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Survival of probiotics encapsulated in calcium alginate and resistant starch beads in drinking yoghurt produced with essential oils during storage and in simulated gastrointestinal juice conditions

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Abstract

The aim of this study was to investigate the effect of microencapsulation with calcium alginate and resistant starch on viability of *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* in drinking yoghurts and the survival rate in simulated gastrointestinal conditions. Two types of probiotic drinking yoghurt, with free and microencapsulated probiotic bacteria with and without essential oils [Mint (*Mentha spicata*) and Ziziphora (*Ziziphora tenuior* L) (0.001 %)], were manufactured in triplicate under the same conditions. The number of viable cells during 28 days of storage in refrigerated conditions and in 0.6% bile salt solution and simulated gastrointestinal condition was evaluated. Results showed that essential oils affected pH, acidity, sensory properties and viability of probiotic bacteria in drinking yoghurt samples. The number of viable cells of probiotic bacteria was reduced significantly during storage period in both types of drinking yoghurt, but reduction in the drinking yoghurt samples containing free cells was significantly ($p < 0.05$) higher than the drinking yoghurt containing microencapsulated cells. The results showed that, microencapsulation process was able to increase the survival rate of probiotic bacteria in drinking yoghurt after during storage period. Survivability of microencapsulated cells in a simulated gastrointestinal condition was significantly more than free cells (p<0/05).

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Introduction

Dairy drinks are produced from milk or its derivatives, with or without the addition of other ingredients, in which the dairy base represents at least 51% (v/ v) of the formulation, and can be submitted to a fermentation process using yogurt cultures (Brazil, 2005). From the technological view point, the main difference between yogurt and fermented dairy beverages is the addition of water to the latter, which results in lower viscosity. Supplementation with probiotic bacteria and prebiotic ingredients represents a new option to add further value to dairy beverages, as reported in various studies on their adequacies as a food matrix (Castro *et al.,* 2009; Zoellner *et al.*, 2009). Drinking yoghurts are made by diluting a fermented yoghurt base with water (and often essential oils as well in Iran). Use of essential oils in the production of dairy drinks could be a promising alternative for dairy industries, because dairy drinks are viewed positively by consumers (Krešic *et al.,* 2010). Many spices and herbs exert sensory properties and antimicrobial activity due to their essential oil fractions. Nychas (1995) reported antimicrobial activity of essential oils from oregano, thyme, sage, rosemary, clove, coriander, garlic and onion against both bacteria and molds.

Foods containing probiotic bacteria fall within the "functional foods" class and these foods should contain at least 10^7 cfu/g probiotic bacteria and consumed at levels higher than 100 g/day to have helpful effects on health (Homayouni, 2008).

Very high levels of probiotic bacteria do not survive in fermented dairy products (Hekmat and McMahon, 1992). In addition, Lankaputhra and Shah (1995) found that survival of *L. cidophilus* 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 food products and the intestinal tract. This has been proposed for various dairy fermentations such as fermentation of whey and continuous inoculation of milk for yoghurt manufacture. Additional benefits of microencapsulation of cells include: Greater stability during storage and during transit through the human gastro-intestinal tract (Wenrong and Griffiths, 2000). Calcium alginate has been used widely for the immobilization 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 microencapsulation of bacteria for fermentation purposes or for incorporation into products, (Jankowski *et al.,* 1997; Khalil and Mansour, 1998). Alginate encapsulation has been used successfully to immobilize bacterial cultures for incorporation into mayonnaise (Khalil and Mansour, 1998). Despite the suitability 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 (Smidsrod and Skjak-Braek, 1990). Thus, stability 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 improve the properties of encapsulated beads. Coated beads not only prevent cell release but also increase mechanical and chemical stability. Mixing with starch can improve stability of beads. Alginate/starch liquid core capsules offer the ability to encapsulate *L. acidophilus* without loss of viability and fermentation ability (Jankowski *et al.,* 1997). Capsule membranes allow sufficient diffusion of nutrients and metabolites to maintain growth of encapsulated cells.

The objectives of this study were to evaluate the survival of encapsulated cultures under simulated gastrointestinal conditions and in drinking yoghurt produced with and without essential oils over a period of 4 weeks during storage at 5˚C.

Material and methods

Essential oils

The essential oils used in this work were purchased from Barij Essence (Iran), which supplies food grade oils. The oils used were Mint (*Mentha spicata*) and Ziziphore (*Ziziphora tenuior).*

Preparation of free and encapsulated probiotics

Pure probiotic culture of *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* was obtained from CHR-Hansen (Harsholm, Denmark) and inoculated into MRS-broth (de Man-Rogosa-Sharpe) and incubated at 37˚C for 24 h under aerobic conditions. 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. The distance between the syringe and CaCl2 solution was 25 cm. Diameter of the resultant beads was 200–500 µm.

Preparation of drinking yoghurt

Pasteurized milk (1.5% fat) and skim milk powder were used to formulate the probiotic drinking yoghurt. The inoculum was prepared using reconstituted (11% w/v) skim milk *Streptococcus salivarius ssp. thermophilus* (TA-40, Cristian hansen, Denmark), *Lactobacillus delbrueckii ssp. bulgaricus* (LB-340, Cristian hansen, Denmark), and a probiotic culture of *Lactobacillus acidophilus LA-14* (DuPont) and *bifidobactrium animalis subs lactis* were also added.

The production of drinking yoghurts with essential oils added after fermentation followed the method of Sun-Waterhouse *et al.* (2013) with some modifications. Low fat milk and skim milk powder were mixed and heated (95 ℃ for 10 min), cooled to 40 ℃ and inoculated with starter culture. Incubation was conducted at 40 ℃ in a Memert incubator (Germany), until the pH reached 4.4 (when the yoghurt was stored at 5 ℃). The coagulum was broken and mixed with water + pectin (0.5 %) (50 mL per 200 g yoghurt) simultaneously using T. T. T (China) mixer (100 rpm). Then essential oils (0.001 %) and probiotic bacteria (free and encapsulated bacteria, 10 g) were added to samples and were mixed completely. Finally, they were filled into 250 ml bottles, sealed and stored at 5 ℃ for 28 days. These samples were manufactured at a pilot scale and their approximate dry matter, fat and pH were11.00, 1.50, and 4.4, respectively. The experiments were done in triplicate.

Measurement of acidity and pH

Acidity of samples was determined according to titration method and based on lactic acid percentage. 10 ml of sample was titrated against N/10 NaOH in presence of phenolphthalein. The values of pH were measured by a digital pH meter (Knick 766, Germany).

Serum separation

In order to measure serum separation, the drinking yoghurt samples were filled into 250 ml bottles and stored at 5 ℃. During 30 days of storage, the height of supernatant was measured and its value (divided by the total height of sample in bottle and multiplied by 100) expressed as the serum separation in percent (Azarikia *et al.* 2008).

Sensory analysis

Odor, taste, texture, color and overall acceptability of drinking yoghurt samples were analyzed after 1 day storage at 5 ℃. Sensory analysis was performed by using 10 trained panellists familiar with the product. A 5-point hedonic scale including both the number and verbal scores was provided to the panellists (Chambers *et al.,* 2004). The scores were; Like very much (5), like moderately (4), neither like nor dislike (3), Dislike moderately (2), Dislike very much (1). A section for the panellist's comments was present in

the evaluation sheet. For texture evaluation, an explanation on the evaluation sheet was provided to the panellists stating that the texture would be evaluated for homogeneity of the texture and presence of aggregates or particulate material. Mean scores for each attribute were calculated for comparison of the samples.

Counts of viable bacteria

Bacterial counts were determined immediately after manufacture of drinking yoghurt and during 28 days at 4˚C. Viable probiotic concentrations were measured by pour plate counting on MRS agar (MERCK, Germany). Samples (1.0 ml) were added to 9.0 ml of sterile Ringers solution, then, appropriate dilutions were made (10^{-6}). Subsequently *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* were plated onto MRS agar+ 10 % W/V salicin and MRS agar+5 ml/liter medium Cysteine HCl + 2.5 ml/liter Mupirocin (MERCK, Germany) respectively. 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 (Tharmaraj and Shah, 2003).

To count the microencapsulated bacteria in drinking yoghurt, the entrapped bacteria were released from the beads according to the method of Mirzaei *et al.* (2012). Ten grams of drinking yoghurt were re-suspended in 100 ml of phosphate buffer (0.1 M, pH 7.0) followed by 15 min shaking on a shaker (IKA-Model Janke and Kunkel GMBH. Type VX5-Germany). The drinking yoghurt sample containing free bacteria was treated in a similar way so as to maintain the same treatment conditions. All experiments were done in triplicate.

Bile salt solution tolerance of free and encapsulated L. bulgaricus

The stability of encapsulated and free *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* was tested in porcine bile salt solution. Suspensions of probiotic bacteria(1 mL) or 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 encapsulated probiotic bacteria were collected at each 30 min time intervals. Assay of the viability of free and encapsulated probiotic bacteria was carried out as described above. After the microspheres were broken in sodium phosphate solution, the viable amounts of *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* were determined according to procedures described in previous section (Krasaekoopt *et al.,* 2004).

Survival of free and microencapsulated probiotic bacteria in simulated gastrointestinal juice

Suspensions of probiotic bacteria (1 mL) or alginate–milk microspheres 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: 7.43) and incubated at 37˚C for 150 min Assay of the viability of free and encapsulated probiotic bacteria was carried out as described above. After the microspheres were broken in sodium phosphate solution, the viable amounts of *Lactobacillus acidophilus* and *bifidobactrium animalis subs lactis* were determined according to procedures described in previous Section (Krasaekoopt *et al.,* 2004).

Statistical analysis

Results were expressed as mean ± SD values which were the average of triplicate experiments. Significant differences between the results were calculated by analysis of variance (ANOVA) using SAS software version 8. Differences at $P<0.05$ were considered to be significant.

Result and discussion

The initial cell count of probiotics beforewas in the range of $7.5\pm3.1\times10^{11}$ cfu/ ml. High cell loading in the range of $2.1\pm1.2\times10^{9}$ –$3.5\pm2.1\times10^{10}$ cfu/ g beads was

achieved in resistance starch-coated beads, which had an average diameter of 200–500 μm. The loss during encapsulation and coating was very low due to the gentle methods used. The coated beads containing probiotics were incorporated into the drinking yoghurt contained essential oils (0.001%) on the day of their preparation. The probiotics in the drinking yoghurts were enumerated periodically after 1 day in the cold storage until 4 weeks.

PH and acidity

The pH changes in the control and experimental drinking yoghurts during storage at 5 ˚C for a period of 4 weeks are shown in Table 1. The drinking yoghurt with the Ziziphora essential oil showed the lowest pH. The final pH (at end of 4 week storage) of yoghurt with encapsulated probiotic bacteria was greater than the drinking yoghurts inoculated with free probiotic bacteria. Probiotic bacteria are slow acid producers. The results of this study showed that post acidification in drinking yoghurt with encapsulated probiotic bacteria was slower compared to drinking yoghurt with free probiotic bacteria and control.

The falls of the pH values were faster in the drinking yoghurt samples that were not contained essential oil. This suggests a greater rate of production of organic acids in the samples without essential oils than others. The drinking yoghurt with the mint essential oils showed low changes in pH The level of acidity in the drinking yoghurt during the storage period ranged from 0.9 to 1.29 (table 2). The acidity of drinking yoghurt without essential oils was higher than drinking yoghurt with essential oils. Drinking yoghurt samples with Mint essential oil had higher pH and lower acidity than other samples. These differences may be attributed to the microbial compounds of essential oils that prevent starter cultures growth and acid production (Hadad Khodaparast *et al.,* 2007).

Sensory analysis

The average sensory scores of all panellists are shown in Table 3. The results showed that there were no significant differences (P>0:05) in the color and odor of the yogurt samples. It was expected that addition of alginate capsules to drinking yogurt could slightly colour the drinking yoghurts, however, the panellist could not identify the differences in the odor and color between drinking yoghurts with encapsulated cultures from the other treatments. The alginate probiotic capsules contained starch and this could have been the reason that the probiotic capsules appeared white and did not impart a difference in color. The texture including smoothness of the drinking yoghurt samples, however, showed significant differences (P>0:05) between the drinking yoghurts containing free probiotic bacteria and encapsulated bacteria. The panellists, however, reported slight grittiness and therefore scored the drinking yoghurts with probiotic capsules as more undesirable as compared to the other three types of yoghurts. The addition of encapsulation material (alginate) and filler material (Starch) with encapsulated cultures could also have influenced the texture of the drinking yoghurts. The capsular matrix and the starch used in the encapsulation process may have influenced the grittiness (mouth feel) of the drinking yoghurt. Sodium alginate used in the encapsulation of the probiotic cultures has been reported to form a gel with a number of cations such as calcium which is present in the yoghurt gel (Kailasapathy, 1996). Williams *et al.* (2004) reported that there is potential for using starch to improve textural properties of stirred yoghurts.

The panellists also could differentiate the five types of yoghurts in flavour. The drinking yoghurt with added ziziphora essential oil had higher taste scores compared to other samples. The essential oils are regarded as natural alternatives and have been used to improve sensory characteristics and extend the shelf life of food. This components was mainly composed of terpenoid compounds (67 of the 95 compounds identified) as well as ketones, aldehydes, alcohols, esters, alkanes, and benzenic compound. It has been well-known that essential oils have antimicrobial and antioxidant effects (Özer *et al.,* 2007). The most common herbs in the dairy industries of Iran are Mint, Bee balm and ziziphore,

widely used as a source of essential oils for flavouring agent.

Kailasapathy (2006) Addition of probiotic bacteria (free or encapsulated) reduced acid development in yogurt during storage. Post acidification in yogurt with encapsulated probiotic bacteria was slower compared to yogurt with free probiotic bacteria and this has affected flavour and taste of products.

Serum separation

Serum separation occurs in fermented milk products due to the aggregation and sedimentation of casein particles during storage. The use of the stabilizers was found necessary to prevent serum separation in fermented milk beverages (Lucey *et al.,* 1999). When the HMP were added to drinking yoghurt, serum separation was reduced compared to that in drinking yoghurt without any HMP (Table 4). Lucey *et al.* (1999) reported that use of pectin at concentrations higher than 0.3% prevented the serum separation in acidic milk beverages. However, use of HMP at a level of 0.50% reduced but did not prevent serum separation in drinking yoghurt in this study. All samples showed separation into two layers, but samples with encapsulated probiotic bacteria had lower serum separation than others. The starch used as the filler material in the capsules was a modified resistant starch. It will swell and absorb water but will not gelatinize fully during the heating step in yoghurt making. The swollen starch therefore will contribute to increased viscosity and decreased serum separation by water absorption. It is reported (Smidsrod and Skjak-Braek, 1990) that alginate hydro-gels are susceptible to disruption in the presence of excess chelating agents such as calcium ions. The porous nature of the sodium alginate capsules may have allowed diffusion of the starch granules from within the capsule into the yoghurt.

Samples with mint essential oils had lower serum separation. This agrees with pH measurements of the yoghurts (Table 1).

Table 1. Variation of pH in drinking yoghurt produced contained free and encapsulated probiotic bacteria with essential oils during cold storage for 28 days.

Essential oils (0.001%)		Storage days				
		1	7	14	21	28
	Control (without essence)	4.4^{a}	3.98^{b}	3.9^{b}	3.85^{b}	3.8^{b}
mint	Free probiotic bacteria	4.3^{a}	4.12^{a}	4.02^{ab}	3.95^{b}	3.93^{b}
	Encapsulated probiotic bacteria	4.39^{a}	4.34^{a}	4.23^{a}	4.18^{a}	4.1^{a}
Ziziphora	Free probiotic bacteria	4.43^{a}	4.04^{a}	3.89^{b}	3.78^{b}	3.7^{b}
	Encapsulated probiotic bacteria	4.31^{a}	4.25^{a}	4.17^{a}	4.17^{a}	4.07^{ab}

(Each observation is a mean ± SD of 3 replications. In each tables means with same superscripts had no significant difference with each other (P >0.05).

Table 2. Acidity changes of drinking yoghurt contained free and encapsulated probiotic bacteria with essential oils during cold storage for 28 days.

Essential oils (0.001%)		Storage days				
		1	7	14	21	28
	Control (without essence)	0.9^{b}	0.98^{ab}	1.0^{a}	1.1^{a}	1.26^{a}
mint	Free probiotic bacteria	0.78^{cd}	0.78^{cd}	0.81^{c}	0.81^{c}	0.84^{c}
	Encapsulated probiotic bacteria	0.76^{cd}	0.76^{cd}	0.78^{cd}	0.83^{c}	0.83^{c}
Ziziphora	Free probiotic bacteria	0.83^{c}	0.99^{ab}	1.09^{a}	1.22^{a}	1.29^{a}
	Encapsulated probiotic bacteria	0.8^{c}	0.86^{bc}	0.89^{bc}	0.92^{b}	0.98^{ab}

Viability of probiotic bacteria during cold storage

The results showed that there were significant losses in the cell numbers of both free and encapsulated probiotic bacteria (*L. acidophilus and B. animalis subs lactis*) over a period of 4 weeks (table 5). There were approximately 3and more than 4 log cycle losses in number of cells of both free *L. acidophilus* and *B. animalis subs lactis*, respectively. The encapsulated bacteria, however, showed only 2 log decrease in cell numbers of drinking yoghurt samples with mint and Ziziphora essential oils. As outlined in Table 5, drinking yoghurt probiotic cultures were affected by all treatments. Control and drinking yoghurt samples treated with encapsulated probiotic bacteria and Mint essential oils exhibited the lowest and highest probiotics activity, respectively. Bayoumi (1992) reported that essential oils of clove, cinnamon, cardamom and peppermint reduce final population of Lactic acid cultures in flavoured yoghurt till 1.5-3 cycles but Agboola and Tesic (2002) were found that Lactic acid bacteria counts in all cheese samples with various spices (mint, lemon myrtle and bush tomato) were not significantly changed during maturation.

Table 3. Sensory properties of drinking yoghurt contained free and encapsulated probiotic bacteria with essential oils.

Essential oils (0.001%)	samples	taste	odor	color	texture	Overall acceptability
	Control (without essence)	3^{c}	4.98^{ab}	4.95^{ab}	4.0^{b}	4.0^{b}
mint	Free probiotic bacteria	4.3^{b}	5.0^{a}	5.0^{a}	4.6^{ab}	4.6^{ab}
	Encapsulated probiotic bacteria	4.8^{ab}	5.0^{a}	4.4^{b}	4.7^{ab}	4.8^{ab}
Ziziphora	Free probiotic bacteria	4.83^{ab}	4.99^{ab}	4.8^{ab}	4.87^{ab}	4.9^{ab}
	Encapsulated probiotic bacteria	5.0^{a}	4.8^{ab}	4.98^{ab}	4.5^{b}	4.8^{ab}

Table 4. Serum separation (%) of drinking yoghurt contained free and encapsulated probiotic bacteria with essential oils and HMP (0.5%) at storage days at 4 ℃.

Essential oils (0.001%)	samples	Storage days				
		1	7	14	21	28
	Control (without pectin)	0^{g}	34^{b}	37.5^{ab}	38.46^{ab}	39.28^{a}
mint	Free probiotic bacteria	0g	6.66^{de}	7.0^{d}	7.3^{d}	7.9^{cd}
	Encapsulated probiotic bacteria	0g	3.3^{f}	3.3^{f}	4.1^{f}	5.8^{f}
Ziziphora	Free probiotic bacteria	0g	7.5^{cd}	8.09^{c}	8.61^{c}	8.8^{c}
	Encapsulated probiotic bacteria	0g	3.9^{f}	3.7^{f}	3.9^{f}	6.6^{de}

Haddad Khodaparast *et al.* (2007) were studied effect of Essential Oil and Extract of *Ziziphora clinopodioides* on Yoghurt Starter Culture Activity. Their results showed that effect of the essential oil of *Ziziphora clinopodioides* was not significantly different ($p<0.01$) from the control. The extract of *Ziziphora clinopodioides* at high concentration (4000 µg LG) significantly ($p<0.01$) decreased the viability of starter culture after 19 days of storage.

Sarabi-Jamab and Niazmand (2009) were investigated effect of Essential Oil of *Mentha piperita* and *Ziziphora clinopodioides* on Lactobacillus acidophilus Activity as Bio yoghurt Starter Culture. The results indicated that the number of starter culture significantly decreased ($P<0.05$) after 7 days storage. Also there was no significant difference in viability of Lactobacillus acidophilus among samples contained various concentration of essential oil of *Mentha piperita*, *Ziziphora clinopodioides* and control ($P< 0.05$).

The low viability in yoghurt is mainly attributed to the lower pH in drinking yoghurt and further reduction of pH in drinking yoghurt during post-acidification. Microencapsulation seems to be the most promising

technology to protect bacterial cells from adverse environment (Kailasapathy, 2002). This study showed that microencapsulation is able to keep the number of probiotic bacteria above the threshold level for therapeutic minimum (P10^7 cfu /ml) in drinking yoghurt. Mirzaei *et al.* (2012) showed that microencapsulation in calcium alginate gel and resistant starch was able to increase the survival rate of L. acidophilus La5 in Iranian white brined cheese after 6 months of storage.

Table 5. Effect of essential oils on viability of free and encapsulated probiotic bacteria (*L. acidophilus and B. animalis subs lactis*) in drinking yoghurt samples (cfu/ ml).

Essential oils (0.001 %)			storage days				
			1	7	14	21	28
mint	Free probiotic bacteria	*B. animalis subs lactis*	2.9×10^9	1.2×10^7	6.7×10^6	8.1×10^5	6.5×10^5
		L. acidophilus	2.6×10^9	1.9×10^7	2.9×10^6	$>10^5$	$>10^5$
	Encapsulated bacteria	*B. animalis subs lactis*	2.7×10^9	2.3×10^9	1.9×10^9	1.8×10^8	1.5×10^7
		L. acidophilus	2.8×10^9	7.2×10^8	6.8×10^8	4.1×10^7	1.3×10^7
Ziziphora	Free probiotic bacteria	*B. animalis subs lactis*	2.4×10^8	1.8×10^6	1.5×10^6	1.3×10^5	4.2×10^5
		L.s acidophilus	2.7×10^8	1.7×10^6	$>10^5$	$>10^5$	$>10^5$
	Encapsulated bacteria	*B. animalis subs lactis*	2.7×10^8	2.1×10^8	2.1×10^7	7.5×10^6	7.1×10^6
		L. acidophilus	2.6×10^8	2.4×10^7	2.0×10^7	1.8×10^6	2.7×10^6
Control(without essential oils)		*B. animalis subs lactis*	2.7×10^9	3.8×10^7	9.7×10^5	7.4×10^5	5.7×10^5
		L. acidophilus	2.3×10^8	1.2×10^8	$>10^5$	$>10^5$	$>10^5$

Table 6. Survival of free and encapsulated probiotic bacteria (*L. acidophilus and B. animalis subs lactis*) bile salts solution (0.6 %, pH: 8.25).

Essential oils (0.001 %)			Incubation times (min)				
			1	30	60	90	120
mint	Free probiotic bacteria	*B. animalis subs lactis*	2×10^7	8.7×10^6	3.1×10^6	1.9×10^6	1.2×10^6
		L. acidophilus	6×10^8	5×10^7	2.9×10^7	8.6×10^6	6.7×10^6
	Encapsulated bacteria	*B. animalis subs lactis*	4×10^9	3×10^8	9×10^7	8×10^7	5×10^7
		L. acidophilus	8×10^9	2×10^9	8×10^8	4×10^8	3×10^8
Ziziphora	Free probiotic bacteria	*B. animalis subs lactis*	4×10^6	2.1×10^6	2.9×10^6	2.9×10^6	$>10^6$
		L.s acidophilus	9×10^8	7×10^7	3×10^7	1.9×10^6	1.1×10^6
	Encapsulated bacteria	*B. animalis subs lactis*	2×10^8	5×10^7	2×10^7	6.1×10^6	4.2×10^6
		L. acidophilus	6×10^9	2.1×10^9	9.1×10^8	8.6×10^7	1.9×10^7
Control(without essential oils)		*B. animalis subs lactis*	3×10^7	1.5×10^7	3.9×10^6	2.1×10^6	$>10^6$
		L. acidophilus	3.6×10^8	2.8×10^7	1.4×10^7	2.9×10^6	1.7×10^6

Godward and Kailasapathy (2003) reported that microencapsulation improves the viability of probiotic bacteria in yoghurt and therefore makes it a better probiotic food vehicle. There is increasing evidence that microencapsulation is helpful in protecting the probiotic cultures destined to be added to acidic foods such as yoghurt (Krasaekoopt *et al.,* 2004). Kailasapathy (2006) showed that there was an increased survival of 2 and 1 log cell numbers of *L. acidophilus* and *B. lactis*, respectively due to protection of cells by microencapsulation.

Survival of free and encapsulated probiotic bacteria (L. acidophilus and B. animalis subs lactis) bile salts solution (0.6 %, pH: 8.25).

The effect of bile salt solution on the viability of free and encapsulated L. bulgaricus was presented in

Table 6. Survival of *L. acidophilus and B. animalis subs lactis* was monitored after exposure to 0.6% bile salts. Survivals were recorded at 30- min intervals and for the maximum exposure up to 2 h. Both the tested strains, free *L. acidophilus and B. animalis subs lactis*, showed a decrease of more than 2 log cycles as compared to the initial cell count and encapsulated probiotic bacteria showed a decrease of only 2 log cycles, while *L. acidophilus* showed only a slight decrease in numbers as compared to *B. animalis subs lactis* cell count. Free *L. acidophilus and B. animalis subs lactis* were totally lost their viability in bile salt solution after 2 h exposure, probably due to the loss of cell wall integrity as a result of action of the bile salts. Many references mentioned probiotics were sensitive to bile salt solution. Clark and Martin (1994) observed a five log decrease in viable cell counts of *Bifidobacterium adolescentis* (*B. adolescentis*) in 2% bile salt solution at 37˚C after 12 h incubation. Truelstrup Hansen *et al.* (2002) found that *B. adolescentis* was decreased about 2 logCFU/mL after 2 h incubation in 0.5% bile salt at 37˚C.

Table 7. Survival of free and encapsulated probiotic bacteria (*L. acidophilus and B. animalis subs lactis*) in simulated gastrointestinal solution.

Essential oils (0.001 %)			Incubation times (min)				
			1	30	60	90	120
mint	Free probiotic bacteria	*B. animalis subs lactis*	6×10^{6}	$>10^{6}$	$>10^{6}$	$>10^{6}$	$>10^{6}$
		L. acidophilus	4.2×10^{7}	1.4×10^{7}	$>10^{6}$	$>10^{6}$	$>10^{6}$
	Encapsulated bacteria	*B. animalis subs lactis*	7.4×10^{8}	2.9×10^{8}	4.7×10^{7}	1.3×10^{7}	3.9×10^{6}
		L. acidophilus	9.1×10^{8}	4×10^{8}	8.5×10^{7}	3.6×10^{7}	1.5×10^{7}
Ziziphora	Free probiotic bacteria	*B. animalis subs lactis*	2.3×10^{6}	$>10^{6}$	$>10^{6}$	$>10^{6}$	$>10^{6}$
		L.s acidophilus	5.4×10^{7}	2.2×10^{7}	5.8×10^{6}	$>10^{6}$	$>10^{6}$
	Encapsulated bacteria	*B. animalis subs lactis*	6.7×10^{8}	7.6×10^{7}	1.6×10^{7}	4.3×10^{6}	2.3×10^{6}
		L. acidophilus	8.9×10^{8}	7.4×10^{8}	5.1×10^{7}	2.6×10^{7}	9.2×10^{6}
Control(without essential oils)		*B. animalis subs lactis*	2.5×10^{6}	$>10^{6}$	$>10^{6}$	$>10^{6}$	$>10^{6}$
		L. acidophilus	3.6×10^{7}	1.7×10^{7}	$>10^{6}$	$>10^{6}$	$>10^{6}$

Probiotic drinking yoghurt samples with Ziziphora essential oils had lower viable cell than probiotic drinking yoghurt samples with mint essential oils, while control samples had the lowest viability. In this study, high bile salt solution tolerance was observed for encapsulated cells when compared with free cells. It was difficult to make any comparison with our findings because different researchers used different concentrations and sources of bile salts. Chandramouli *et al.* (2004) and Kailasapathy (2005) found that encapsulated probiotic bacteria could survive better than free probiotic cells in 1–3% bile salts solution.

Survival of free and encapsulated probiotic bacteria (L. acidophilus and B. animalis subs lactis) in simulated gastrointestinal solution

Encapsulated and free probiotic bacteria were exposed to in vitro simulated gastrointestinal conditions of high acid and to bile salts as described in above. The data of survivals of bacteria in acid and bile are represented in table 7. The viability of all free and encapsulated *L. acidophilus* and *B. animalis subs lactis* were reduced dramatically when those were exposed to acidic conditions. When *L. acidophilus* and *B. animalis subs lactis* were exposed to simulated gastrointestinal solution, none of the free microorganism survived after 120 min. but encapsulated probiotic bacteria survived after 120 min. In general, the pore size of calcium alginate gel with resistant starch microspheres ranges 200–500 μm in diameter and a increasing in pore size of calcium alginate gel microspheres can be achieved more viability exposing the gels to low pH, pepsin

molecule and other conditions (Gombotz *et al.,* 1998). Therefore, *L. acidophilus* and *B. animalis subs lactis* would be microencapsulated to ensure high survival rate in the gastrointestinal environment. The amount of bacteria in alginate micro particles gradually declined with incubation time after 120 min. However, the number of *B. animalis subs lactis* was reduced more than *L. acidophilus* and these bacteria were very sensitive to low pH.

In order to exert positive health effects, probiotics should resist the stressful conditions of the stomach. Therefore, one main purpose of encapsulation is to improve the low pH tolerance of probiotics. The pH of gastric juices is about 1.5–3.0 (Kos *et al.,* 2000).

Excellent pH tolerance of encapsulated *L. acidophilus* and *B. animalis subs lactis* probably was due to the buffering ability of milk in microspheres. Guerin *et al.* (2003) reported that the buffering ability of whey protein contributed to high survival rate for bifidobacteria encapsulated in alginate–pectin–whey protein microspheres when exposed to simulated gastric fluid pH 2.5. Heidebach *et al.* (2009) reported that viability of *Lactobacillus paracasei spp. paracasei* F19 and *Bifidobacterium lactis* Bb 12 encapsulated in casein microcapsule could be improved when exposed to simulated gastric fluid pH 2.5. The current results also demonstrated that mint essential oils tended to provide better protection than Ziziphora essential oils.

Conclusions

This study showed that drinking yogurts incorporating probiotic cultures could be made without much modification from the traditional yoghurt making technology. The addition of probiotic cultures either in the free or encapsulated forms tend to slow down the post acidification during storage of drinking yogurt. Microencapsulation enhanced the survival of probiotic cultures compared to free cells in drinking yogurts stored over 4 weeks. Sensory analysis of the yoghurts showed that addition of probiotic capsules did not significantly alter the odor and color of the drinking yoghurts, however, significantly altered the textural properties of yoghurts. The panellists reported grittiness in drinking yoghurts with encapsulated probiotic cultures. Serum separation reduced in samples with encapsulated probiotic bacteria compared to control and drinking yoghurt with free probiotic bacteria. Addition of polysaccharides (alginate and starch as encapsulation material) tends to strengthen the drinking yogurt gel. According to the results of this study, microencapsulation of *L. acidophilus* and *B. animalis subs lactis* cells with calcium alginate gel and resistant starch can successfully keep the count of this probiotic bacterium high enough for the therapeutic minimum in simulated gastrointestinal conditions in the drinking yoghurt.

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