a method to improve the stability of probiotic organisms in functional food products [39]. Moreover, according to Ding and Shah (2009), microencapsulation may improve the survival of these microorganisms, during both processing and storage, and also during passage through the human gastrointestinal tract. Very high levels of probiotic bacteria do not survive in dairy products [18]. In addition, Lankaputhra and Shah (1995) found that survival of *L. casei* and *Bifidobacterium* spp. is low in the presence of acid and bile salts. Protection of probiotics by microencapsulation in hydrocolloid beads has been investigated for improving their viability in milk products and the intestinal tract. Calcium alginate has been used widely for the capture of lactic acid bacteria due to its ease of handling, its non-toxic nature, and due to its low cost. Model studies are available where alginates have been used for the encapsulation of bacteria for fermentation purposes or for incorporation into products, [21, 25]. Alginate microencapsulation has been used successfully to immobilize bacterial cultures for incorporation into mayonnaise [27]. Despite the appropriateness of alginate as the entrapment matrix material, gel entrapment in alginate has some limitation due to low stability in the presence of chelating agents.

The chelating agents share affinity for calcium and destabilize the gel [40]. Thus, instability problems are encountered during lactic acid fermentation and cause cell release from the beads. In the case of other matrix material, such as chitosan, the entrapped cells can be released from the beads during fermentation and cause low initial loading for the next fermentation. Therefore, special treatments, such as coating the beads, are applied in order to develop the properties of microencapsulated beads. Coated beads not only prevent cell release but also increase mechanical and chemical stability. Mixing with starch can develop stability of beads. Alginate/starch liquid core capsules offer the ability to microencapsulate *L. acidophilus* without loss of viability and fermentation ability [21]. Capsule membranes allow sufficient diffusion of nutrients and metabolites to maintain growth of microencapsulated cells.

Therefore, the objective of this study was to evaluate the survival rate of free and microencapsulated *L. casei and B. animalis subsp.lactis* by calcium alginate and resistant starch in chocolate milk during storage time and simulated gastrointestinal conditions.

MATERIAL AND METHODS:

1. Preparation of free and microencapsulated probiotics.

Freeze-dried of *Lactobacillus casei* and *Bifidobactrium animalis subsp. lactis* was obtained from Iranian research organization for science and technology (IROST) and inoculated into MRS-broth (de Man-Rogosa-Sharpe) and incubated at 37˚C for 24 h. The probiotic biomass in late-log phase was collected by centrifugation (Centrion Centrifuge, Model 2010, West Sussex, BNI8OHY, UK) at 10,000 rpm for 10 min and then it was washed twice in sterile saline before using in the microencapsulation procedure. In this study extrusion technique was performed for microencapsulation process described earlier by Mirzaei et al. (2012). A 2% Na–alginate mixture in distilled water containing 2% Hi-maize resistant starch (Merck, Darmstadt, Germany) and 0.1% culture was prepared. Then, the mixture of cell suspension and Na–alginate and resistant starch were injected into a 0.1 M CaCl2 solution. The droplets formed gel spheres immediately. Diameter of the resultant beads was 300–500 µm.

2. Preparation of chocolate milk

Sterilized chocolate milk (1/5% fat, 3/2% protein, 11/9% carbohydrate) was obtained from Pegah dairy Company in Tabriz, Iran.

Chocolate milk was poured in sterile containers 100ml under sterile conditions. Free and microencapsulated probiotics were added in order to achieve approximately10^9 cfu.g-1. As controls, Chocolate milk with free probiotics and microencapsulated probiotics were produced. Each type of Chocolate milk was produced in two repeat.

3. Measurement of pH

Acidity of samples was resolute according to titration technique and based on lactic acid percentage. 10 ml of sample was titrated beside N/10 NaOH in presence of phenolphthalein. The values of pH were measured by a digital pH meter.

4. Enumeration of probiotic bacteria

Bacterial counts were determined immediately after manufacture of chocolate milk and during 21days at 5˚C. Viable probiotic concentrations were measured by pour plate counting on MRS agar. Samples (1.0 ml) were added to 9.0 ml of sterile Ringers solution, then, appropriate dilutions were made. Subsequently *Lactobacillus casei* and *Bifidobactrium animalis subsp. lactis* were plated onto MRS agar+ 10 % W/V salicin. The colonies were counted after 72 h of incubation at 37°C. Colony forming units (CFU) were enumerated in plates containing 15 to 300 colonies and cell concentration was expressed as cfu/ml [42]. To count the encapsulated bacteria in chocolate milk, the entrapped bacteria were released from the beads according to the method of Pourjafr et al. (2011). Ten ml of chocolate milk were re-suspended in 100 ml of phosphate buffer (0.1 M, pH 7.0) followed by 10 min shaking on a shaker. The chocolate milk sample containing free bacteria was treated in a similar way so as to maintain the same treatment conditions. All experiments were done in two repeat.

5. Bile salt solution tolerance of free and microencapsulated probiotic bacteria

The stability of free and microencapsulated *Lactobacillus casei* and *Bifidobactrium animalis subsp. lactis* was experienced in porcine bile salt solution. Alginate–milk microspheres containing probiotic bacteria (1 g) were placed in a tube containing 9 mL bile salt solution (0.6%, w/v and pH: 8.25) and incubated at 37˚C for 2 h. Free and microencapsulated probiotic bacteria were collected at each 30 min time intervals. Assay of the viability of encapsulated and free probiotic bacteria was carried out as described above. After the beads were broken in sodium phosphate solution, the viable amounts of probiotic bacteria were determined according to procedures described in previous section [35].

6. Survival of free and encapsulated probiotic bacteria in simulated gastrointestinal juice

Beads containing probiotic bacteria (1 g) were placed in a tube containing 9 mL Simulated gastric solution containing (0.08 mol/L HCl solution contained 0.2% NaCL and pH: 1.55 without pepsin) and incubated at 37˚C for 0, 30, 60, 90 and 120 min. After incubation, 1.0 mL of these solutions was added in 9 mL of simulated intestine solution containing (0.05 mol/L KH_2PO_4 solution with 0.6 % bile salts and pH: 7.42) and incubated at 37˚C for 2 h. Assay of the viability of free and encapsulated probiotic bacteria was carried out as described above. After the beads were broken in sodium phosphate solution, the viable amounts of probiotic bacteria were determined according to procedures described in previous Section [35].

7. Sensory examination

Colour and appearance, flavour and taste, body and texture, total acceptability of chocolate milk samples were analyzed. Sensory analysis was performed on days one, three, seven, fourteen and twenty one by using 32 trained panellists familiar with 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 comparison of the samples.

8. Statistical analysis

Significant differences between the results were calculated by analysis of variance (ANOVA) using SAS software version 8. $P < 0.05$ considered to be as significant. The normality of the overall acceptability score was tested and confirmed by Kolmogorov–Smirnov test. The mean of overall acceptability score were compared between two chocolate milk type using independent samples t-test. These comparisons were carried out for colours and appearance, for texture and for odor and taste by Mann–Whitney and Sign test, respectively.

RESULT AND DISCUSSION

The primary cell count of probiotic bacteria before was in the range of 6.4×10^{10} cfu/ ml. High cell loading in the range of 3.1×10^{9} – 4.5×10^{10} cfu/ g beads was achieved in resistance starch-coated beads, which had an average diameter of 300–500 µm. The loss during microencapsulation was very low due to the gentle process used. The coated beads containing probiotic bacteria were included into the chocolate milk on the day of their preparation. The probiotic bacteria in the chocolate milk were counted periodically in 1day in the cold storage until 3 weeks.

1. Survey of PH

Table 1show the pH changes in the control and experimental chocolate milk during storage at 5 ˚C for a period of 3 weeks.The ending pH (at end of 3 week storage) of chocolate milk with microencapsulated probiotic bacteria was bigger than the chocolate milk inoculated with free probiotic bacteria. Probiotic bacteria are slow acid producers. The results of this study confirmed that acidification in Chocolate milk with microencapsulated probiotic bacteria was slower compared to chocolate milk with free probiotic bacteria.

3.2. Viability of probiotic bacteria during cold storage

Table 2 show variations in counts of free and microencapsulated *L. casei* and *B.animalis subsp. lactis* during 21 days storage period at 5 ˚C as (cfu gr^{-1}). Live cell counts in chocolate milk samples were determined from 1 until 21 days with 7 days of intervals. The results showed that there were approximately 2 log cycle losses in number of cells of both free *L.casei* and *B.animalis subsp. lactis*. The encapsulated bacteria, however, showed only 1 log decrease in cell numbers of chocolate milk samples.

This study showed that encapsulation is able to keep the number of probiotic bacteria above the threshold level for therapeutic minimum ($\geq 10^7$ cfu /ml) in chocolate milk. Mirzaei et al. (2012) showed that microencapsulation in calcium alginate and resistant starch was able to enhance the survival rate of *L. acidophilus La5* in Iranian white brined cheese after 6 months of storage.

3.3. Survival of free and microencapsulated probiotic bacteria (*L. casei* and *B. animalis subsp. lactis*) bile salts solution (0.6 %, pH: 8.25).

Table 3 showed survival of *L. casei* and *B. animalis subsp. lactis* in 0.6% bile salts. Survivals were recorded at 30- min intervals after 120 min exposure. Probiotic bacteria, free *L. casei* and *B. animalis subsp. lactis*, showed a decrease of more than 2 log cycles. Probiotic bacteria, microencapsulated *L. casei* and *B. animalis subsp. lactis*, showed a decrease of only 2 log cycles, while *L. casei* showed only a slight decrease in numbers as compared to *B. animalis subsp. lactis* cell count. Probiotic bacteria of free *L. casei* and *B. animalis subsp. lactis* were lost their viability in bile salt solution after 120 min exposure, perhaps due to the loss of cell wall integrity as a result of action of the bile salts. Many references mentioned probiotic bacteria were sensitive to bile salt solution. Clark and Martin (1994) reported a 5 log decrease in viable cell counts of *Bifidobacterium adolescentis* (*B. adolescentis*) in 2% bile salt solution at 37˚C after 12 h incubation. Truelstrup et al. (2002) showed that *B. adolescentis* was decreased about 2 log CFU/mL after 2 h incubation in 0.5% bile salt at 37˚C.

3.4. Survival of free and microencapsulated probiotic bacteria in simulated gastrointestinal solution

Microencapsulated and free probiotic bacteria were exposed to in vitro simulated gastrointestinal conditions of high acid. Table 4 shows the data of survivals of bacteria in acid. The viability of all free and microencapsulated *L. casei* and *B. animalis subsp. lactis* were diminished significantly when those were exposed to acidic conditions. When *L. casei* and *B. animalis subsp. lactis* were exposed to simulated gastrointestinal solution, none of the free bacteria survived after 2 h. but microencapsulated probiotic bacteria survived after 2 h. Therefore, *L. casei* and *B. animalis subsp. lactis* would be encapsulated to ensure high survival rate in the gastrointestinal environment. The amount of probiotic bacteria in alginate micro particles little by little declined with incubation time after 2 h. The number of *B. animalis subsp. lactis* was reduced more than *L. casei* and these bacteria were very sensitive to low pH. In order to exert positive health effects, probiotic bacteria should resist the stressful conditions of the stomach. Therefore, one main purpose of microencapsulation is to improve the low pH tolerance of probiotics. The pH of gastric juices is about 1.5–3.0 [28].

Excellent pH tolerance of encapsulated *L. casei* and *B. animalis subsp. lactis* perhaps was due to the buffering ability of milk in microspheres. Guerin et al. (2003) showed that the buffering ability of whey protein contributed to high survival rate for *Bifidobacteria* microencapsulated in alginate–pectin–whey protein microspheres when exposed to simulated gastric fluid pH 2.5.

3.5. Sensory analysis

Table 5 show the average sensory scores of all panellists. The points allocated for colour,flavour and taste showed that there were significant differences (P<0:05) in the colour and flavour and taste of the chocolate milk during 21days of storage at at 5 ˚C. It was expected that addition of alginate capsules contained starch to chocolate milk could alter colour, flavour and taste the chocolate milk, however, the panellist could recognize the differences in the flavour , colour and taste between chocolate milk produced contained free and microencapsulated during cold storage. The body and texture including smoothness of the chocolate milk samples, however, showed significant differences (P>0:05) between the chocolate milk containing free probiotic bacteria and microencapsulated bacteria. The panellists, however, reported slight grittiness and therefore scored the chocolate milk with probiotic capsules as more disagreeable as compared to the other of chocolate milk. Kailasapathy (1996) reported that sodium alginate used in the microencapsulation of the probiotic cultures form a gel with a number of cations such as calcium which is present in the milk gel.Total acceptability of chocolate milk samples containing microencapsulated *L.casei* and *B.animalis subp.lactis* was better than samples with free one. Acidification in chocolate milk with microencapsulated probiotic bacteria was slower compared to chocolate milk with free probiotic bacteria and this has affected flavour and taste of products.

Table 1: Variation of pH in chocolate milk produced contained free and microencapsulated probiotic bacteria during cold storage for 21 days

bacteria	samples	Day1	Day7	Day14	Day21
No bacteria	Control	6.70	6.70	6.70	6.70
Lactobacillus casei	Free	662	4.75	3.94	3.73
	Microencapsulated	6.62	6.26	5.33	4.25
Bifidobactrium animalis	Free	6.67	6.0	5.60	5.10
	Microencapsulated	6.67	6.40	5.92	5.35

Table 2: Survival of free and encapsulated probiotic bacteria *(L.casei* and *B. animalis subsp. lactis*) in chocolate milk samples (cfu/ ml).

Bacteria	Samples Samples	storage days 1	7	14	21
No bacteria	control	0	0	0	0
0*Lactobacillus casei*	Free	1.6×10^9	2.6×10^9	2.8×10^8	7×10^7
	Encapsulated	2.3×10^9	2.4×10^9	1.8×10^9	1.6×10^9
Bifidobactrium animalis subsp.lactis	Free	4.1×10^9	8.6×10^9	5.2×10^8	5.6×10^7
	Encapsulated	3.6×10^9	5.4×10^9	3.4×10^9	1.0×10^9

Table 3: Survival of free and microencapsulated probiotic bacteria (*L.casei* and *B.animalis subsp. lactis*) bile salts solution (0.6 %, pH: 8.25).

	Bacteria	Incubation time (min) 1	30	60	90	120
Free probiotic	*L.casei*	8.8×10^{7}	4.5×10^{7}	8.6×10^{6}	4.6×10^{6}	7.6×10^{5}
	B.animalis	9.6×10^{8}	6.3×10^{7}	9.3×10^{6}	7.4×10^{6}	4.6×10^{6}
Encapsulated probiotic	*L.casei*	2.4×10^{9}	8.2×10^{8}	3.6×10^{8}	9.1×10^{7}	5.6×10^{7}
	B.animalis	4.2×10^{9}	9.4×10^{8}	4.7×10^{8}	1.6×10^{8}	8.4×10^{7}

Table 4: Live cell counts of free and encapsulated probiotic bacteria (L. casei and B. animalis subs lactis) in simulated gastrointestinal solution.

	Bacteria	Incubation time (min) 1	30	60	90	120
Free probiotic	*L.casei*	7.8×10^{7}	4.5×10^{7}	7.6×10^{6}	$<10^{6}$	$<10^{6}$
	B.animalis	9.5×10^{7}	3.3×10^{7}	1.3×10^{6}	$<10^{6}$	$<10^{6}$
Encapsulated probiotic	*L.casei*	1.7×10^{9}	7.6×10^{8}	3.6×10^{8}	4.1×10^{7}	7.6×10^{6}
	B.animalis	5.4×10^{9}	6.4×10^{8}	2.7×10^{8}	4.5×10^{7}	1.4×10^{6}

Table 5: Sensory scores of 7-day-old chocolate milk and 14-day-old chocolate milk: samples A and C (containing probiotic bacteria in the free state); samples B and D (containing probiotic bacteria in the microencapsulated state).

Samples (1-5)	Colour and appearance (1-5)	Body and texture	flavour and taste (1-10)	total accptability (1-20)
		Lactobacillus case		
A (7 day)	2 (2-3)	2 (2-3)	1 (0-1)	5 .10 ±0.357
B (7 day)	5 (4-5)	4 (4-5)	8 (8-9)	18 .20± 0.567
C(14 day)	1 (1-2)	0 (0-1)	1 (1-2)	2.00 ±0.214
D(14 day)	2 (2-3)	1 (1-2)	2 (1-2)	5.80 ±0.421
		Bifidobactrium animalis		
A(7 day)	5 (4-5)	5 (4-5)	7 (6-7)	17.30±0.514
B(7 day)	5 (4-5)	4 (4-5)	9 (9-10)	19.10 ±0.854
C(14 day)	4 (4-5)	3 (3-4)	4 (4-5)	10.50±0.354
D(14 day)	4 (4-5)	4 (4-5)	5 (4-5)	13.90±0.620

CONCLUSIONS:

Microencapsulation enhanced the survival of probiotic cultures compared to free cells in chocolate milk stored over 3 weeks. Microencapsulation of *L.casei* and *B. animalis subsp. lactis* cells with calcium alginate and resistant starch can successfully keep the count of this probiotic bacteria high enough for the therapeutic minimum in the chocolate milk and can serve as agood carrier for delivering the probiotic bacterial cells into human gut. Sensory analysis of the chocolate milk showed that addition of probiotic capsules delay change in odor and colour in the chocolate milks, however, significantly altered the textural properties of chocolate milk. The panellists reported grittiness in chocolate milks with microencapsulated probiotic cultures.

ACKNOLEDGMENT

The authors would like to express their thanks to Dr.H.Pourjafar for his assistance in data analysis .This study was supported by Pegah Company in Tabriz, Iran. The authors are grateful from Food Research and Development unit of Pegah Company.

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