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Research Paper

Survival and release of probiotic bacteria from chitosan-coated alginate-starch capsules in mice gastro-intestinal tract and effect on faecal flora and immune parameters in mice

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ABSTRACT

Chitosan-coated alginate-starch (CCAS) capsules containing approximately 10^8 CFU of *Lactobacillus casei* Shirota strain cells (LCS) were fed to mice and the release profile of bacteria from CCAS capsules in murine GI tract was investigated at different time intervals. There was a complete release (10^8 CFU) of LCS from capsules within 12 h in mice ileal region. There was only a partial release of encapsulated LCS, in duodenal, jejunal and colon regions, while there was no release of encapsulated LCS in stomach from the CCAS capsules even after 24 h. The effect of feeding (14 days) CCAS encapsulated LCS on faecal microbial population and immune parameters in mice were also determined. The numbers of LCS recovered from the faeces confirmed adequate percentage of LCS survived passage through gastrointestinal tract. A concomitant increase in the LCS and total lactobacilli count during the test period was observed in the group fed with CCAS encapsulated bacteria compared to the one fed with free non-encapsulated bacteria. This experiment also showed that the CCAS microencapsulated LCS did not affect or mask the probiotic bacteria's immunomodulatory activity. The effect on the specific immune response was assessed by measuring cytokine production of the mouse splenocytes. There was significant increase ($p<0.05$) in interleukin (IL-10) and interferon (IFN)-γ production between groups fed with CCAS encapsulated LCS and free LCS compared to the control and the one fed with empty CCAS alginate capsules. Chitosan coated alginate-starch capsules can be effectively used to deliver viable bacteria safely to animal intestine for increased immunomodulatory activity.

Keywords: Microencapsulation, Chitosan-coated alginate-starch capsules, Cytokines, *Lactobacillus casei,* Immunomodulation

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INTRODUCTION

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer health benefits on the host" (Reid *et al*., 2003). One of the requirements for probiotic bacteria to be used as dietary adjuncts is the need to retain viability and activity during food processing and transit through gastro-intestinal tract (Kailasapathy and Chin, 2000; Lourens-Hattingh and Viljoen, 2001). Poor survival of probiotic bacteria in dairy products and in the gastro-intestinal tract is a major concern for dairy industries all over the world (Dave and Shah, 1997a; Kailasapathy and Rybka, 1997; Rybka and Fleet, 1997; Shah, 2000; Lourens-Hattingh and Viljoen, 2001). A number of techniques have been used to improve the survival of probiotic bacteria: addition of growth promoting factors, such as cysteine, whey protein concentrate, casein hydrolysate, prebiotics, such as oligo-saccharides, buffering of yoghurt mixes with whey proteins and modulating packaging conditions (Modler *et al*., 1990; Tanaka and Hatanaka, 1992; Kailasapathy and Supriadi, 1996; Dave and Shah, 1997b; Gomes *et al*., 1998; Ravula and Shah, 1998; Crittenden *et al*., 2001; Talwalkar *et al*., 2004). However, these have had only limited success. Microencapsulation of probiotic living cells with alginate as a means of improving viability is an approach that has received considerable interest in the recent past (Kailasapathy, 2002; Krasaekoopt *et al*., 2003). Microencapsulation has shown to protect probiotic bacteria not only during food-fermentation and storage conditions but also from adverse gastro-intestinal environment. A variety of bio-polymers have been used as a capsular wall material, including alginate, gelatin, carrageenan, cellulose, maize starch (Rao *et al*., 1989; Hunik and Tamper, 1993; Hyndman *et al*., 1993; Sheu and Marshall, 1993; Khalil and Mansour, 1998; Wang *et al*., 1999; Lee and Heo, 2000; Yang *et al*., 2000; Koo *et al*., 2001; Truelstrup Hansen *et al*., 2002; Adhikari *et al*., 2003; Krasaekoopt *et al*., 2003). Among the available techniques for immobilizing living cells, entrapment in calcium alginate beads has been frequently used for the immobilization of probiotic bacteria due to its ease of handling, low cost, non-toxic to the cells being immobilized and alginate is an accepted food additive (Prevost and Divies, 1992). Although calcium-induced alginate encapsulation has been widely used for probiotic bacteria, there is no uniformity in the reported literature as to the protective nature of capsule against adverse gastrointestinal conditions and also in products shelf-life (Chandramouli e al., 2004). An improved method of chitosan coated alginate-starch (CCAS) encapsulation for probiotic bacteria has been reported by Iyer *et al*.(2004b), which was effective in protecting the viability of probiotic bacteria under *in vitro* gastric conditions and also during yoghurt (set yoghurt) shelf-life. Although CCAS encapsulation has been used to improve the survival of bacteria under *in vitro* gastric condition, there is no information regarding its efficacy in *in vivo* conditions.

We conducted two different experiments to investigate the release profile and immunomodulatory activity of *Lactobacillus casei* Shirota (LCS) from CCAS

capsules. In the first experiment CCAS capsules containing LCS was fed to mice and its release in different parts of GI tract was monitored up to 24 h. In the second experiment the efficacy of released bacteria in terms of its interaction with intestinal microbiota and also its immunomodulatory activity was investigated. In both these experiments we used LCS as a model probiotic strain because of the specific selective media available that allowed us to differentiate the inoculated bacteria from food and gastrointestinal flora and also due to its documented effects on the immune system.

MATERIALS AND METHODS

Bacterial strains, growth conditions and preparation of cell suspensions

Pure culture of probiotic bacteria LCS was isolated from fermented milk drink, Yakult® (Yakult Australia, Dandenong, Victoria, Australia). The bacteria were cultured at 37°C for 24 h under anaerobic conditions in *lactobacillus* selective broth modified by replacing glucose with lactitol and by adding vancomycin (lactitol-*Lactobacillus*-vancomycin medium: LLV) to increase the selectivity for LCS as described by Yuki *et al*. (1999). Anaerobic conditions were achieved using an anaerobic glove box (95% N_2 and 5% H_2, Coy laboratory products inc., Grass Lake, MI, U.S.A.). Cells for survival experiments were propagated in 500 ml LLV for 20 h at 37°C**.** Cells were harvested by centrifugation at 3000 x g for 10 min at 4°C and washed twice with phosphate buffer (0.1 M, pH 7.0). The cell suspensions was subsequently used either directly (free cells) in assays or microencapsulated as described below.

Microencapsulation

The capsules were prepared aseptically using an Inotech Encapsulatorâ (Inotech AG, Dottikon, Switzerland) as described by Iyer *et al*. (2004b) modified by the addition of prebiotics (Hi-maizeä; Starch Australasia Ltd., Lane cove, Australia) and chitosan coating. The standard conditions used for encapsulation were 1.8 % (w/v) alginate (from brown algae, viscosity of 2 % solution at 25°C is ~250 cps, Sigma chemical Co., St. Louis, Mo., U.S.A), probiotic culture (approx. 10^{10} cfu ml^{-1}), 1.0 % (w/v) prebiotics (Hi-maize) and 30 min hardening in 0.1M calcium chloride solution and further coated with 0.4 % (w/v) chitosan solution by immersing them for 20 min.

Animal feeding

Six-week old male BALB/c mice were obtained from the Animal Resource Centre, Perth, Australia. Mice were randomly assigned into one of 4 groups (n = 7), the animals consumed: Group 1: 50 ml skim milk (control), Group 2: Free LCS culture (containing approx. 10^8 CFU 50 ml^{-1}) in Skim milk, Group 3: 50mg of CCAS encapsulated LCS culture (containing approx. 1.7 x 10^8 CFU) in Skim milk along

with 50 µl of 10 % reconstituted skim milk, Group 4:50µg of empty CCAS capsules along with 50 µl of 10 % reconstituted skim milk.

Survival and release of co-encapsulated LCS in the murine GI tract

Seven BALB/c male mice (6 week old) were force-fed 50 µg of encapsulated bacteria (containing approx. 10^8 CFU). The animals were euthanised at different time intervals and tested for the release of encapsulated bacterial cells. The total contents of the stomach, small intestine (divided into three parts corresponding to duodenum, jejunum, and ileum), and colon were immediately collected after euthanising, weighed, and diluted (1/3) in sterile water. *Lactobacillus casei* Shirota counts were estimated from these dilutions for each sample on selective LLV agar plates as described above. The colonies from LLV agar plates were randomly picked and further confirmed for identification by amplifying with casei-specific primer and then running it in Denaturation Gradient Gel Electrophoresis (DGGE).

Fecal microflora

Fresh faecal samples were collected aseptically into a pre-weighed sterile eppendorf tubes. The samples were weighed and stored at 4ºC. Within 6 h the samples were homogenised using 0.1% (w/v) sterile buffered peptone water (Amyl Media Pty. Ltd., Dandenong, Australia) in an anaerobic glove box. Aliquots of 0.1 ml of the appropriate dilution were spread onto the following agar media: Reinforced clostridial Agar (Oxoid CM0151, Australia) supplemented with 5g l^{-1} glucose, 75 ml l^{-1} sterile horse blood and 75 ml l^{-1} (0.4%) china blue (Reinforced-China blue; RCB agar) for total anaerobic bacteria; RCB agar containing 80 mg/l kanamycin and 1mg l^{-1} vancomycin for *Bacteroidaceae*; MacConkey agar (Oxoid) for enumeration of presumptive Coliforms; MRS-lithium propionate (MRS-LP) for *bifidobacterium*. The culture media were incubated in an anaerobic chamber at 37 ºC for 48 to 72 h.

Outside the anaerobic glove box, aliquots of 100 µl were spread plated on to the following agar media: Rogosa agar (Oxoid) for *Lactobacillus*; LBS agar (Oxoid) containing 10mg l^{-1} vancomycin and 2 % lactitol for LCS (large white colonies) and perfringens agar base (oxoid) with 2 vials/l perfringens SPF selective supplement (oxoid) and 50 ml l^{-1} egg yolk emulsion for *Clostridium*. These culture media were incubated anaerobically (anaerobic chamber) or aerobically at 37 ºC or 24 ºC. After incubation, the specific colonies on the selective media were counted and the number of viable microorganisms per gram faecal sample (CFU g^{-1}) was calculated.

PCR conditions

Species-specific PCR primers casei-f GC (GCclamp CAG ACT GAA AGA TTC AAT TTC AC) and casei-r (GCG ATG CGA ATT TCT TTT TC) were used to amplify the 16S-23S spacer region of *L. casei* for DGGE analysis. A 40-nucleotide GC clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG

GGG G) was incorporated onto the 5^1 end of the casei-f primer to ensure the DNA remained partially double-stranded during DGGE. A reaction mixture (50 µl) consisted of reaction buffer (10 mM Tris-HCl [final concentration], 2.0 mM $MgCl_2$ concentration, and 50 mM KCl [pH 8.3]), a 200 µM concentration of each deoxynucleoside triphosphate, 10 pmol of each primer, 50 ng of bacterial DNA, and 1.75 U of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany). Amplification of DNA was performed in a Hybaid express thermal cycler programmed for an initial denaturation of 92°C for 5 min followed by 30 cycles (95°C ×30 s) + (55°C×30 s) + (72°C× 30 s) and a final extension of 72°C for 1 min. PCR products were analysed on 2 % agarose gels, stained with ethidium bromide, visualised and photographed using a Bio-Rad, Gel Doc imaging system.

Denaturation Gradient Gel Electrophoresis (DGGE)

DGGE was performed with a DCode universal mutation detection system (Bio-Rad, Hercules, Calif.) utilizing 16-cm by 16-cm by 1-mm gels. Eight percent polyacrylamide gels were prepared and run with 1× TAE buffer diluted from 50× TAE buffer (2 M Tris base, 1 M glacial acetic acid, and 50 mM EDTA). The denaturing gradient was formed with two 8 % acrylamide (acrylamide-bis, 37.5:1) stock solutions (Bio-Rad). The gels contained a 30 to 50 % gradient of urea and formamide increasing in the direction of electrophoresis. A 100 % denaturing solution contained 40% (v/v) formamide and 7.0 M urea. The electrophoresis was conducted with a constant voltage of 130 V at 60°C for about 4. The run was stopped when a xylene cyanol dye marker reached the bottom of the gel. Gels were stained with ethidium bromide solution (5 µg ml^{-1}; 20 min), washed with deionized water, and viewed by UV transillumination.

Cytokine production

For measurement of the cytokine production by splenocytes from the mice (after 14 days of feeding), 2 $x10^6$ splenocytes/ml were cultured with 1.25 µg/ml ConA (Sigma chemical Co., St. Louis, Mo., U.S.A), in 24-well plates (total volume: 1 ml $well^{-1}$) in duplicate for 24 hrs, the supernatant harvested by centrifugation for 4 min at 3000 x *g* and stored at -80°C until cytokine analysis. Interferon-γ (Assay Designs Inc., Ann Arbor, MI, USA) and IL-10 (Chemicon International, Temecula, CA, USA) Enzyme-linked immunosorbent assays (ELISA's) were performed according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Release of encapsulated bacteria in murine gastrointestinal tract (in vivo)

The release of LCS from chitosan-coated alginate-starch capsules in different regions of murine gastrointestinal tract is shown in Table 1. There was no release of LCS from capsules in gastric and duodenal contents up to 24 h. There was very low release of LCS from capsules in jejunal contents (approx. 10^3 cfu g^{-1}) while there

while there was greater, but incomplete release of LCS ($2.4x10^5$ cfu g^{-1}) from capsules in colon contents after 24 h of feeding. There was complete release of LCS from CCAS capsules in ileal region after 12 h where as there was only a partial release of encapsulated bacteria in duodenal, jejunal and colon contents. There was no release of LCS in mice stomach region.

Smidsrod and Skajak-Braek (1990) reported that alginate capsule is susceptible to disintegration in the presence of excess monovalent ions, Ca^{2+}-chelating agents and harsh chemical environments. A cross-linked alginate matrix system at very low pH is reported to undergo a reduction in alginate molecular weight causing a faster degradation and release of active ingredients (Gombotz and Wee, 1998). Poly-cations, such as chitosan or poly-amino acids (for example, poly-l-lysine (PLL), form strong complexes with alginates which are stable in the presence of Ca^{2+} chelators and reduce the porosity of the gel (Smidsrod and Skjak-Braek, 1990; Gombotz and Wee, 1998).

Iyer *et al*. (2004a) reported a complete release of encapsulated *E. coli* GFP^+ from alginate capsules with in 1 h incubation in *ex vivo* porcine intestinal contents. Results from the current study shows that it took nearly 12 h to completely release the encapsulated LCS from CAAS capsules in murine intestine. The slow release of encapsulated bacteria in the current study is due to chitosan coating over the alginate capsules. Chitosan, a positively charged polyamine, forms a semi-permeable membrane around a negatively charged polymer such as alginate. This membrane does not dissolve in the presence of Ca^{2+} chelators or antigelling agents and thus enhances stability of the gel (Smidsrod and Skjak-Braek, 1990), and provides a barrier to cell release. Drug release from chitosan-alginate microcapsules occurs primarily by diffusion from the calcium alginate core through the semi-permeable chitosan-alginate membrane. Sawayanagi *et al*. (1982) studied the release of several drugs through a chitosan membrane and compared the results to that of a cellulose membrane. They concluded that the bulk of the drug release was due to diffusion through pores, and that the charge state of the chitosan had an influence (i.e. greater permeabilities were observed for acidic drugs than basic drugs). Thus, coating alginate beads with poly-cations can improve the chemical and mechanical stability of the alginate beads, consequently improving the effectiveness of encapsulation.

Lee *et al*. (2004) reported that the average doubling time of LCS measured in mice duodenum, jejunum, ileum and colon were 4.10, 4.78, 4.56 and 5.59 days, respectively. Since the LCS doubling time in mice intestine is nearly 4-5 days, the increase in LCS counts at different time intervals in different intestinal regions indicates the actual release of encapsulated bacteria from the capsules and not the multiplication of already released cells. After complete release of LCS from capsules in ileal and colon contents the viable counts remained constant up to 24 h. This indicates the ability of newly added probiotic bacteria (LCS) to survive in the gastrointestinal contents. This result is contrary to our previous study in which there was a significant decrease ($p<0.05$) in *E. coli* GFP^+ in ileal and colon contents after complete release form capsules (Iyer *et al*., 2004a). This decrease of *E. coli*

GFP^+ viable count was due to inability of newly added strain to survive in porcine gut contents. Also, *E. coli* GFP^+ strain K12 is not a native gut bacterium and is therefore relatively intolerant to gut conditions.

It is well known that environmental conditions vary greatly between the stomach and the colon. The initial stress encountered by bacteria in the GI tract is due to acidic gastric secretions. It was proposed that these constitute a major defence mechanism against the majority of ingested microorganisms (Marteau and Rambaud, 1993). The ability of lactic acid bacteria to survive in gastric juices varies according to the species (Marteau and Rambaud, 1993; Conway, 1996). The viability of lactic acid bacteria in the GI tract was studied for many different species, including two yoghurt bacteria *Lactobacillus delbrueckii* subspp. *bulgaricus* and *Streptococus thermophilus* (Marteau and Rambaud, 1993; Pochart *et al*., 1992). Pochart *et al*. (1992) reported that the survival rate of these organisms was very low (only 1 %). The results from our study show that LCS cells are more adaptable to gut environment and can survive longer. Increases in viable cell counts of LCS in gastric, duodenal and jejunal contents after addition of phosphate buffer (after 24 h) indicate that bacteria were alive but not released completely from the capsules. Where as, the encapsulated bacteria was released completely in ileal contents even before addition of phosphate buffer. All the colonies randomly picked up from LLV agar showed a positive for LCS in PCR/DGGE analysis and an example of the gel is shown in Fig. 1.

The results of the *in vivo* experiment demonstrated that the most likely site of bacterial release in the gut would be the ileum. This is a significant finding in that

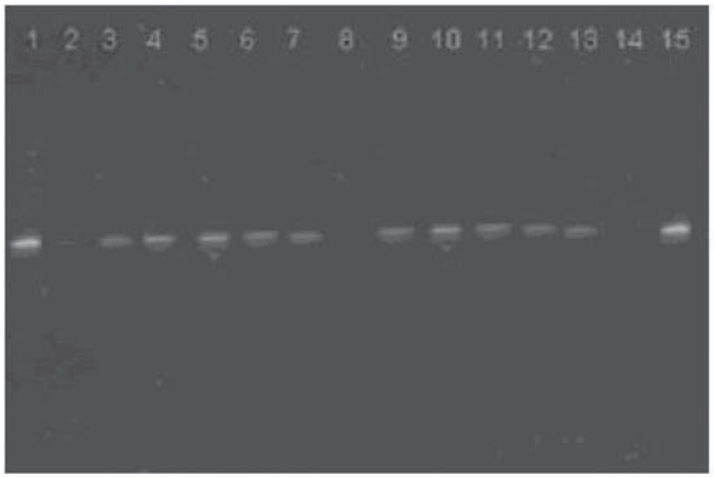

Fig. 1: Denaturation gradient gel electrophoresis of 16S V2-V3 rDNA sequences from isolates grown on Lactitol-*Lactobacillus*-Vancomycin agar with an 8% polyacrylamide 30 to 50% denaturing gradient gel. Lane 1 and 15, pure LCS (control); lane 3,4,5,6,7 isolates from gastric, duodenum, jejunum, ileum and colon respectively(sample 1); lane 9,10,11,12,13 isolates from gastric, duodenum, jejunum, ileum and colon respectively (sample 4).

many important functions attributed to probiotics are reliant on the presence of the selected bacteria in the small bowel. For example, this segment of the gut is important due to the presence of Peyer's patches and other mucosa associated lymphatic tissues that are thought to play a critical role in immunomodulation (Perdigon *et al*., 2000; Rescigno *et al*., 2001).

Table 1: Release profile of *Lactobacillus casei* Shirota from chitosan-coated alginate-starch capsules in mice gastrointestinal tract (GIT) at different time intervals

GIT Regions	Time (h)				
	2	4	8	12	24
Stomach	ND	ND	ND	ND	ND
Duodenum	ND	$1.3 \pm 0.2 \times 10^1$	ND	ND	ND
Jejunum	ND	$2.1 \times \pm 0.6 \times 10^1$	$7.3 \pm 0.4 \times 10^3$	$4.2 \pm 0.6 \times 10^3$	$3.3 \pm 0.1 \times 10^3$
Ileum	ND	$4.1 \times \pm 1.0 \times 10^2$	$3.0 \pm 0.1 \times 10^5$	$0.7 \pm 0.5 \times 10^8$	$1.0 \pm 1.1 \times 10^8$
Colon	ND	ND	$5.4 \pm 0.9 \times 10^3$	$4.6 \pm 0.3 \times 10^4$	$2.4 \pm 1.0 \times 10^5$

ND= Not detectable

[a] Results expressed as $\log_{10}$ CFU g^{-1} of tissue samples (mean ± S.E) (n=7)

The effect of consumption of CCAS encapsulated LCS on faecal flora and immune parameters in mice

There was a significant increase ($p<0.05$) in the counts of LCS in faecal sample of mice consuming free and encapsulated bacteria during the test period reaching the level of 10^7 CFU per g of wet faeces compared to the control (Table 2). The numbers of LCS recovered from the faeces confirmed adequate percentage of LCS survives passage through gastrointestinal tract. Although not statistically significant, a concomitant increase in the LCS and total *lactobacillus* count during the test period was observed in the group fed with encapsulated bacteria compared to the one fed with free non-encapsulated bacteria. There was absence of indigenous LCS in the mice fed with control diet. This confirms the release of *L. casei* from the ca-alginate encapsulated capsules.

Table 2: Effect of feeding free and chitosan-coated alginate-starch encapsulated *Lactobacillus casei* Shirota on fecal flora (after 14 day feeding).

	Control	***Free LCS***	***Enc. LCS***	***Empty capsule***
Total anaerobes	9.1 ± 0.8	9.1 ± 0.6	9.1 ± 1.0	9.1 ± 0.1
Bacteroidaceae	8.91 ±0.27	8.39±0.08	8.44 ±0.12	8.64 ±0.14
Enterobacteriaceae	7.32 ±0.3	7.12 ±0.2	7.36 ±0.26	7.28 ±0.10
Clostridium	2.6 ±0.5	2.4 ±0.07	2.1 ±0.91	2.2 ±0.2
Lactobacillus (total)	8.9±0.1	9.0±0.04	9.7±0.09	9.0±0.07
LCS (Shirota)	ND	7.02±0.27	8.1±0.01	ND
Bifidobacteria	<3.00	4.3±0.62	4.7±0.27	<3.00
Streptococci	7.87±0.22	7.99±0.01	7.90±0.09	7.49±0.10

Coliform	8.7±0.2	8.4±0.07	8.1±0.03	8.6±0.11

ND= Not detectable

[a]Results expressed as $\log_{10}$ CFU g^{-1} of tissue samples (mean ± S.E) (n=7)

This study clearly indicates the protective effect of CCAS capsules in delivering viable bacteria to the animal intestine. Also, a significant increase in the *Bifidobacterium* count was observed in the treatment groups consuming both free and encapsulated LCS but did not have any significant effect on other faecal populations determined. The counts of *Bacteroidaceae*, *Enterobacteriaceae*, *Streptococcus*, *Clostridium* and *Enterococcus* were not significantly different in the free and encapsulated groups compared to control.

Similar results were also observed by Spanhaak *et al*. (1998). It has been suggested that an increase in the *Bifidobacterium* count may indicate a beneficial effect on the stability of the intestinal flora (Mitsuoka, 1992). Since the faecal flora may not accurately reflect the microbial composition in other parts of the GI tract, we cannot exclude the possibility of more pronounced effects of LCS administration on the microbial composition in specific parts of the ileum, caecum or colon. Results from our experiment also indicate the fact that the CCAS encapsulation does not affect the antagonistic properties of released bacteria with gut microflora.

The effect of free and CCAS encapsulated bacteria on mice Interferon gamma (IFN-γ) and Interleukin –10 (IL-10) over a period of 14 days is given in Figures 2 and 3 respectively. The alginate coating may both enhance and attenuate bacterial immunomodulation, by improving viability and by shielding immunologically active

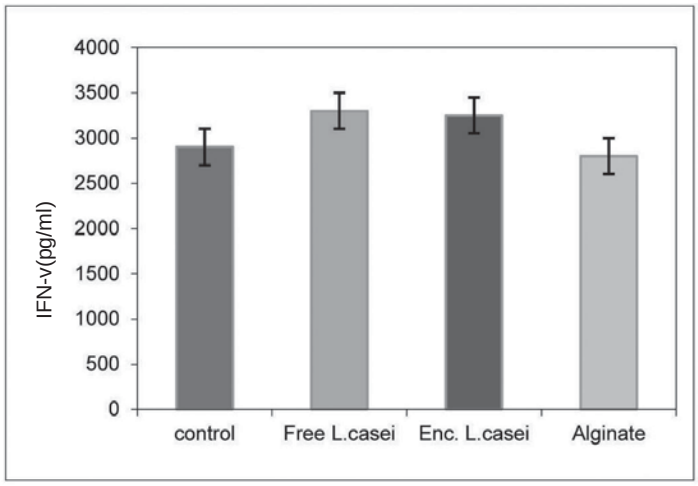

Fig. 2: Effect of free and chitosan-coated alginate-starch encapsulated *Lactobacillus casei* Shirota on mice Interferon-gamma production. The error bars represent group mean ± SD (n=7).

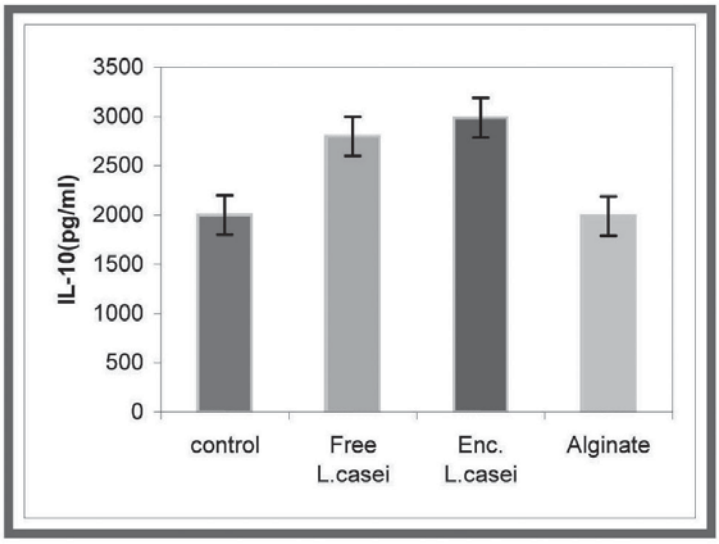

Fig. 3: Effect of free and chitosan-coated alginate-starch encapsulated *Lactobacillus casei* Shirota on mice Interleukine-10 production. The error bars represent group mean ± SD (n=7).

components of the bacteria, respectively. The aim of this experiment was also to examine the effect of feeding with free and microencapsulated LCS on the production of IFN-**γ** and IL-10 by murine splenocytes stimulated with 1.25 µg/ml ConA. These cytokines were chosen because they promote a T_h1 and a $T_h2/3$ response, respectively. The concentrations of IFN-γ were significantly different between groups fed various diet supplements. There was a tendency towards higher IFN-γ concentrations in groups fed with free or encapsulated LCS compared to control. There was no significant difference ($p<0.05$) in the amount of IFN-γ or IL-10 in the groups fed with free and encapsulated. In a study performed by Kato *et al*. (1999), mice were fed 10^9 live LCS daily for seven consecutive days, the IFN-γ production by splenocytes was found to increase significantly for mice fed LCS compared to mice fed a control diet, although the variation in IFN-γ levels between mice fed LCS was considerable. In native nasal lymphocytes from mice fed LCS the IFN-γ in response to ConA was also increased compared to control mice (Hori *et al*., 2002). Matsuzaki (1992) has reviewed studies, in which LCS has been injected intraperitoneally as a possible treatment for tumours.

In conclusion, CCAS effectively protects probiotic bacteria from adverse gastric conditions and releases them in host intestine without detrimentally affecting immunomodulatory activity of probiotic bacteria. Further studies need to be carried out using a least acid resistant probiotic strain to further confirm the efficacy of CCAS encapsulation in terms of protecting the viability as well as the immunomodulatory activity of bacteria under *in vivo* conditions.

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