



ISSN NO. 2320-5407

Journal homepage: <http://www.journalijar.com>

INTERNATIONAL JOURNAL
OF ADVANCED RESEARCH

RESEARCH ARTICLE

In Vitro Characterization of *Lactobacillus paracasei* HML1 Microencapsulated with Alginate Containing Prebiotics Inulin and Psyllium

Hemal Sadrani, Bhoomi Patel, Jayantilal Dave and Bharatkumar Rajiv Manuel Vyas*
Department of Biosciences, Saurashtra University, Rajkot 360005, Gujarat State, India.

Manuscript Info

Manuscript History:

Received: 22 May 2015
Final Accepted: 19 June 2015
Published Online: July 2015

Key words:

Inulin, *Lactobacillus*,
Microencapsulation, Prebiotics,
Probiotics, Psyllium

*Corresponding Author

Bharatkumar Rajiv
Manuel Vyas

Abstract

Synbiotics, combination of probiotics and prebiotics, are able to promote host health by positively modulating the intestinal microflora. *Lactobacillus paracasei* HML1 was encapsulated with alginate containing prebiotic psyllium and inulin, stored for 4 weeks and its survival in simulated gastrointestinal conditions was evaluated. The alginate (ALG), alginate-psyllium (ALG-PSY) and alginate-inulin (ALG-INU) beads with encapsulation efficiency 88, 84 and 74% respectively was achieved using extrusion method. Average diameter of the ALG, ALG-PSY and ALG-INU beads was 553.12, 537.83 and 570.96 mm respectively. Survival of encapsulated *L. paracasei* HML1 after incubation in simulated intestinal fluid was better with psyllium (4.25 log CFU/ml) and inulin (4.15 log CFU/ml) than beads without prebiotic (3.15 log CFU/ml) and free cells (3.24 log CFU/ml). The encapsulated cells hit maximum release in simulated colonic pH solution in 1 h, and remained constant, thereafter. The incorporation of the prebiotics also increased viability during heat treatments. Inulin and psyllium can be potential prebiotic candidates and can deliver a better functional food product with high added value, suitable for consumers with dairy sensitivities.

Copy Right, IJAR, 2015., All rights reserved

INTRODUCTION

“Probiotics are live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host” (1). Most of the probiotic strains belong to the *Lactobacillus* and *Bifidobacterium* genera, which are health-promoting bacteria forming part of the balanced intestinal microbiota (2). The health promoting effects of lactobacilli have been widely explored and include stabilisation of the indigenous microbial population, protection against intestinal infection, alleviation of lactose intolerance, increased nutritional value of foods, reduction of serum cholesterol levels and non-specific enhancement of the immune systems (3–6). Other health benefits of probiotic organism include antimicrobial activity, antimutagenic properties, anticarcinogenic properties, anti diarrhoeal properties, and improvement in inflammatory bowel disease. Antimicrobial activity of *Lactobacillus* strains against Gram-positive and Gram-negative organisms, which are major food spoilage organisms and gastrointestinal pathogens has also been reported earlier (7-9). Probiotic *L. rhamnosus* 231 cells has ability to bind, biotransform and detoxify different mutagens like acridine orange (AO), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 2-amino-3, 8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx) (10).

It is suggested that probiotics should be formulated in products with a minimum count of 10^{6-7} CFU/g or ml of viable probiotic bacteria (1). *Lactobacillus* spp. lack the ability to survive harsh acidity and bile concentration commonly encountered in the gastrointestinal tract (GIT) and also the high temperature of dairy processing (11-16). Various techniques have been utilized to improve viability and stability of probiotics and efficient delivery of the cells to their active sites (17). However, the stabilization of probiotics using a carrier may improve survival of these microbes in products, both during processing and GIT transition (18). In this regard, encapsulation of probiotics in a variety of polymers is the most frequently applied method that is cited in numerous studies (19).

Probiotics are less stable in non-dairy than in dairy products. Various solutions to this problem, such as durable strain selection in adverse environments (20) and addition of prebiotics (21), have been evaluated. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (22, 23). A novel approach combines probiotics and prebiotics in an association defined as synbiotic (24, 25) in an attempt to obtain synergistic effects of the two by an improving the probiotic colonisation or metabolic effect. The most frequently studied examples are inulin-type fructans and fructooligosaccharides (26). Inulin and its partially hydrolyzed derivative oligofructose are made up of linear β -(2 \rightarrow 1) glycosidic bonds of D-fructose, often with a terminal glucose moiety that is linked by an α -(1 \rightarrow 2) glycosidic bond, as in sucrose (27). Psyllium, the common name used for several members of the plant genus *Plantago*, is gel-forming mucilage composed of a highly branched arabinoxylan. The backbone consists of xylose units, while arabinose and xylose form the side chains (28, 29). Psyllium as a soluble fiber has a potential to stimulate bacterial growth in digestive system and has also been used as prebiotic (30-33).

Encapsulation has been investigated for protecting probiotics in food products and the GIT (34); advantages include prevention of interfacial inactivation, stimulation of production and excretion of secondary metabolites, and continuous utilization. Alginate, a polymer extracted from seaweed, is a favoured encapsulation agent because it is non-toxic, biocompatible, and inexpensive. The ease of solubilizing alginate gel (by Ca⁺⁺ sequestration) and its release of entrapped cells within the human intestine are additional benefits. Researchers have investigated the use of encapsulated lactic acid bacteria (LAB) in dairy products (35, 36); Preparation of alginate bead, with well retained bacteria in the matrix, can be easily achieved by simple techniques like extrusion or emulsion methods. Incorporating both prebiotics and calcium alginate in coating materials may better protect probiotics in food systems and the GIT due to symbiosis (37, 38).

In the present work, the probiotic *Lactobacillus paracasei* HML1 was microencapsulated within an alginate-inulin and alginate-psyllium matrix and evaluated for (i) encapsulation efficiency, (ii) viability of encapsulated and free cells during Simulated Gastrointestinal Transit, (iii) release in simulated colonic pH solution during 4 weeks of storage at 4°C, and (iv) survival of free and encapsulated cells during heat treatment.

Materials and Methods:

Bacterial inoculum

The probiotic *Lactobacillus paracasei* HML1 was isolated from curd using De Man Rogosa and Sharpe (MRS, Himedia, Mumbai, India) agar medium and preserved in 10% skim milk at 4°C. The GenBank accession number for *Lactobacillus paracasei* HML1 is KC884268. *L. paracasei* HML1 was sub-cultured twice in MRS broth at 37°C for 24 h. The cell density of culture was adjusted to an OD₆₂₀ of 1.0 (10⁸ cfu/ml) and inoculated in 10 ml of MRS broth and incubated at 37°C. 18 h cells were harvested by centrifugation (5000 rpm, 10 min, 4°C), washed twice with sterile phosphate buffer saline (PBS, pH 7, 0.1 M, 0.85% NaCl) and resuspended in 1 ml Tris-HCl buffer.

Chemicals

Inulin, calcium chloride, sodium alginate, pancreatin, pepsin, MRS broth and sodium hydroxide were purchased from Himedia, Mumbai, India. Lactulose was purchased from Sigma-Aldrich (UK); Tris chloride was purchased from Merck (Germany). Psyllium seed husk was supplied by Unjha Laxmi brand Sat-Isabgol (India).

Preparation of Prebiotic solutions

0.2% psyllium husk was partially hydrolyzed in 1 M HCl at 80°C with continuous stirring at 50 rpm for 1 h. The homogenous solution was neutralized to pH 7.0 by 5 M NaOH and sterilized at 121°C for 15 min. Aqueous inulin solution (0.2% w/v) was filter sterilized using 0.22 μ filter.

Microencapsulation

The alginate (ALG), alginate-psyllium (ALG-PSY) and alginate-inulin (ALG-INU) beads were prepared by extrusion technique as described by Krasaekoopt *et al.* (39) with minor modifications. Sodium alginate (3%) was sterilized at 121°C for 15 min. Cooled ALG, ALG-PSY or ALG-INU solutions (25 ml) were mixed with 1 ml bacterial suspension (10⁸ cfu/ml) and gently stirred for 30 min to obtain a homogeneous suspension and extruded drop-wise through a 10 ml syringe into sterilized hardening solution (4% CaCl₂). The beads were shaken gently for 30 min, isolated by aseptic filtration (Whatman No. 1), washed twice with sterile water, and kept in Tris-HCl (5 mM) at 4°C. Free cells (OD₆₂₀ of 1.0) were also stored in the same solution at 4°C.

Size of Beads

The particle size of beads was assessed using optical microscopy (Zeiss Stemi DV4). Data were collected from 50 beads, and mean particle size was reported.

Encapsulation efficiency

The colony forming units (CFU)/ml of free and encapsulated cells were determined by aerobic culturing on MRS agar plates (37°C, 48 h). Encapsulation efficiency (EE) (1) was determined by disintegrating encapsulated cells in phosphate buffer (pH 6.8) and comparing the number of released cells with the number of cells pre-encapsulation and calculated as

$EE = (\text{Log}_{10}N/\text{Log}_{10}N_0) \times 100$, where, N is the number of viable entrapped cells released from the beads and N_0 is the number of free cells added to the biopolymer mixture immediately before the production procedure.

Viability of free and encapsulated cells during Simulated Gastrointestinal Transit

Viability of free and encapsulated cells (ALG, ALG-PSY and ALG-INU) of *L. paracasei* HML1 was determined during Simulated Gastric Fluid (SGF, NaCl 125 mM, KCl 7 mM, NaHCO₃ 45 mM, pepsin 3 g/L, pH 2.5 adjusted with 1 M HCl) and Simulated Intestinal Fluid (SIF, pancreatin 0.1% w/v, bovine bile salts 0.15% w/v, pH 8.0 adjusted with 1 M NaOH) transit as described by Zarate *et al.* (40) initially at 24 h intervals (0, 24, 48 and 72 h), and later at weekly interval for 4 weeks (1-4 week). 100 mg stored beads with entrapped bacteria were mixed with 1 ml of SGF and incubated for 2 h at 37°C with continuous agitation at 200 rpm, washed with distilled water, removed, and incubated in 1 ml of SIF for 3 h at 37°C with continuous agitation at 200 rpm. The SGF and SGF-SIF treated beads were harvested, disintegrated in phosphate buffer (pH 6.8), serially diluted, and plated on MRS agar for the determination of cell viability. The control constituted of beads treated with Tris-HCl buffer instead of SGF and SIF. The percentage of bacterial survival was calculated as follows: $\text{CFU}_{\text{assay}} / \text{CFU}_{\text{control}} \times 100$, (41)

where $\text{CFU}_{\text{assay}}$ represents CFU/ml after 3 h exposure to SGF or SIF and $\text{CFU}_{\text{control}}$ the CFU/ml after incubation in Tris-HCl buffer as a control.

Release of encapsulated cells in simulated colonic pH solution

In vitro release of encapsulated cells (ALG, ALG-PSY and ALG-INU) of *L. paracasei* HML1 at simulated colonic pH solution was examined as described by Mandal *et al.* (42). The beads were mixed with 1 ml of simulated colonic pH solution (0.1 M KH₂PO₄, pH 7.4 ± 0.2), mixed gently and incubated for 3 h at 37°C with continuous agitation at 50 rpm. Samples were taken at different time intervals, and CFU was assayed using pour plate method.

Survival of free and encapsulated cells during heat treatment

Effect of heat treatment was determined by using 1 g of encapsulated cells or 1 ml of the free cell suspension (10⁸ cells/ml) of *L. paracasei* HML1. Encapsulated and free cells were transferred in 10 ml of distilled water and heat treated at 55, 60 or 65°C for 20 min, cooled to room temperature and enumeration of viable cells was carried out (42).

Statistical Analysis:

All the experiments were performed in triplicate and repeated at least once. Appropriate controls were also conducted. The data collected were statistically analyzed using a completely randomized design. The means were compared using a least significant difference test. The correlation coefficients between a pair of trait were determined and the significance of the correlation determined using ANOVA. All the statistical tests were performed at $p < 0.05$, using the software INSTAT.

Results:

Size of Beads

The mean diameters of beads containing Psyllium were higher (570.96 mm) than beads containing inulin (537.83 mm) and beads having only alginate (553.12 mm) (Table 1) because of the viscosity of the resultant gel.

Encapsulation efficiency (EE)

The initial cell count of *L. paracasei* HML1 before alginate (ALG), alginate-psyllium (ALG-PSY) and alginate-inulin (ALG-INU) bead preparation was 8.32 ± 0.37 , 7.56 ± 0.44 and 8.10 ± 0.37 log CFU/ml, number of cells entrapped in the resultant bead was 7.63 ± 0.32 , 7.08 ± 0.90 and 6.46 ± 0.24 , and EE was 88, 84 and 74% respectively (Table 2). Results indicated very little loss of viability for all preparations.

Viability of free and encapsulated cells during Simulated Gastrointestinal Transit

The protective effects of different coats of ALG, PSY-ALG, and INU-ALG after 2 h exposure to acid condition (pH 2.5) and after a consecutive 3 h of exposure to bile juice (pH 8) were compared to free cells; results were expressed as viability (log CFU/ml) of cells in Figure 1. The viability of free cells of *L. paracasei* HML1 after 2 h incubation in SGF reduced from 6.4 CFU/ml to 5.7 CFU/ml and after 10 days reduced to 4.88 CFU/ml. Viability of 3, 17 and 31 days old *L. paracasei* HML1 free cells upon SGF-SIF transit reduced to 5.29, 4.59 and 3.24 CFU/ml respectively. The viability of 3, 17 and 31 days old ALG encapsulated cells was similar to free cells after SGF treatment but that of SGF-SIF treated cells reduced to 4.90, 4.0 and 3.15 CFU/ml respectively. Viability of PSY-ALG and INU-ALG reduced marginally when incubated with SGF for 31 and 24 days respectively. When treated with SGF-SIF, cells entrapped with psyllium reduced to 4.5 CFU/ml and remained stable till 31 days while cells entrapped with inulin remained stable till 24 days and then reduced to 4.15 CFU/ml. It is clear that the survival of bacterial cells after SGF and SGF-SIF exposure, in all prepared beads was significantly ($P < 0.05$) higher than that of the free cells. The ALG microencapsulated cells showed less reduction in viability as compared to free cells. Coating of *L. paracasei* HML1 cells with PSY-ALG and INU-ALG showed negligible reduction in viability in comparison to ALG beads and free cells.

Release of encapsulated cells in simulated colonic pH solution

Count of the released bacterial cells after 30 min was 4.03, 4.50 and 4.20 log CFU/ml (53, 70 and 59%) for ALG, INU-ALG and PSY-ALG respectively (Figure 2). As incubation time increased, the release of cells increased to 6.34, 6.30 and 6.42 log CFU/ml (83, 98 and 91%) for ALG, INU-ALG and PSY-ALG respectively. In all preparations, the count of released cells reached maximum in one hour, and thereafter remained constant. Data were significant for one hour ($P < 0.05$) but beyond 60 minutes there was no significant change ($P > 0.05$).

Survival of free and encapsulated cells during heat treatment

Viable count upon heat treatments at 55, 60 and 65°C, (7.65 log CFU/ml) of free cells was 3.92, 3.59 and 2.43 log CFU/ml, and the ALG encapsulated cells was 4.18, 3.96 and 3.60 log CFU/ml respectively. Beads containing prebiotic (PSY or INU) showed higher survival than the others. Beads with inulin showed highest survival (>4.78 log CFU/ml) while beads containing psyllium showed >4.00 log CFU/ml. Reduction in viability with increased temperature was negligible in the case of prebiotic-containing beads, but it was higher in free cells and alginate beads not containing prebiotics.

Table 1: Dimensions of the beads prepared by extrusion method

Parameter of beads	Area (mm)	Perimeter (mm)	Radius (mm)	Diameter (mm)
Alginate (ALG)	240614.8	1737.68	276.56	553.12
Psyllium-Alginate (PSY-ALG)	256197.5	1793.71	285.48	570.96
Inulin-Alginate (INU-ALG)	228073.6	1689.64	268.92	537.83

The mean diameter of 40 beads in each sample is reported; The mean diameter of PSY-ALG beads were significantly higher than the others ($P < 0.05$)

Table 2: Encapsulation of probiotic *Lactobacillus paracasei* HML1 with Alginate (ALG), Psyllium-Alginate (PSY-ALG) and Inulin-Alginate (INU-ALG)

Formulation	Free <i>L. paracasei</i> HML1 log CFU/ml	Encapsulated <i>L. paracasei</i> HML1 log CFU/ml	Encapsulation efficiency (%)

ALG bead	8.32 ± 0.37	7.63 ± 0.32	88
PSY-ALG bead	7.56 ± 0.44	7.08 ± 0.90	84
INU-ALG bead	8.10 ± 0.37	6.46 ± 0.24	74

Figure 1: Viability (log CFU/ml) of *Lactobacillus paracasei* HML1 after 2 h of exposure to simulated gastric fluid and after a consecutive 3 h of exposure to simulated intestinal fluid. (A) free cells, cells encapsulated in (B) ALG (Alginate), (C) INU-ALG (Inulin-Alginate) and (D) PSY-ALG (Psyllium-Alginate). Error bars indicate standard error of the mean (n = 3)

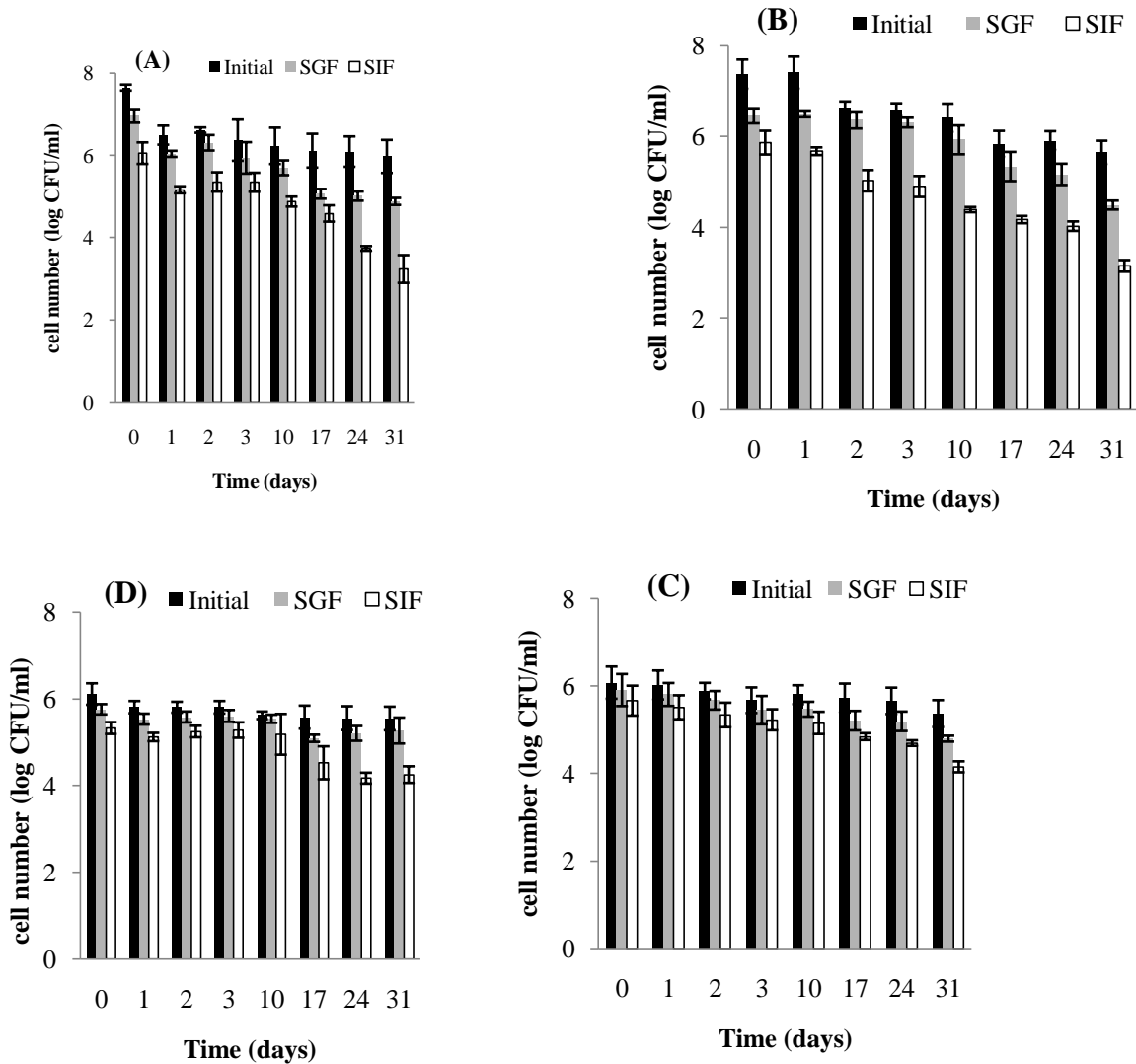


Figure 2: Release of encapsulated *Lactobacillus paracasei* HML1 cells (log CFU/ml) after 0-3 h in simulated colonic pH solution. Error bars indicate standard error of the mean (n = 3)

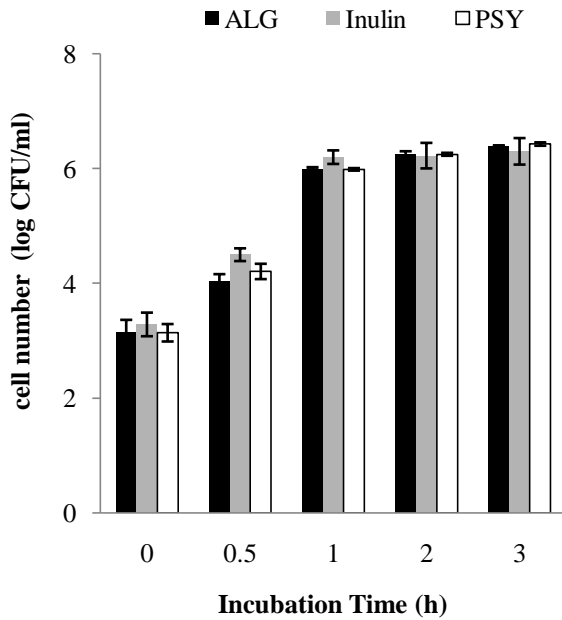
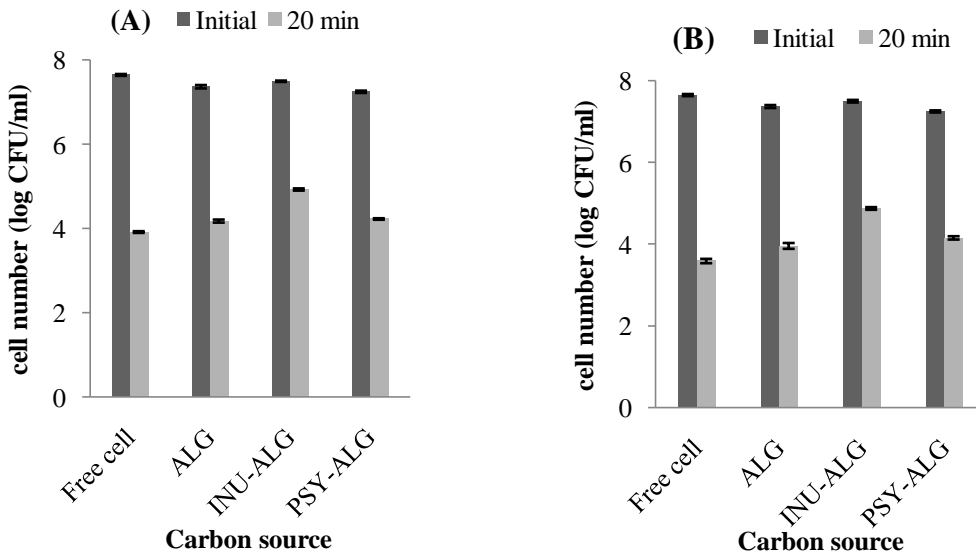
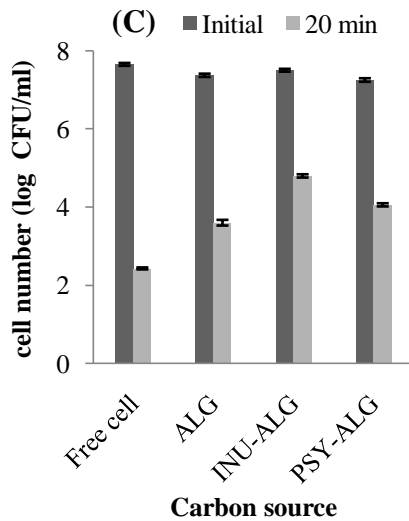


Figure 3: Survival of free and encapsulated cells of *Lactobacillus paracasei* HML1 (log CFU/ml) after heat treatment of 20 minutes: (A) 55°C (B) 60°C and (C) 65°C. Error bars indicate standard error of the mean (n = 3)





Discussion

There is considerable interest in the development of dietary supplements that benefit the composition and activity of health-promoting gut microflora. The main fermentative substrates of dietary origin are non-digestible carbohydrates (e.g. resistant starch, non-starch polysaccharides and fibers of plant origin and non-digestible oligosaccharides) and protein which escape digestion in the small intestine. Carbohydrate fermentation is energetically more favorable, leading to a gradient of substrate utilization spatially through the colon (43). Many non-digestible oligosaccharides, such as inulin (INU) and fructooligosaccharide, in the gut act as prebiotics enhancing specifically the growth of certain lactic acid bacteria (LAB) and other beneficial gut microbes (44, 45). Microencapsulation has many benefits including enhancement of microbial survival and efficiency during fermentation (46), and overcome inactivation during drying or exposure to artificial gastric conditions (47, 48). Microencapsulation of probiotics in hydrocolloid beads has been tested for improving their viability in food products, and during gastrointestinal tract (GIT) transit (49-51).

In this study, we first aimed to encapsulate *Lactobacillus paracasei* HML1 in alginate with inulin (INU) and psyllium (PSY) using extrusion technique and characterized the beads in terms of size, encapsulation efficiency (EE), viability in simulated gastric and intestinal fluids, release in simulated colon pH conditions and survival of microencapsulated probiotic bacteria after heat treatment.

Beads, were prepared in 3% alginate (ALG) and 4% w/v CaCl_2 , as viscosity was insufficient at 1% (w/v) alginate conc because of low ionic sites for cross-linkage (47). As compared to inulin, addition of PSY into ALG gel increases the viscosity and adherence of resultant gel. Increased viscosity of the starter gel results into bigger beads in the extrusion method. The ALG, ALG-PSY and ALG-INU beads showed $\geq 70\%$ encapsulation efficiencies. Farzaneh Lotfipour et al. (17) entrapped more than 98.9% cells with different formulations of alginate and psyllium. The retention of viability of *L. paracasei* HML1 cells upon SGF-SIF transit was less compared to encapsulated cells while in case of free cells, it was higher in INU-ALG and PSY-ALG than ALG encapsulated cells. ALG beads containing prebiotics INU and PSY enhance the protection to SGF and SGF-SIF fluidity.

The probiotics should retain viability and activity during transit through host's GIT, the natural barrier. Exposure to gastric and intestinal fluids along the digestive tract is the main stress that could decrease the viability of ingested probiotics. About 2.5 l of gastric juice of approximately pH 2 is secreted each day in the stomach (52), which causes destruction of most microorganisms ingested. Bile juice produced in the intestine also decreases the survival rate of microorganisms. In this sense, resistance to human gastric and intestinal transit is an important selection criterion for probiotic microorganisms (53). The cells were encapsulated in ALG, INU-ALG and PSY-ALG gel to increase their activity and stability of probiotic product. The viability of free SGF-SIF exposed *L. paracasei* HML1 cells stored for 3 d decreases little. The viability of cells decreases with increase in storage time. Viable count in beads with PSY

and INU was essentially constant. As the storage time increases the viability of stored cells decreases in free cells, followed by ALG bead and negligible reduction occurs in beads with Inulin and Psyllium. Beads containing inulin showed better results as compared to others. Most preparations were resistant to SGF as compared to SGF-SIF due to the rapid pH shift from acidic to basic conditions. Our results show that alginate protect LAB during GI transit by retarding permeation of acidic fluid into the beads.

There are contradicting reports on the viability of free and microencapsulated cells under stress conditions. Sohail *et al.* (54) showed that encapsulation of probiotic bacteria in cross-linked alginate beads is of major interest for improving the survival in harsh acid and bile environment. Chan and Zhang (55) used the prebiotics fructooligosaccharide or isomaltooligosaccharide and sodium alginate to microencapsulate different probiotics and achieved a remarkable increase in survival under acidic conditions relative to free cells. Goderska *et al.* (56) reported that encapsulation of *L. rhamnosus* in alginate improved survival at pH 2.0 up to 48 h, while the free cells were destroyed completely. Moreover, microencapsulation of bifidobacteria also exhibited a lower population reduction during exposure to simulated gastric environment and bile solution (57). However, Sultana and coworkers (58) reported that encapsulation of bacteria in alginate beads did not effectively protect the organisms from high acidity.

Encapsulated probiotic bacteria will be washed out from the body without exerting any beneficial effect to the host, if they fail to release out from the beads in proper time (42). The release of probiotic bacteria from the beads, their subsequent growth and colonization in colon is critical to derive the advantages of the beneficial cells. The release of cells from the beads increases up to 1 h and remains constant thereafter. The results were same for ALG, PSY-ALG, and INU-ALG beads. Mandal *et al.* (42) found that the count of *L. casei* in simulated colonic pH solution rose to its highest point in 60 min and after that remained constant. Picot and Lacroix (57) also reported a progressive release of viable cells from whey protein-based microcapsules in simulated intestinal conditions. Moreover, according to Suita-Cruz & Goulet (59) an efficient release of viable and metabolically active cells in the intestine is one of the aims of microencapsulation.

Last aim of our study was to evaluate influence of heat on free and encapsulated cells. Very little research has been done for incorporation of probiotics into heat-treated foods due to destruction of live culture during heat treatment. We found that as compared to encapsulated cells, viability of free cells drastically reduced on heat treatments. Selmer-Olsen reported that encapsulation of lactobacilli in calcium-alginate beads improved their heat tolerance (60). Lower diffusion of glucose and ethanol in concentrated alginate gels was attributed to decreased number and length of pores rather than decrease in pore diameter (61). Mandal *et al.* (42) concluded that due to the slower diffusion of water in 4% alginate matrix during heat treatment led to the higher survival of *L. casei* NCDC-298.

This study has identified some potential synbiotics, a combination of probiotic *Lactobacillus paracasei* HML1 and prebiotic inulin or psyllium. Microencapsulation of *L. paracasei* HML1 along with prebiotic (psyllium and inulin) enhanced cell viability in simulated harsh conditions of GIT, during storage and heat treatment as compared to beads without prebiotics and free cells. The results indicated that addition of prebiotic in beads improved its delivery to the active site as they maintained their release in simulated colonic pH solution. Inulin and psyllium, suitable polymer candidates, can enhance probiotic bacterial viability, activity, stability and functional properties of food products. These associations need to be further assayed *in vitro* and *in vivo* models to demonstrate their potential probiotic properties and interaction with human cells.

Acknowledgements

UGC-Meritorious Fellowship to Hemal Sadrani is gratefully acknowledged.

References:

1. FAO/WHO, "Guidelines for the evaluation of probiotics in food," Food and Agriculture Organization of United Nations and World Health Organization Working Group Report, World Health Organization, London, UK, 2002.
2. Ventura, M., O'Flaherty, S., Claesson, M.J., Turrone, F., Klaenhammer, T.R. and van Sinderen, D, *et al.* (2009): Genome-scale analyses of health-promoting bacteria: probiogenomics. *Nat Rev Microbiol.*, 7:61-71.

3. Kim, Y., Kim, S.H., Whang, K.Y., et al. (2008): Inhibition of *Escherichia coli* O157:H7 attachment by interactions between lactic acid bacteria and intestinal epithelial cells. *J Microbiol Biotechnol.*, 18: 1278–1285.
4. Hooper, L.V., Xu J., Falk P.G., et al. (1999): A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. *Proc Natl Acad Sci U S A.*, 96: 9833–9838.
5. Perdigon, G., Maldonado, Galdeano, C., Valdez, J.C., et al. (2002): Interaction of lactic acid bacteria with the gut immune system. *Eur J Clin Nutr* 56., Suppl. 4: S21–S26.
6. Suvarna, V.C., and Boby, V.U. (2005): Probiotics in human health: a current assessment. *Curr Sci.*, 88: 1744–1748.
7. Sadrani, H., Dave, J.M., and Vyas, B.R.M. (2014): Screening of potential probiotic *Lactobacillus* strains isolated from fermented foods, fruits and of human origin. *Asian J Pharm Clin Res.*, Vol 7 Suppl 2: 216–225.
8. Pithva, S.P., Shekh, S., Dave, J.M., and Vyas, B.R.M. (2014): Probiotic attributes of autochthonous *Lactobacillus rhamnosus* strains of human origin. *Appl Biochem Biotechnol.*, 173: 259–277.
9. Ambalam, P., Prajapati, J.B., Dave, J.M., Nair, B.M., Ljungh, A., and Vyas, B.R.M. (2009): Isolation and characterization of antimicrobial proteins produced by a potential probiotic strain of human *Lactobacillus rhamnosus* 231 and its effect on selected human pathogens and food spoilage organisms. *Microb Ecol Health Dis.*, 21:211–220.
10. Ambalam, P., Dave, J.M., Nair, B.M., and Vyas, B.R.M. (2011): *In vitro* Mutagen Binding and Antimutagenic Activity of Human *Lactobacillus rhamnosus* 231. *Anaerobe.*, 17: 217–222.
11. Conway, P.L., Gorbach, S.L. and Goldin, B.R. (1987): Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J Dairy Sci.*, 70: 1–12.
12. Gardiner, G.E., O’Sullivan, E., Kelly, J., Auty, M.A., Fitzgerald, G.F. and Collins, J.K., et al. (2000): Comparative survival rates of human derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. *Appl Environ Microbiol.*, 66: 2605–2612.
13. Hood, S.K. and Zottola, M.L. (1988): Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. *J Food Sci.*, 53: 1514–1516.
14. Lankaputhra, W.E.V. and Shah, N.P. (1995): Survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in the presence of acid and bile salts. *Cultured Dairy Products Journal*, 30: 2–7.
15. Shah, N.P. and Jelen, P. (1990): Survival of lactic acid bacteria and their lactases under acidic conditions. *J Food Sci.*, 55: 506–509.
16. Silva, J., Carvahlo, A.S., Teixeira, P. and Gibbs, P.A. (2002): Bacteriocin production by spray dried lactic acid bacteria. *Letters in Appl Microbiol.*, 34: 77–81.
17. Farzaneh, L., Shahla, M. and Maryam M. (2011): Preparation and Characterization of Alginate and Psyllium Beads Containing *Lactobacillus acidophilus*. *The Scientific World Journal.*, 2012: 1–8.
18. Goderska, K., Zybals, M. and Czarnecki, Z. (2003): Characterization of microencapsulated *Lactobacillus rhamnosus* LR7 strain. *Pol J Food and Nut Sci.*, 12/53: 21–24.
19. Ouyang, W., Chen, H., Jones M.L. et al. (2004): Artificial cell microcapsule for oral delivery of live bacterial cells for therapy: design, preparation, and in-vitro characterization. *J Pharm Pharm Sci.*, 7/3, 315–324.
20. Chou, L.S. and Weimer, B. (1999): Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. *J Dairy Sci.*, 82: 23–31.
21. Topping, D.L., Fukushima, M. and Bird, A.R. (2003): Resistant starch as a prebiotic and synbiotic: State of the art. *Proc Nutr Soc.*, 62:171–176.
22. Gibson, G.R., Probert, H., Van Loo, J., Rastall, R. and Roberfroid, M.B. (2004): Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.*, 17:259–275.
23. Gibson, G.R. and Roberfroid, M.B. (1995): Dietary modulation of the human 6536 MAKRAS ET AL. APPL. ENVIRON. MICROBIOL. colonic microbiota: introducing the concept of prebiotics. *J. Nutr.*, 125:1401–1412.
24. Roberfroid, M.B. (1998): Prebiotics and synbiotics: concepts and nutritional properties. *Br J Nutr.*, 80: 197–202.
25. Ziemer, C.J. and Gibson, G.R. (1998): An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies. *Int Dairy J.*, 8: 473–9.

26. Filomena, N., Florinda, F., Raffaele, C., Alfonso S. and Pierangelo, O. (2009): Fermentative ability of alginate-prebiotic encapsulated *Lactobacillus acidophilus* and survival under simulated gastrointestinal conditions. *J Funct Food.*, 1: 319-323.
27. Roberfroid, M.B. and Delzenne, N.M. (1998): Dietary fructans. *Annu. Rev. Nutr.*, 18:117–143.
28. Fischer, M.H., Yu, N., Gray, G.R., Ralph, J., Anderson, L. and Marlett, J.A. (2004): The gel-forming polysaccharide of psyllium husk (*Plantago ovata* Forsk). *Carbohydr Res.*, 339/11: 2009–2017.
29. Guo, Q., Cui, S.W., Wang, Q. and Christopher Young, J. (2008): Fractionation and physicochemical characterization of psyllium gum. *Carbohydr Pol.*, 73/ 1: 35–43.
30. Fujimori, S., Gudis, K. and Mitsui, K. *et al.* (2009): A randomized controlled trial on the efficacy of synbiotic versus probiotic or prebiotic treatment to improve the quality of life in patients with ulcerative colitis. *Nutr.*, 25/ 5: 520–525.
31. Damaskos, D. and Kolios, G. 2008: “Probiotics and prebiotics in inflammatory bowel disease: microflora ‘on the scope’,” *Brit J Clin Pharmacol.*, 65/ 4: 453–467.
32. Fujimori, S., Tatsuguchi, A. and K. Gudis et al., 2007: “High dose probiotic and prebiotic cotherapy for remission induction of active Crohn’s disease,” *J Gastroen Hepatol.*, 22/8: 1199–1204.
33. Rishniw, M. and Wynn, S.G. (2011): Azodyl, a synbiotic, fails to alter azotemia in cats with chronic kidney disease when sprinkled onto food. *J Feline Med Sur.*, 13/6: 405–409.
34. Favaro-Trindale, C.S. and Grosso, C.R.F. (2002): Microencapsulation of *L. acidophilus* (La-05) and *B. lactis* (Bb-12) and evaluation of their survival at the pH values of the stomach and in bile. *J Microencapsul.*, 19: 485–494.
35. Adhikari, K., Mustapha, A., Grun, I.U. and Fernando, L. (2000): Viability of microencapsulated bifidobacteria in set yogurt during refrigerated storage. *J Dairy Sci.*, 83: 1946–1951.
36. Sultana, K., Godward, G., Reynolds, N., Arumugaswamy, R., Peiris, P. and Kailasapathy, K. (2000): Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. *Int J Food Microbiol.*, 62: 47–55.
37. Bielecka, M., Biedrzycka, E. and Majkowska, A. (2002): Selection of probiotics and prebiotics for synbiotic and confirmation of their in vivo effectiveness. *Food Res Intrnl.*, 35: 125–131.
38. Chen, K.N., Chen, M.J., Liu, J R., Lin, C.W. and Chiu, H. Y.(2005): Optimization of incorporated prebiotics as coating materials for probiotic microencapsulation. *J Food Sci.*, 70: 260–266.
39. Krasakoopt, W., Bhandari, B. and Deeth, H. 2004: “The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria,” *Int Dairy J.*, 14/8: 737–743.
40. Zarate, G., Chaia, A.P., Gonzalez, S. and Oliver, G. (2000): Viability and beta-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. *J Food Prot.*, 63: 1214-21.
41. Julien, G., Henri, D., Céline, C., Pierre, M., Françoise, O., Vassilia, T. and Christine, R. (2010): In vitro screening of probiotic lactic acid bacteria and prebiotic glucooligosaccharides to select effective synbiotics. *Anaerobe.*, 30: 1-8.
42. Mandal, S., Puniya, A K. and Singh, K. (2006): Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298,” *Int Dairy J.*, 16/ 10: 1190–1195.
43. Macfarlane, G. T., Gibson, G. R. and Cummings, J. H. (1992): Comparison of fermentation reactions in different regions of the human colon. *J Appl Bacteriol.*, 72:57–64.
44. Licht, T. R., Ebersbach, T. and Frøkiær, H. (2012): Prebiotics for prevention of gut infections. *Trends of Food Sci Technol.*, 23:70-82.
45. Roberfroid, M. (2007): Prebiotics: the concept revisited. *J Nutr.*, 137:830S-7S.
46. Champagne, C. P., Lacroix, C. and Sodini-Gallot, I. (1994). Immobilized cell technologies for the dairy industry. *Cr Rev Biotechn.*, 14:109–134.
47. Chandramouli, V., Kailasapathya, K., Peirisb, P. and Jones, M. (2004): An improved method of microencapsulation and its evaluation to protect *Lactobacillus spp.* in simulated gastric conditions. *J Microbiol Meth.*, 56: 27–35.
48. De Giulio, B., Orlando, P., Barba, G., Coppola, R., De Rosa, M., Sada, A., De Prisco, P. P. and Nazzaro, F. (2005): Use of alginate and cryo-protective sugars to improve the viability of lactic acid bacteria after freezing and freeze-drying. *World J. Microbiol. Biotechnol.*, 21: 739–746.

49. Kebary, K. M. K., Hussein, S. A. and Badawi, R. M. (1998): Improving viability of bifidobacteria and their effect on frozen ice milk. *Egypt J Dairy Sci.*, 26: 319–337.
50. Khalil, A. H. and Mansour, E. H. (1998): Alginate encapsulated bifidobacteria survival in mayonnaise. *J Food Sci.*, 63: 702–705.
51. Krasaekoopt, W., Bhandari, B. and Deeth, H. (2003): Evaluation of encapsulation techniques of probiotics for yoghurt. *Int Dairy J.*, 13: 3–13.
52. Charteris, W. P., Kelly, P. M., Morelli, L. and Collins, J. K. (1998a): Development and application of an in vivo methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *J Appl Microbiol.*, 84:759–768.
53. Charteris, W. P., Kelly, P. M., Morelli, L. and Collins, J. K. (1998b). Ingredient selection criteria for probiotic microorganisms in functional dairy foods. *Int J Dairy Technol.*, 51(4):123–136.
54. Sohail, A., Turner, M. S., Coombes, A., Bostrom, T. and Bhandari. B. (2011): “Survivability of probiotics encapsulated in alginate gel microbeads using a novel impinging aerosols method,” *Int J Food Microbiol.*, 145/1: 162–168.
55. Chan, E. S. and Zhang, Z. (2002): Encapsulation of probiotic bacteria *Lactobacillus acidophilus* by direct compression. *Food and Bioproducts Processing*, 80: 78–82.
56. Goderska, K., Zybals, M. and Czarnecki, Z. (2003): Characterization of microencapsulated *Lactobacillus rhamnosus*LR7 strain. *Pol J Food Nutr Sci*, 12/53, 21–24.
57. Picot, A. and Lacroix, C. (2004): Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. *Int Dairy J.*, 14: 505–515.
58. Sultana, K., Godward, G., Reynolds, N., Arumugaswamy, R., Peiris, P. and Kailasapathy, K.(2000). “Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt,” *Int J Food Microbiol.*, 62/1-2: 47– 55.
59. Suita-Cruz, P. and Goulet, J. (2001): Improving probiotic survival rates. *Food Technol.*, 55: 36–42.
60. Selmer-Olsen, E., Sorhaug, T., Birkeland, S. E. and Pehrson, R. (1999): Survival of *Lactobacillus helveticus* entrapped in Ca-alginate in relation to water content, storage and rehydration. *J Ind Microbiol Biot*, 23: 79–85.
61. Hannoun, B. and Stephanopoulos, G. (1986): Diffusion coefficient of glucose and ethanol in cell-free and cell-occupied calcium alginate membranes. *Biotechnol Bioeng.*, 28: 829–835.