

Viability of microencapsuls containing lactic acid bacteria under stimulated gastrointestinal conditions while incorporated in steamed rice cake

Abstract

The microencapsulation of *Lactobacillus acidophilus* NCIM2902 with alginate offers an effective means of delivery of viable bacterial cells in levels appropriate to the colon and helps in maintaining their survival during simulated gastric and intestinal juice. Non-encapsulated cells were completely destroyed when exposed to artificial gastric juice (AGJ) of pH 1.2 after 2h of incubation at 37°C, while the treatment declined the viable count of encapsulated samples only by 4log. Encapsulated cells exhibited a significantly higher resistance to artificial intestinal juice (AIJ) than non encapsulated samples. Sensory properties of SRC were improved by the addition of encapsulated *L. acidophilus*.

Keywords: *Lactobacillus acidophilus*, microencapsulation, AGJ, AIJ, HPLC, texture

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Abbreviations: AGJ, artificial gastric juice, AIJ, artificial intestinal juice; CFU, colony forming unit, HPLC, high performance liquid chromatography, LAB, lactic acid bacteria; SEM, scanning electron microscope; SRC, steamed rice cake

Introduction

Beneficial effects of probiotics on the human gut flora include antagonistic effects and immune effects. The use of probiotic bacterial cultures stimulates the growth of preferred microorganisms, crowds out potentially harmful bacteria and reinforces the body's natural defense mechanisms.¹ Recently, probiotics have been proposed for various treatments of human intestinal barrier dysfunctions such as lactose intolerance, acute gastroenteritis, food allergy, atopic dermatitis, Crohn's disease, rheumatoid arthritis, and colon cancer.^{2,3} Such beneficial microorganisms have been added to various food stuffs to create "functional food or nutraceuticals." In order to exert positive health effects, lactic acid bacteria (LAB) have to resist gastric juice and bile salts. After the LAB pass through the stomach and upper intestinal tract, LAB should attach to the epithelium of the intestinal tract and grow.⁴ Providing probiotic living cells with a physical barrier against adverse environmental conditions is an approach currently receiving considerable interest.⁵ Studies have used cellulose acetate phthalate gelatin, vegetable gum,⁶ fats⁷ or κ-carrageenan⁸ as encapsulating agents, alginate remains the most commonly used bio polymer for microencapsulation. The advantages of using alginate as an encapsulating agent include: non-toxicity, formation of gentle matrices with calcium chloride to trap sensitive materials such as living microbial cells, simplicity in entrapping living microbial cells and low cost. Alginate is also an accepted food additive and can be safely used in food.^{8,9} It is indigestible for humans, and behaves much like a dietary fiber.¹⁰ Microencapsulation techniques have been successfully used to enhance dairy fermentation for the production of concentrated lactic acid bacteria¹¹ and to improve the survival of microorganisms in dairy products,¹² mayonnaise,¹³ and gastric juice, but till now no attempt has been made to fortify cooked product by utilizing microencapsulated

LAB. Therefore, this study was performed to determine the survival of encapsulated and free cells of *L. acidophilus* in steamed rice cake during exposure to artificial gastrointestinal juice and to evaluate their effect on the product quality.

Materials & methods

Preparation of encapsulated culture

Lactobacillus acidophilus (NCIM 2902) was collected from culture collections of the National Collection of Industrial Microorganisms (NCIM), Pune, India. Microorganisms were grown at 37°C for 24h in MRS broth (Himedia, India). Calcium alginate beads of immobilized cells were prepared according to the procedure of Sheu and Marshall.⁹ The beads were harvested by gentle centrifugation (350×g, 10min) and washed with sterile distilled water.

Preparation of steamed rice cake (SRC)

SRC were prepared with parboiled rice and split dehusked black gram dhal in 2:1 ratio. The ingredients, were washed and soaked separately for 4h at 30±1°C and ground in a kitchen mixer blender separately, then both were mixed together with common salt (2.0%). The combined mixture was then allowed to ferment for 20h at 30±1°C in glass beakers covered with cotton cloths. One batch was the control (without *L. acidophilus*) and encapsulated and nonencapsulated *L. acidophilus* was added separately to the other two batches to give average initial concentrations of 1.2×10⁸cfu/ml respectively. Test samples include preparation three types of samples, including without any microorganism designated as "A", non encapsulated microorganism enriched SRC designated as "B", and encapsulated microorganism enriched SRC designated as "C". While preparing SRC, in case of encapsulated LAB, sodium alginate beads containing LAB were added and in case of non-encapsulated in SRC, same amount of LAB is added from MRS medium. The final product is obtained by cooking the fermented batter in a mould on steam for 20min.

Morphology and bacterial enumeration

The morphology of the microcapsules was also examined by scanning electron microscope (SEM). The encapsulated samples were mount on the stub with the aid of double side tape and coated by sputter coater (*Sputter Coater S150*, Edwards, Germany) for 6min. Observations were made using the scanning electron microscope (FEI QUANT 200, Hillsboro, USA) at an accelerating voltage of 20.0 kV. Non-encapsulated *L. acidophilus* was enumerated in the MRS agar. Peptone water was used to prepare the serial dilutions and culture was plated by the pour plate technique. The plates were incubated at 37°C for 48h. To determine the viable counts of the encapsulated *L. acidophilus*, 0.1g of capsules were resuspended in 10ml of phosphate buffer (pH 7.4) and stirred for 30min using a magnetic stirrer. The colony forming units (CFU) were determined by aerobic plating on MRS agar plate and incubating at 37°C for 48h.

Preparation of artificial gastric juice (AGJ) and artificial intestinal juice (AIJ)

The AGJ was prepared with pH adjusted MRS broth which was adjusted to 1.2, 2 and 7.0 (control) with 5mol/l HCl or 1mol/l NaOH solution and sterilized. Suspending pepsin (1000 unit/ml) in MRS was sterile-filtered through a membrane filter (0.45µm, Lot No. BM6SM0117H, Millipore, Bengalore, India) and 0.1ml of suspending pepsin was inoculated to 9.9ml of AGJ. Three and 5g/l oxgall solutions were used as AIJ representing biles with 3 and 5g/l concentrations. The oxgall solutions were prepared by dissolving 0.03 and 0.05g of oxgall in 10ml of MRS broth. All solutions were sterilized at 121°C for 15min.

Viability of probiotic microorganism in AGJ and AIJ

The encapsulated *L. Acidophilus* beads from SRC sample and non-encapsulated *L. acidophilus*(1.2×10^8 cfu/ml) samples of individual treatments were completely dispersed in 10ml of AGJ and AIJ. Nonencapsulated and encapsulated *L. acidophilus* in AGJ was incubated at 37°C and sampled every 60min. Sampled capsules in AGJ were neutralized with NaOH solution, and centrifuged at $2000 \times g$ for 10min. The obtained capsules were resuspended in 10ml of phosphate buffer (pH 7.4) and viable cells of non-encapsulated and encapsulated *L. Acidophilus* was incubated in 37°C for 3h. Samples were taken hourly for determination of the cell count. An aliquot of 100µl was diluted 1/10 and then serially diluted to obtain a sensible dilution for plating. The plates were incubated under anaerobic conditions at 37°C for 48h. To determine the viability in AIJ, samples were withdrawn after incubation at 37°C for 3h. Determination of cell count was done as described above.

Physico-chemical analysis of SRC

The total lactic acid concentration was determined by the technique described by Balasubramanian et al.,¹⁴ Diacetyl production and hydrogen peroxide production was determined using a method described by Edema et al.,¹⁵ The SPE method of Cho et al.,¹⁶ was used for the extraction of water-soluble vitamins. A method given by Sreelatha et al.,¹⁷ was used to detect the DPPH radical scavenging activity. SRC samples were coded and presented to 30 panel members for sensory scoring. Water was used for mouth rinsing before and after each sample testing. Samples were scored for appearance, taste, colour, texture, aroma and overall acceptability according to numerical scoring system. The model used in this analysis was an acceptance test on the hedonic scale, with values ranging from “1” (extremely disliked) to “9” (extremely liked).

Results and discussion

Morphology

The size and shape of the beads was determined from the electron photomicrographs, as well as by using the light microscope. The encapsulation procedure used in this study resulted in bead size of 1–1.4mm. Size segregation was carried out using 14-16 mesh size sieves. Figure 1 shows the shapes and surface morphologies of alginate capsule. The shape of the beads was generally spherical; sometimes elliptical shaped capsules were observed as well. There was a large variation in capsule size depending of the probiotic strain and prebiotic contents. The size of calcium alginate microencapsulation can be affected by various factors such as alginate concentration, probiotic cell load, and hardening time in calcium chloride etc. According to the morphological analysis of the freeze-dried microcapsule, they generally were spherical with a wrinkled surface and a collapsed centre containing bacteria (Figure 1). The wrinkled surface was probably due to the loss of water content during the freeze-drying process.¹⁸ Alginate microparticles usually had a heterogeneous structure with a dense surface layer and a loose core due to the heterogeneous gelation mechanism, which resulted in the collapsed center during the drying process.¹⁹

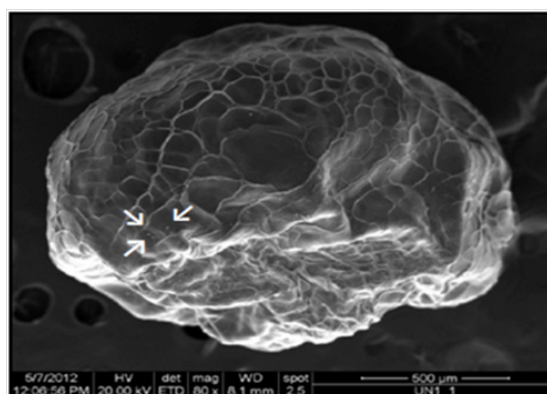


Figure 1 Micrograph of the microcapsule containing *L. acidophilus* (NCIM 2902) encapsulated in alginate with entrapped bacteria (arrows).

Viability of *L. acidophilus*(NCIM 2902) in AGJ

To be used as probiotics, bifidobacteria must survive the transit through the stomach to be able to reach the intestine.²⁰ In order to determine the influence of the pH on the survival of non-encapsulated and encapsulated *L. acidophilus*, *in vitro* system was used. According to Berrada et al.,²¹ for the acidity resistance, there was only a slight difference between *in vitro* and *in vivo* results. When inoculated in AGJ at pH 1.2 at 37°C a dramatic decline in non-encapsulated *L. acidophilus* was observed 5 log reduction after 1h and complete destruction after 3h. On the other hand, encapsulated *L. acidophilus* in AGJ maintained above 10^4 cfu/ml at pH 1.2 after 3h (Figure 2). At pH 2, non-encapsulated *L. acidophilus* decreased from 1.1×10^{10} to 16 cfu/ml after 3h of incubation while encapsulated *L. acidophilus* decreased from 1.1×10^{10} to $9 \times 10^5 \text{ cfu/ml}$. At pH 7.0 (control), viability of *L. acidophilus* in AGJ remained almost constant after 3h incubation at 37°C whether encapsulated or not. SEM micrograph of the microcapsule containing *L. acidophilus* after 3h incubation in artificial gastric conditions is shown in Figure 3. For lactic acid bacteria to exert positive health effects, they have to colonize on the colon in large quantities.²² Free *L. acidophilus* was very feeble in the low-pH environment. This is in good agreement with the similar

study done by Rao et al.,²³ who reported that no *Bifidobacterium pseudolongum* survived in the simulated gastric environment of pH 1.33 for 60min, but at pH 6.06 and 7.13 survival was fully sustained. Sheu et al.,¹² reported that calcium alginate could provide good protection (90%) for lactobacilli in frozen ice milk. Favaro-Trindale and Grosso²⁴ showed that none of *L. acidophilus* (La-05) survived in the artificial gastric environment of pH 1.0 after 1h, but microencapsulated *L. acidophilus* (La-05) suffered a reduction of 1 log at pH 1.0 after 2h incubation. According to Chandramouli et al.,⁴ a higher survival of *L. acidophilus* CSCC2400 and CSCC2409

immobilized in alginate bead in low pH environments. Our results suggested that non-encapsulated *L. acidophilus* was sensitive to the acidic environment (pH 1.2 and 2) and the ingestion of unprotected lactic acid bacteria might result in reduced viability. Therefore, the survival of encapsulated *L. acidophilus* in the gastric environment was significantly ($P \leq 0.05$) better than that of non-encapsulated *L. acidophilus*. This indicates that the product with encapsulated cells, which initially contained 1.2×10^{10} cfu/ml, could reach the small intestine with a good probiotic concentration (9×10^7 cfu/ml) at pH 7.

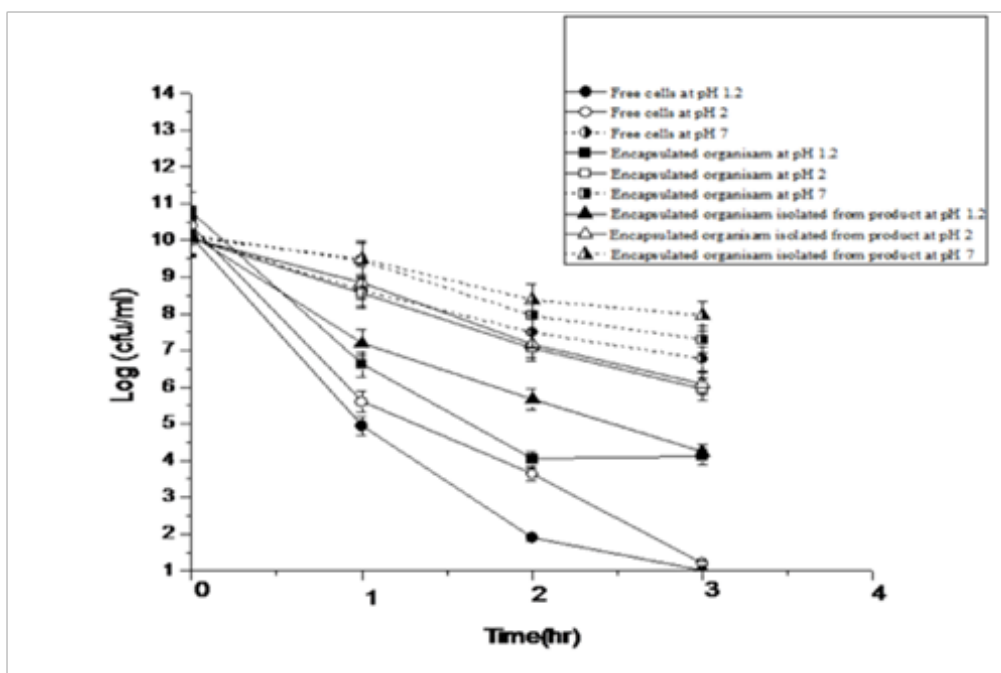


Figure 2 Survival of non-encapsulated, encapsulated and encapsulated organism isolated from the product *L. acidophilus* NCIM 2902 after sequential incubation in artificial gastric conditions.

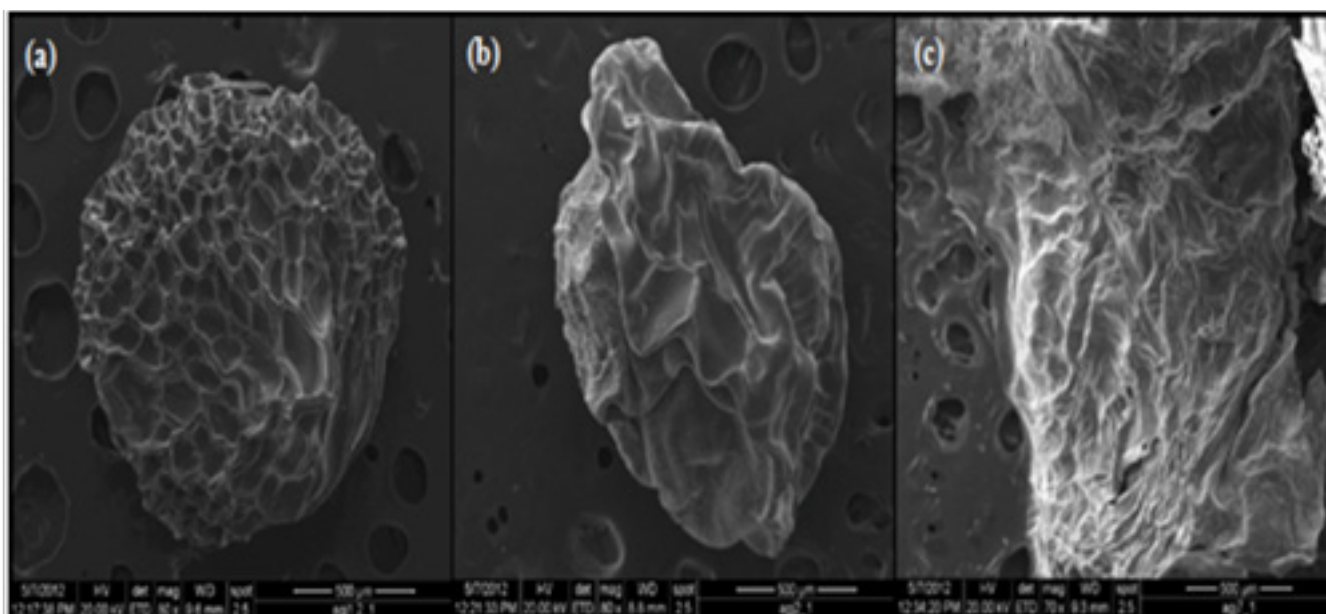


Figure 3 SEM micrograph of the microcapsule containing *L. acidophilus*: after 3h incubation in artificial gastric conditions (A) pH 1.2 (B) pH 2 (C) pH 7.

Viability of *L. acidophilus*(NCIM 2902) in AIJ

Chou & Weimer²⁵ reported that in order for LAB to exert positive health effects, they should resist the stressful conditions of the stomach and upper intestine that contain bile. According to Mituoka²⁶ *L. acidophilus* is most active in the small intestine and *B. bifidum* is most active in the large intestine of humans. Therefore, *L. acidophilus* should resist bile acid. Survivals of non-encapsulated and encapsulated *L. acidophilus* were monitored up to 6h after exposure to 0.3% and 0.5% AIJ (Figure 4). The viability of non-encapsulated *L. acidophilus* decreased from 1.2×10^{10} to 8×10^5 and from 1.3×10^{10} to 5×10^4 , respectively, at 0.3% and 0.5% bile concentration after 6h incubation at 37°C. However, the viabilities of encapsulated and encapsulated organism isolated from final product, *L. acidophilus* were not decreased significantly at 0.3% and 0.5% AIJ after 6h. Initial

cell numbers of *L. acidophilus* in alginate microparticles dropped from 1.4×10^{10} to 2×10^5 cfu/ml and initial cell numbers of *L. acidophilus* in encapsulated organism isolated from final product dropped from 1.3×10^{10} to 3×10^6 cfu/ml in 0.5% bile concentration after 6h incubation at 37°C. SEM micrograph of the microcapsule containing *L. acidophilus* after 3h incubation in artificial intestinal conditions is shown in Figure 5. The present results agreed with previous reports²⁷ that acid and bile resistance of free cells varies greatly among strains within a species and among species. Microencapsulation of various bacterial cultures including probiotics has been a common practice for expanding their shelf life.⁸ It has been reported that *L. acidophilus* was significantly better with regard to bile tolerance than other cultures.^{28,29} Our study demonstrated microcapsulation using alginate may be an effective way to increase the survival of *L. acidophilus* in bile solution. Sultana et al.,²² also reported similar type of results.

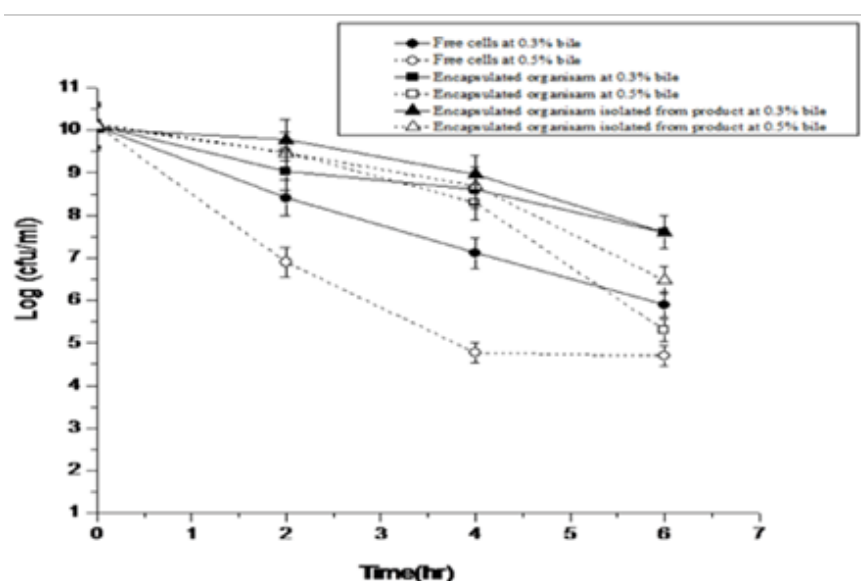


Figure 4 Survival of non-encapsulated, encapsulated and encapsulated organism isolated from the product *L. Acidophilus* NCIM 2902 after sequential incubation in artificial intestinal conditions.

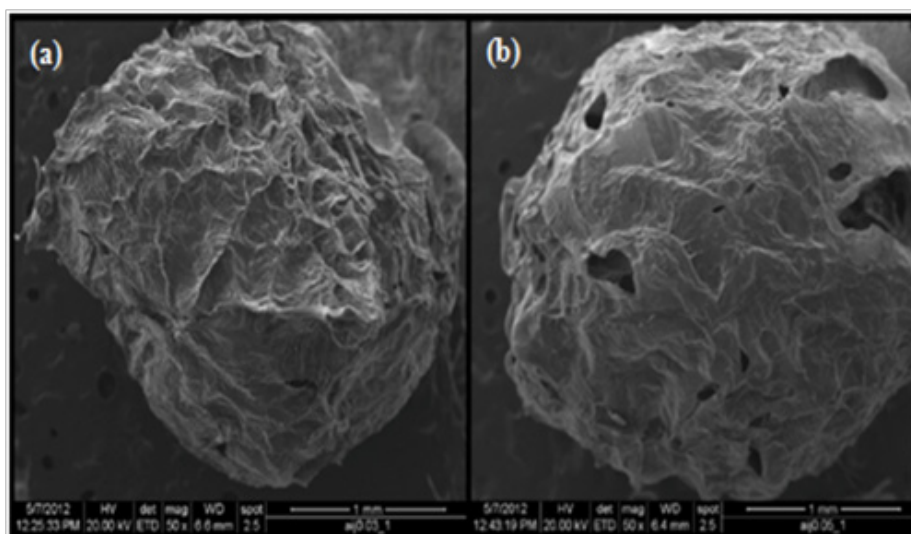


Figure 5 SEM micrograph of the microcapsule containing *L. acidophilus*: after 3h incubation in artificial intestinal conditions (A) 0.03% bile solution (B) 0.05% bile solution.

Physico-chemical analysis of SRC samples

Total lactic acid concentration and pH of SRC batter was affected significantly ($p \leq 0.05$) by free and encapsulated *L. acidophilus*. SRC batter containing encapsulated *L. acidophilus* had highest ($P \leq 0.05$) diacetyl and hydrogen peroxide values than those of controls and samples containing free cells of *L. acidophilus*. SRC containing encapsulated *L. acidophilus* had highest ($P \leq 0.05$) antioxidant potential than those of controls and samples containing free cells of *L. acidophilus*. The concentration of water-soluble vitamin such as panthothenic acid (vitamin B5), thiamine (vitamin B1) and folic acid (vitamin B9) at 20h after fermentation in different test samples is shown in Table 1. It is evident from Table 1 that sample C provides maximum production of all vitamins. Sensory data of the test, texture and overall acceptability of different SRC samples were presented (Table 1) at significant difference level $p \leq 0.05$. The increase in total acidity could be attributed to acid production by surviving lactic acid bacteria. The pH value of SRC batter containing encapsulated lactic acid bacteria was lower ($P \leq 0.05$) than other treatments. LAB have been known to take part in bread fermentations such as in the production

of the Swedish rye sourdough³⁰ and the Indian Idli³¹ wherein they improve flavor, texture and keeping quality through the production of metabolites such as diacetyl, hydrogen peroxide and bacteriocins, these compound give fermented food their characteristic flavor and also impart improved safety and rheology to the food. The increase in antioxidant potential could be attributed by surviving LAB. Ghosh et al.,³² found enhanced amount of vitamin B production during fermentation and 7h is the recommended time for the production of maximum amount of B vitamins, possibly because they use *L. mesenteroides*, whereas in our study the organisms involved was *L. acidophilus*. On the taste aspect of the SRC samples, SRC containing encapsulated *L. acidophilus* scored the highest appreciation, followed by the samples containing free cells of *L. acidophilus*. The control which is without any LAB scored lowest score as it does not contain characteristic sour aroma produced by LAB. Texture of the SRC containing encapsulated *L. acidophilus* showed lowest score as it contain alginate beads incorporated in it. In terms of overall acceptability index, SRC containing encapsulated *L. acidophilus* was credited to be superior to both control and samples containing free cells of *L. acidophilus*.

Table 1 Physico-chemical characteristics of steamed rice cake samples

	Acidity (%)	Diacetyl (mg)	H ₂ O ₂ (mM)	Antioxidant DPPH Radical Scavenging (%)	Vitamin B Profile (µg/ml)			Sensory Analysis (Hedonic Scale)		
					B1	B5	B9	Test	Texture	Overall Acceptability
A	0.711±0.012 ^a	21.03±1.68 ^b	0.204±0.01 ^b	2.51±0.16 ^a	0.0033±0.001 ^b	0.097±0.002 ^b	0	6±0.5 ^a	7±0.05 ^c	6.5±0.01 ^a
B	0.760±0.01 ^b	28.69±1.93 ^b	0.313±0.21 ^a	6.43±0.21 ^b	0.0053±0.002 ^c	0.12±0.003 ^a	0.016±0.001 ^d	6.5±0.4 ^b	7.5±0.4 ^a	7±0.01 ^b
C	0.840±0.026 ^c	47.23±2.13 ^a	0.430±0.013 ^c	12.67±0.19 ^c	0.0067±0.001 ^c	0.16±0.0015 ^c	0.023±0.002 ^e	7±0.7 ^c	6.5±0.04 ^b	7.5±0.03 ^c

Test samples include preparation three types of samples, including without any microorganism designated as "A", non encapsulated microorganism enriched SRC designated as "B", and encapsulated microorganism enriched SRC designated as "C". Values are Mean±SD, n=3. Mean values followed by different letters within columns are significantly different by Fisher's LSD tests at $p \leq 0.05$

Conclusion

Microencapsulation offers significant benefits to food technology and production. It provides a solution to the low viability of probiotics incorporated in food products. Ideally it can maintain the level of the beneficial probiotic bacteria at the minimum standard amount required. One of the requirements for microorganisms to be used as dietary adjuncts is the need to maintain viability and activity in the carrier food before consumption. Microencapsulation in alginate microparticles successfully improved the survival of *L. acidophilus* in AGJ and AIJ. A good quality SRC could be manufactured by incorporating encapsulated *L. acidophilus* NCIM 2902 in calcium alginate beads. Furthermore, the sensory evaluation of SRC with microencapsulated probiotic bacteria has revealed the consumer response, to the texture and the changes in organo-leptic characteristic of the steamed rice cake.

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Conflict of interest

The manuscript represents original work that is not being considered for publication, in whole or in part, in another journal,

book, conference proceedings, or government publication with a substantial circulation and there is no conflict of interest.

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