International Journal of Fermented Foods: v.2 n.1 p.33-45. June, 2013

Research Paper

Prebiotics in the microencapsulating matrix enhance the viability of probiotic *Lactobacillus acidophilus* LA1

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Received: 09th January 2013 Accepted: 14th May 2013

ABSTRACT

The viability of microencapsulated *Lactobacillus acidophilus* LA1 beads containing different levels of fructo-oligo-saccharides (FOS - 1.5%, 2.5% and 3%) and partially hydrolysed guar gum (PHGG - 3% and 5%**)** were studied under different conditions of pH (1.0, 2.0), bile salt concentrations (1%, 1.5% and 2%) and high temperature exposure (75° and 85° C). The microcapsules had 8-9 log of viable cells of *L. acidophilus* LA1. Cells entrapped in alginate beads with no prebiotic (CAB) and containing prebiotic (PAB) were incubated at 37°C under the test conditions of pH, bile and temperature for different lengths of time. Survival rates decreased with increasing incubation periods at the experimental levels of pH, bile concentrations and severity of heat. PAB survived significantly better than CAB at higher levels of the prebiotic concentrations. The matrix with 3% PHGG, 3% alginate, and 2% starch performed well at all test conditions.

Keywords: Microencapsulation, Probiotic, Prebiotic, Synbiotic, Lactobacilli, Microgel, Atomization

INTRODUCTION

Probiotics are live microorganisms that are used as dietary supplements with the aim of benefitting the health of consumers by positively influencing the intestinal microbial balance (Fuller, 1989). The maintenance of the viability and functionality of probiotics until they reach their target site in the human gut is a key requirement. While it is generally proposed that dairy products should contain at least 10^7 viable probiotic cells per ml, the actual levels detected in yoghurt and fermented milk products are often much lower due to the adverse conditions during product storage. It has also been clearly demonstrated that the extreme acidic environmental conditions

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in the human stomach can seriously decrease the number of living cells reaching the intestine (Krasaekoopt *et al*., 2003; Ross *et al*., 2005). Microencapsulation has been attempted to enhance the survival of probiotic bacteria during processing, storage and gastric transit (Augustin, 2003; Krasaekoopt *et al*., 2003). By adopting improved methods to enhance the survival and stability of bacteria including their protection in biopolymeric structures, an increased delivery of viable cells in the human intestine can be achieved. Wide range and choice of biopolymers are available presently with multitude of beneficial attributes. Among all these alternatives alginate still dominates and enjoys prime place. It is an accepted food additive which can be safely used in foods and is a popular choice for microencapsulation techniques based on emulsion and extrusion. However, the use of alginate is limited due to its low stability in the presence of chelating agents and in acidic conditions below pH 2.0 (Ding and Shah, 2007). Incorporating prebiotics into calcium alginate as wall materials may give better protection to probiotics in food systems and the gastrointestinal tract due to synbiosis (Bielecka *et al*., 2002; Chen *et al*., 2005).

Currently there has been interest in introducing food products combining probiotics with prebiotics or fibres into the market (Tallon, 2004). Due to the potential synergistic relationship between probiotics and prebiotics, food products containing a combination of these ingredients are often referred as synbiotics. However, the technological performance of prebiotic substances as probiotic protectants has been relatively less studied. Prebiotics identified thus far are nondigestible carbohydrates, including lactulose, inulin, and a range of oligosaccharides. Fructo-oligosaccharides (FOS) and inulin are among the most popular prebiotics (Cardarelli *et al*., 2007). Chen *et al*. (2006) demonstrated that the addition of FOS, isomalto-oligosaccharides and peptides in the walls of probiotic microcapsules provided improved protection for the active organisms. Some prebiotics may also act as thermoprotectants enhancing probiotic viability during spray-drying or powder storage (Ross *et al*., 2005).

Little research has been conducted with an aim to incorporate prebiotics as wall materials into calcium alginate beads, and their effect on the survival of probiotic organisms under harsh processing and gastrointestinal environments. Though encapsulating lactobacilli in calcium alginate beads reportedly improves their heat tolerance and *in vitro* acidic and bile salt tolerance, there are no systematic reports on survival of encapsulated probiotics in the presence of different prebiotics at varying concentrations. Since alginate and partially hydrolysed guar gum (PHGG) are acid-resistant and heat-stable, combining both gels as coating materials for probiotic capsules may extend the stability of probiotics under food processing or as a new functional additive for application in synbiotic food and dairy products. This paper reports the effect of varying concentrations of prebiotics (FOS and PHGG) as co-encapsulating agents in alginate-starch beads on survival of microencapsulated probiotic bacteria during heat treatment, low pH and high bile salt concentration. The term co-encapsulation in this article is with reference to encapsulation of both probiotic bacteria and prebiotics together.

MATERIALS AND METHODS

Microorganism and culture conditions

Probiotic *Lactobacillus acidophilus* LA1 was obtained from the National Collection of Dairy Cultures of National Dairy Research Institute (Karnal, India). The freeze-dried culture was activated in chalk litmus milk at 37°C for 24 h and grown in 100 ml of MRS broth (proteose peptone 5 g/l, beef extract 10 g/l, yeast extract 5 g/l, dextrose 20 g/l, polysorbate 80 g/l, ammonium citrate 2 g/l, sodium acetate 5 g/l, magnesium sulphate 0.10 g/l, manganese sulphate 0.05 g/l, dipotassium phosphate 2 g/l, pH at 25°C 6.5±0.2) at 37°C for 24–48 h. The culture was transferred 2 to 3 times before cell encapsulation. The reactivated cultures were centrifuged at 2500×g for 10 min at 4°C using a refrigerated bench top centrifuge (Hermle Z 382 K, Maschinenfabrik Berthold Hermle AG, Gosheim, Germany) and washed in distilled water twice. The cell concentration was adjusted to 10^{11}.

Prebiotics and other chemicals

Fructo-oligosaccharide (FOS) was gifted by M/s Alchem Laboratories, Pune (India). Partially hydrolysed guar gum (PHGG) was purchased from M/s Taiyo-Lucid Pvt. Ltd., Mumbai (India). All other chemicals and reagents were procured from standard manufacturers.

Microencapsulation of the probiotic organism

The organisms were microencapsulated by air atomization method (Kwok *et al*., 1991; Cui *et al*., 2000, 2001) using a spray nozzle for generating microgel droplets using a single fluid nozzle atomizer. To begin with, a 100 ml matrix solution consisting of 3% sodium alginate and 2% corn starch was prepared in distilled water. To this matrix solution, PHGG (3%, 5%) and FOS (1.5%, 2.5%, 3%) were added separately to get different concentrations of these prebiotics in the gel matrix. The final matrix was then sterilized and cooled to 38-40°C, and used fresh for encapsulating the microbial cell biomass. To prepare the matrix-prebiotic-cell mixture (MPCM), 100 ml of the matrix and 20 ml of the cell suspension (10^{10} to 10^{11}) were mixed and stirred with a magnetic stirrer for 10 min. MPCM thus prepared was fed to the supply column of previously sterilized pneumatic spray nozzle.

A simple, low cost equipment (similar to the spray gun used for automobile painting) consisting of a double tube construction with separate inlet points was assembled for the purpose. The inner tube carried the MPCM at ~ 0.5 kg/cm^2 pressure, which was adjusted to suit the viscosity of the material. The discharge area of the outer tube was wider and just above that of the inner tube. Inert gas at a pressure of 2.5-3 kg/cm^2 was applied through the outer tube. An adjustable nozzle at the discharge end controlled the flow of the inert gas. As the MPCM was fed into the inner tube, the high pressure of the inert gas carried it forward, atomizing it as very small droplets. The pressurised MPCM was extruded as a fine spray into chilled 0.1 M calcium chloride bath and left undisturbed for 30 min to facilitate the formation of

micro-gel beads. The beads were separated by filtration (Wattman No. 1). In order to make the matrix resistant to water penetration (Redenbaugh and Reyes, 1985), the beads obtained were re-filtered with distilled water and aseptically transferred to a sterile flask for storage at 4°C. The bead size was measured using phase contrast microscopy under 10X objective (Kearney *et al*., 1990). For each trial 100-200 microcapsules were measured randomly and size distribution of capsules were expressed as percent frequency distribution. Microencapsulated cells in control alginate beads without the prebiotic (CAB) were prepared for comparison at each experimental stage with those entrapped in alginate beads containing prebiotic (PAB).

Depolymerization and enumeration of microcapsules

To determine the viability, the entrapped *L. acidophilus* LA1 were released from the microcapsules by the method suggested by Sheu and Marshall (1993). One g of the microencapsulated beads was mixed in test tubes containing 10 ml of the depolymerization solution (28 ml of 0.2 M NaH_2PO_4 and 72 ml of 0.2 M Na_2HPO_4 adjusted to 200 ml with distilled water, pH 7.1± 0.1, sterilized). After incubation at 37°C for 10 min, the mixture was vortexed at high speed for breaking the polymer formed and releasing completely the encapsulated culture into the buffer. The released cells were enumerated using MRS media at 37°C for 24 h. All enumeration was done by the pour-plate method described by Houghtby *et al*. (1993). The population, in cfu, were recorded for every enumeration. A comparison was made at each enumeration between the cells entrapped in control alginate beads (CAB) with those entrapped in alginate beads containing prebiotic (PAB).

Viability under heat stress

The microencapsulated cultures were tested for their survival during exposure to high temperature. One g of microencapsulated biomass was transferred to 10 ml of sterile distilled water (pH 6.4± 0.2) in a thin walled test tube. The contents were heated to 75°C and 85°C for 30 sec in a water bath and immediately cooled by immersing in chilled water. The remaining viable cells were enumerated after the appropriate depolymerization of beads in phosphate buffer.

Viability at gastric pH

Survival of the probiotic organism at low pH was tested by the method suggested by Lee and Heo (2000). Simulated gastric solutions containing 0.2% NaCl at pH 1.0 and 2.0 (pH adjusted with 0.1 N HCl) were prepared as suggested by Rao *et al*. (1989). One g of microencapsulated bacterial cells was mixed in test tubes containing 10 ml of simulated gastric solution of varying pH. The tubes were incubated at 37°C for 1 h and 2 h, removed at hourly intervals, beads filtered, washed, and immediately used for enumeration after depolymerisation.

Viability at high bile salt concentrations

One g of microcapsules was transferred to test tubes containing 10 ml of 1.0%, 1.5%, and 2.0% bile salt (w/v) and adjusted to neutral pH (Lee and Heo, 2000). The tubes were incubated at 37°C for 1, 2 and 3 h intervals, enumerated thereafter.

Analysis of data

The outcomes of study were recorded as mean±SE of three independent replicates carried out on different days with freshly prepared cultures, media, matrix materials and other reagents. The data obtained in the present study were subjected to one-way analysis of variance (ANOVA) using SPSS v.16.0 for Windows 246 software (SPSS South Asia (P) Limited, Bangalore, India).

RESULTS AND DISCUSSION

Microencapsulation of cells

Beads obtained were in the form of clusters and measured 15–180 μm in diameter (average 80 μm). Microscopic examination of individual beads revealed that the organisms were entrapped in the matrix. The beads appeared rough on the surface. The spherical nature of the beads was affected at 5% PHGG, which may be due to increase in polymer viscosity.

Bead particle size is important in terms of probiotic performance during various stress conditions. Lee and Heo (2000) reported that survival of bacterial cells under *in vitro* gastric conditions decreased with decreasing capsule size (diameter 1-2.6 mm). McMaster *et al*. (2005) indicated that particles with a diameter below 3μm were undetected by the tongue and that larger capsules can impart a gritty texture to foods not normally associated with the sensation. Although dried alginate beads with large particle size have been commonly used to incorporate immobilized probiotic bacteria including bifidobacteria (Lee and Heo, 2000), small and controlled size of solid-type microparticles are more desirable in food products due to their easier handling, comfortable taste, better sensory characteristics and higher stability (Cui *et al*., 2000, 2001; Picot and Lacroix, 2003). The bead size achieved in the current study agrees with those suggested by other researchers (Kwok *et al*., 1991; Lee *et al*., 2003).

Survival under heat stress

The results of exposing CAB and PAB to high temperatures (75°C and 85°C for 30 s) are illustrated in Fig. 1. The initial logarithmic count of organisms was 8.84 log. *L. acidophilus* LA1 entrapped in prebiotic-alginate micro-particles showed significantly higher ($P<0.05$) heat stability than encapsulated *L. acidophilus* LA1 without the prebiotic. The difference between the reduction in the counts of CAB and PAB at 75°C was significant ($p<0.05$), though among different levels of prebiotics, the effect was significant ($p<0.05$) only for 3% PHGG. Increasing the level of PHGG to 5% level had no effect on increasing the thermo-tolerance. The

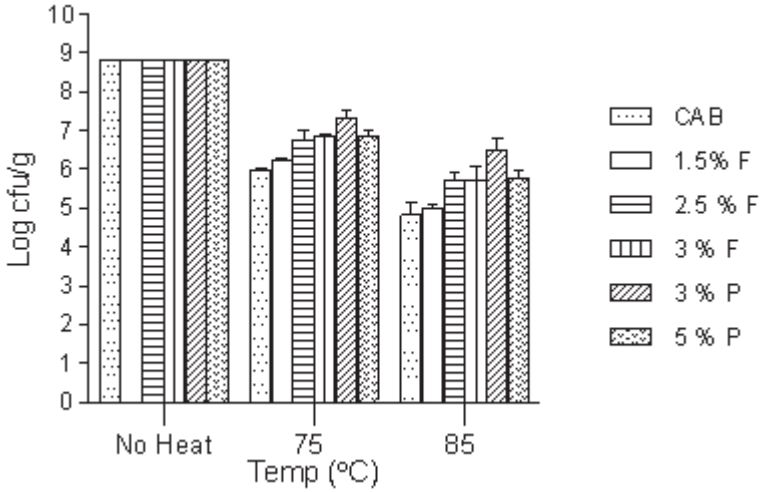

Fig. 1: Effect of FOS and PHGG on survival of microencapsulated *L. acidophilus* LA1 under heat stress. Error bars represent SE of mean (n=3). CAB – no prebiotics, F – FOS, P – PHGG

higher heat tolerance of PHGG-loaded beads than FOS-loaded ones may be due to the denser matrix. At 85°C there was almost 3.0 log reduction in all the combinations studied.

It is important to establish and ensure the heat resistance of beneficial organisms that are used in food products, because the thermal processing treatments that inactivate spoilage and pathogenic microorganisms to enhance the shelf life of food products would also inactivate the beneficial ones. The most thoroughly characterized stress response in bacteria and higher cells is heat shock (Ang *et al*., 1991; Kim *et al*., 2001). The current results agree with the findings of Kim *et al*. (2001) who reported 53°C as the sub-lethal temperature for *L. acidophilus* because cells were still growing at this temperature while 60°C was reported as the lethal temperature. Chen and Chen (2007) reported that optimal thermo-tolerance of *Bifidobacterium bifidum* improved with addition of 1% gellan gum as wall material in alginate. Hannoun and Stephanopoulos (1986) found that the survival of the lactobacilli increased on alginate encapsulation and was proportionate to concentrations of alginate in capsules. Ding and Shah (2007) tested heat tolerance of the probiotic organisms by exposing to 65°C for up to 1 h and found that microencapsulated probiotic bacteria survived better than free probiotic bacteria. However, there are no data available at very high temperatures. The survival of the encapsulated probiotic organisms might be due to the better thermal stability and denser matrix in case of PHGG and high concentration (3%) of alginate and additional protection given by starch and prebiotics, as reported by Mandal *et al*. (2006). Sabikhi *et al*. (2011)

reported that addition of 2% inulin to alginate-starch matrix improved thermal tolerance of *L. acidophilus* NCDC15.

Survival at low pH

The results of evaluation of viability of immobilized control and prebiotic-loaded cells of *L. acidophilus* LA1 in simulated gastric juice are shown in Fig. 2a and Fig. 2b. The results illustrate that the survival of the probiotic organism was lower at lower pH and decreased further as the incubation period increased. *L. acidophilus* LA1 survived in greater numbers and for longer duration than control beads in the beads co-encapsulated with the prebiotics. At pH 1.0 after 1 h, the live cell numbers of *L. acidophilus* LA1 in CAB and PAB containing 1.5%, 2.5%, 3% FOS and 3%, 5% PHGG were 6.09, 6.64, 6.64, 6.65, 6.98 and 6.64 log respectively (initial count - 8.84 log). In the same environment for 2 h the respective numbers reduced to 5.12, 5.61, 5.61, 5.70, 5.92 and 5.80 log for these microcapsules as is evident from Fig. 2a. The results followed a similar trend at pH 2.0. Statistical comparison by post hoc LSD demonstrated that at pH 1 and incubation for one h the CAB differed significantly ($p<0.05$) only to 3% PHGG, whereas after 2 h it differed significantly ($p<0.05$) to 3% FOS as well as 3% and 5% PHGG. At pH 2, the alginate–starch-prebiotic wall matrix could protect *L. acidophilus* LA1 against acid environment effectively as the prebiotics significantly performed better than CAB. There was no significant difference ($p<0.05$) among various prebiotics.

Numerous research findings substantiate that encapsulation protected *L. acidophilus* from exposure to simulated gastric conditions where microencapsulation in alginate beads greatly increased the survival of probiotic bacteria in acidic conditions (Audet *et al*., 1988; Jankowski *et al*., 1997; Krasaekoopt *et al*., 2003; Doleyres and Lacroix, 2004). Although alginate is frequently used to microencapsulate probiotics, it has certain undesirable attributes, such as susceptibility to degradation by acids (Sun and Griffiths, 2000; Truelstrup-Hansen *et al*., 2002; Krasaekoopt *et al*., 2003). Hence alginate micro-capsules are likely to wash out in the hydrochloric acid of the stomach and not reach the colon intact. Addition of prebiotic substances as wall materials or coating may lead to enhanced survivability at low pH. However, literature on the survival rate of probiotic at low pH after co-encapsulation with prebiotic substances is scarce. Results of Chen *et al*. (2005) indicated that microencapsulation with sodium alginate and prebiotics could provide good protection for probiotics with respect to low gastric acid pH and the probiotic counts for the alginate microcapsules with prebiotics remained at 10^5–10^6 CFU/g in simulated gastric fluid after 8 weeks of storage. Iyer and Kailasapathy (2005) reported that co encapsulation of probiotics with prebiotics led to significant increase in viability during *in vitro* acidic conditions. Nazzaro *et al*. (2009) reported that co-encapsulation of *L. acidophilus* in an alginate-xanthan mixture containing the prebiotic inulin represented a good source of this probiotic not only for controlled-release applications, but also for increased viability at low pH. Wood (2010) reported that the addition of 1.0% FOS to pea protein isolate-alginate capsules containing

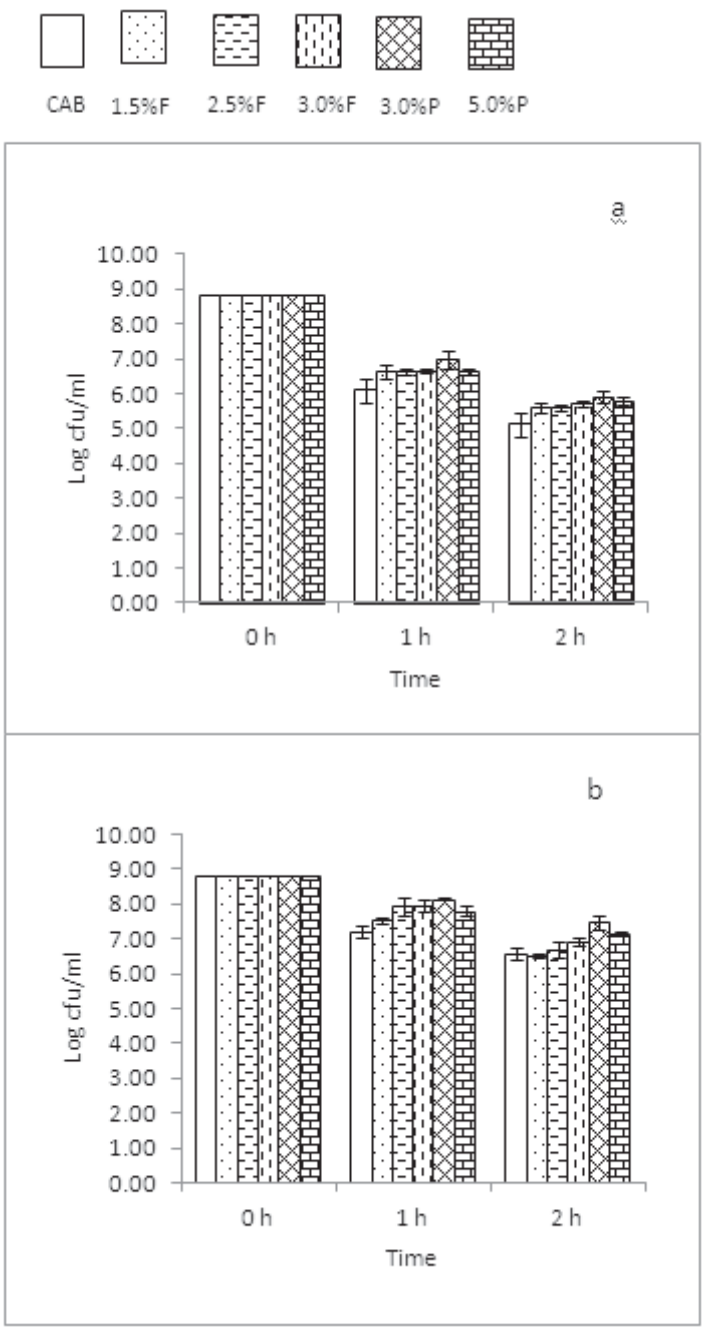

Fig 2: Effect of FOS and PHGG on survival of microencapsulated *L. acidophilus* LA1 under acidic pH. (a) pH 1 (b) pH 2. Error bars represent SE of mean (n=3). CAB – no prebiotics, F – FOS, P - PHGG

Bifodobacterium adolescentis improved survival within simulated gastric juice of pH 2.0 during incubation at 37°C. The enhanced protective nature was attributed to the prebiotic increasing the walls ability to limit simulated gastric juice diffusion into the capsule. Sabikhi *et al*. (2011) also demonstrated similar effect leading to increased viability of *L. acidophilus* NCDC15 at low pH with the addition of 2% inulin in alginate-starch matrix. Klemmer *et al*. (2011) studied capsule design for entrapping *Bifidobacterium adolescentis* and fructoligosacchardes within a pea protein isolate–alginate matrix as a function of total biopolymer concentration (2.5–6.5%), fructoligosacchardes levels (0–3.0%) and needle gauge (G; 20 vs. 27) by extrusion. Capsules were classified on the basis of the levels of entrapped fructoligosacchardes and protein, size, swelling and probiotic survival within simulated gastric juice (SGJ) and release properties within simulated intestinal fluids (SIF). They concluded that that all designs offered sufficient protection to *B. adolescentis* during challenge experiments.

Survival at high bile salt concentrations

The results of subjecting *L. acidophilus* LA1-containing CAB and PAB to different concentrations of bile salts (1%, 1.5%, and 2%) are presented in Fig. 3a, 3b and 3c. It is evident from the figure that these results followed the same trend as those at low pH. From the initial counts, of CAB and PAB, the numbers declined steadily as the bile concentration and time of incubation increased. The rate of decrease was greater in the CAB containing cells. There was significant difference ($p<0.05$) in the reduction of cell numbers between CAB and PAB at higher bile levels (1.5% and 2% w/v). However, the difference at 1% level between CAB and PAB with 1.5%, 2.5% and 3% FOS during 1 and 2 h of incubation was not significant ($p<0.05$), though the difference became significant after 3 h. Among all the prebiotics tested 3% PHGG ($p<0.05$) offered significant protection in comparison to CAB. At 2% bile concentration there was 1.21, 1.67 and 1.93 log reduction in the cell count after 1, 2 and 3 h respectively with PHGG, as against 2.24, 2.61 and 3.21 log in CAB.

The protective effect of microencapsulation against high bile salt concentrations has been tested earlier (Lee and Heo, 2000; Krasaekoopt *et al*., 2004). Wang *et al*. (1999) found that amylomaize improves the survivability of microorganisms by adhesion to starch granules at 0.05% bile acid concentration. Sabikhi *et al*. (2010) reported that alginate-starch encapsulation significantly protected *L. acidophilus* LA1 from high bile salt concentrations. Our results show that addition of prebiotics as wall material in alginate-starch matrix could enhance the survivability of probiotics during exposure to high concentrations of bile salt. Several workers have reported similar findings (Bielecka *et al*., 2002; Chen *et al*., 2005; Drgalic *et al*., 2005; Capela *et al*., 2006; Sabikhi *et al*., 2011). Thus our results are in corroboration with other researchers.

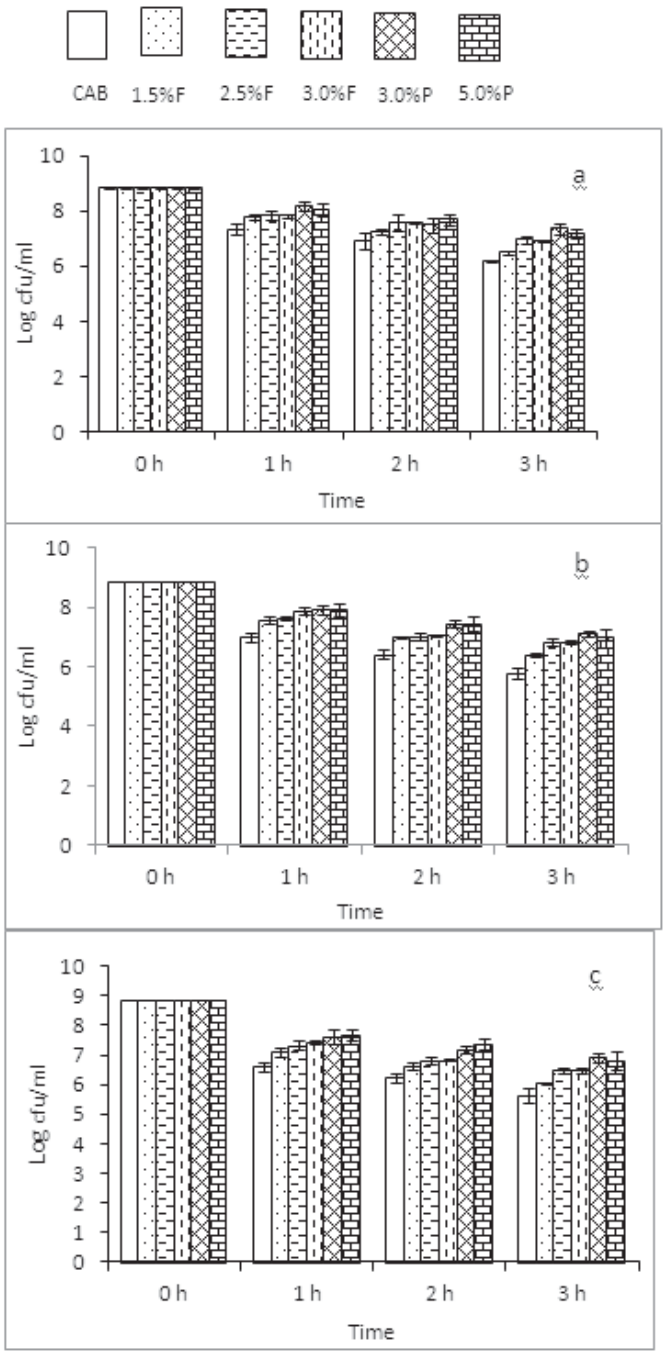

Fig 3: Effect of FOS and PHGG on survival of microencapsulated *L. acidophilus* LA1 in different bile concentrations. (a) 1% (b) 1.5 % (c) 2%. Error bars represent SE of mean (n=3). CAB – no prebiotics, F – FOS, P - PHGG

CONCLUSION

Co-encapsulating prebiotic and probiotic offers new directions in functional food arena by synergistically enhancing the efficacy of microcapsules as delivery vehicles for these bioactive compounds. Addition of FOS or PHGG as a co-encapsulant appears to improve the survival of encapsulated probiotic bacteria under *in vitro* acidic and bile salt environments and during heat processing. The co-encapsulated beads containing prebiotics and probiotics may prove to be useful additives in synbiotic food applications.

ACKNOWLEDGEMENTS

The first author acknowledges National Dairy Research Institute, Karnal (India) for the grant of Institute Fellowship to pursue Ph.D. The authors thank M/s Alchem Laboratories, N.H. No.8, Mandva, Pune-393010 (India) for free samples of fructo-oligosaccharides.

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