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Microencapsulation of *Lactobacillus brevis* and Preliminary Evaluation of Their Therapeutic Effect on the Diarrhea of Neonatal Calf

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Abstract: Microcapsules of *Lactobacillus brevis* were prepared using spray drying technique and the resistance of the microencapsulated form of this microorganism to drying at high temperature and simulated gastrointestinal conditions were evaluated. The therapeutic effect of these microcapsules on the diarrhea of neonatal calf was also evaluated using ERIC-PCR. The results showed that the completely release of the encapsulated bacteria took place after 60 min in contact with simulated intestinal conditions. Microencapsulated bacteria were resistant at least for 90 min to simulated gastric juice (pH 1.2) or simulated intestinal juice (pH 7.2) and microencapsulated form of the microorganisms was more resistant to drying at 85°C than free form. ERIC-PCR profiles of diarrhea calves treated with probiotic capsules were similar to that of control health calves at 3 days post administration suggesting that administration of these probiotic capsules may have strong positive effect on the treatment of neonatal calf diarrhea.

Key words: Microencapsulation, *Lactobacillus brevis*, diarrhea of neonatal calf, ERIC-PCR, administration, evaluate

INTRODUCTION

Probiotics are live microorganisms that are used as dietary supplements with the aim of benefiting the health of consumers by positively influencing the intestinal microbial balance (Limdi *et al*., 2006; Yan and Polk, 2006). The beneficial effects of probiotics on the host gut flora included antagonistic effects and immune effects (Sun *et al*., 2005; Huebner and Surawicz, 2006). 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 (Qin *et al*., 2005; Hou *et al*., 2007). Lactic Acid Bacteria (LAB) are the most important probiotic microorganisms typically associated with the host gastrointestinal tract (Thanantong *et al*., 2006). These bacteria are Gram-positive, rod-shaped, non-spore-forming, catalase-negative organisms that are devoid of cytochromes and of non-aerobic habit but are aero-tolerant, fastidious, acid-tolerant and strictly fermentativel; lactic acid is the major end-product of sugar fermentation (Naidu *et al*., 1999; Ishida-Fujii *et al*., 2004; Sepova *et al*., 2008). However, the ability of probiotic microorganisms to survive and multiply in the host strongly influences their probiotic benefits. Some microbiological analyses have confirmed that probiotic strains exhibit poor survival in traditional probiotic foods (Agarwal *et al*., 2001). The bacteria should be metabolically stable and active in the product, survive passage through the upper digestive tract in large numbers and have beneficial effects when in the intestine of the host. Microencapsulation is a process by which particles are formed containing an active ingredient covered by a layer of another material which provides protection and controlled liberation as well as convenience to the ingredients (Martoni *et al*., 2008). Several reports have focused on the utilization of coacervation methods to coat probiotic strains with calcium alginate and have documented different degrees of success (Favaro-Trindade and Grosso, 2002; Oliveira *et al*., 2007).

Entrapment in calcium alginate beads has been frequently used for the immobilization of lactic acid bacteria because of its easy handing, nontoxin nature and low cost (Muthukumarasamy and Holley, 2006). It was demonstrated that survival of bacteria entrapment in calcium alginate beads depends on the several factors including alginate concentration and bacteria species (Lee and Heo, 2000; Urbanska *et al*., 2007). The aims of this study were to microencapsulate the probiotic microorganisms *Lactobacillus brevis* and to investigate whether the material used as coating, afford an increase

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on strain survival under simulated gastrointestinal conditions and high temperature. Furthermore, the therapeutic effect of prepared capsules on the diarrhea of neonatal calf was also evaluated using ERIC-PCR methods.

MATERIALS AND METHODS

Bacterial strains and culture conditions: *Lactobacillus brevis* (La-11) was isolated from pig feces at the Microbial Ecology Laboratory of Northwest A and F University, Yangling. La-11 strains were selected by their probiotic properties: resistance to simulated gastric medium pH 1.2 and artificial intestinal medium pH 7.4 as well as inhibition of specific pathogen microorganisms. La-11 strains were kept at -20°C in MRS broth medium. La-11 were activated and grown in broth medium. Overnight cultures were harvested by centrifugation washed and resuspended in Phosphate Buffer (PBS) to a final concentration of 10^{12} CFU mL^{-1}.

Preparation of microcapsules: The materials used to obtain alginate capsules were 2% sodium alginate sterile solution, a probiotic bacteria suspension in non fat milk (initial cell load 10.44±0.11), 3% calcium chloride and 2% chitosan.

Efficacy of cellular release from capsules: To determine the complete release of encapsulated bacteria, 1 g capsule were resuspended in 100 mL^{-1} simulated stomach or intestine solution followed by gentle shaking at 37°C for their dissolution. To evaluate the level of released viable bacteria, samples taken at different time intervals were estimated by detection the absorbation at 680 nm.

Survival of encapsulated bacteria in simulated gastrointestinal conditions: To determine the resistance of viable of bacteria in capsules to gastrointestinal conditions, 1 g capsule were placed in 9 mL^{-1} of an acid solution (pH 2.5) that simulates stomach conditions (1.0 M NaOH 0.5 mL^{-1}, pepsin 10 g L^{-1} and 0.1 M HCl 16.4 mL^{-1} and in 9 mL^{-1} of a stimulated intestinal medium (pH 6.8) containing KH_2PO_4 6.8 g L^{-1}, 0.2_N NaOH 250 mL^{-1} and pancreatin 10 g L^{-1}, respectively (Ding and Shan, 2007). Thereafter, samples of capsules taken each 30 min during 2 h were rinsed in sterilized saline solution, drying and dissolved using release solution (0.1 M Na_2HPO_4, 0.05 M citric acid, pH 7.25). Counts of viable bacteria in capsules were determinate by 10 fold dilution series spreading on MRS agar and incubation at 37°C for 24 h.

Survival of encapsulated bacteria in high temperature conditions: The resistance of viable bacteria of microencapsulated form versus free form to high temperature was also compared. Briefly, 1 g capsule placed in 9 mL^{-1} of release solution (free form) or in 9 mL^{-1} of sterilized water (microencapsulated form) were dried at 85°C for 1-3 min. Counts of viable bacteria in capsules were determinate by 10 fold dilution series methods.

Evaluation of therapeutic effect of alginate microcapsules on the diarrhea of calf using ERIC-PCR: To evaluate the therapeutic effect of alginate microcapsules on the diarrhea, structural features within intestinal microbial communities of calf suffering from diarrhea were analysed by using Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR as described previously (Cao *et al*., 2008). Briefly, three calves suffering from acute diarrhea were daily inoculated with capsules (10 g/each calf) prepared in this study. After inoculation, faecal samples were collected at 1-3 days post inoculation (p.i.) and put into 80% glycerol and stored at -70°C before DNA extraction. The faecal samples of diarrhea calves that did not treated with capsules were collected synchronously.

Total DNA extraction procedure modified was used to prepare the DNA templates for ERIC-PCR reactions to ensure that stable, representative and reproducible fingerprints were obtained (Wei *et al*., 2004).

Statistical analysis: Data was analyzed by Kruskal-Wallis one-way Analysis of Variance (ANOVA) using commercially available software (SPSS 12.0, Chicago, IL, USA). Results were logarithmically transformed to obtain geometric means. Comparisons of means were conducted using student's t-test or a Mann-Whitney rank sum test. Results of all statistical analyses were considered significant only if $p<0.05$.

RESULTS

Efficacy of cellular release from capsules: The results of released viable bacteria from sodium alginate capsules are detected by light transmittance measurement. As shown in Fig. 1, the OD_{680} values of capsules increased slightly after 60 min in contact with simulated stomach conditions and then remained unaltered.

However, the OD_{680} values of simulated intestinal solution increased dramatically at this time point and remained elevated until the terminal of experiment (120 min). The results indicated that the complete release of the encapsulated bacteria in simulated intestinal

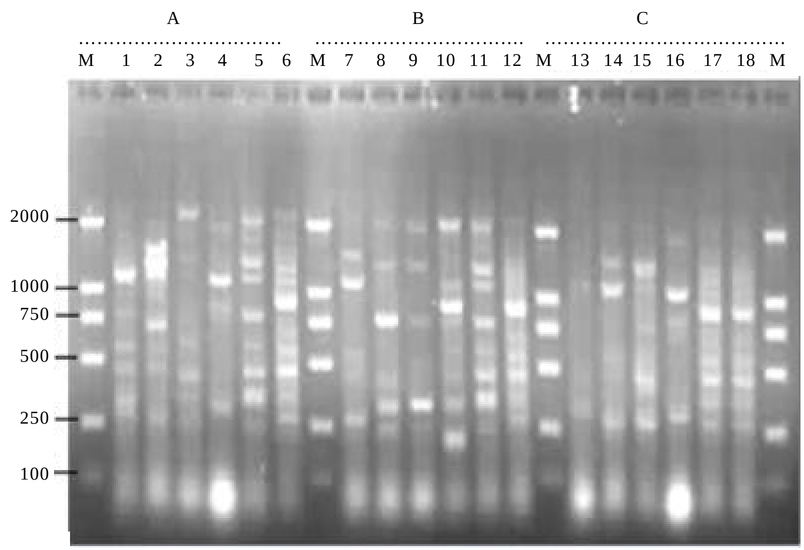

Fig. 1: Efficacy of cell release from calcium alginate capsules. Each point represents the level of released viable bacteria of *Lactobacillus brevis* capsules after contact with simulated gastrointestinal conditions

Table 1: Survival of bacteria in capsules under simulated gastrointestinal conditions

Time (min)	Control (CFU g^{-1})	Capsules (CFU g^{-1})
Simulated gastric juice (pH 1.2)		
0	2.11×10^{9a}	1.77×10^{9a}
30	4.32×10^{5b}	1.51×10^{9a}
60	2.99×10^{4b}	8.81×10^{8b}
90	3.42×10^{4b}	8.37×10^{8b}
Simulated intestinal juice (pH 7.2)		
0	4.24×10^{9a}	2.62×10^{9}
30	5.7×10^{6b}	2.57×10^{9}
60	4.62×10^{5b}	1.89×10^{9}
90	3.79×10^{5b}	1.76×10^{9}

The data represents the mean viable bacteria obtained from three duplicate of each sample collected at 30 60 and 90 min post contact with simulated gastric juice or simulated intestinal medium and means obtained at 0 min express initial cell load. Means with different superscripts letters differ significantly ($p<0.05$)

conditions took place at 60 min post contact. Correspondingly, there was no or less release of the encapsulated bacteria in simulated stomach conditions.

Survival of encapsulated bacteria in simulated gastrointestinal conditions: The survival of viable bacteria in capsule under simulated gastrointestinal conditions was shown by Table 1. The viability of bacteria in capsule showed a significant declination only at 60 min in contact with a simulated gastric juice. Other experience alginate capsules were exposed to simulated intestinal juice. It was not observed a significantly decrease on the number of viable bacteria in capsule within 90 min in contact with the medium, so sodium alginate capsules protected probiotic bacteria against an unfavorable environment.

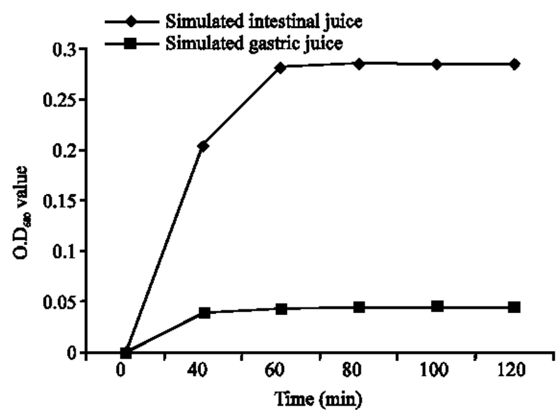

Fig. 2: Resistance of bacteria in capsules under high temperature. Each point represents the cell count of *Lactobacillus brevis* of microencapsulated from or free form at different time after drying at 85°C

Survival of encapsulated bacteria in high temperature conditions: The results of the resistance of encapsulated bacteria to high temperature conditions are shown in Fig. 2. The results showed that the viability of bacteria in capsule declined slightly after drying at 85°C for 3 min.

However, the viability of bacteria of free form decreased markedly. The results in this study indicated that the resistance of viable bacteria to high temperature was improved by sodium alginate encapsulation.

Effect of alginate microcapsules on the diarrhea of calf: The ERIC-PCR fingerprints analysis showed that the

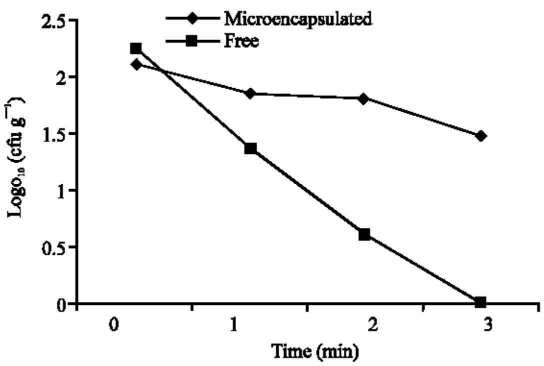

Fig. 3: ERIC-PCR fingerprints analysis of effect of capsules on the diarrhea of calf. A, B and C denotes three pairs of diarrhea calves that including treated with capsules and did not treated each pair and arabic numerals represents ERIC-PCR fingerprints of faecal samples of diarrhea calves at different time post administrated with capsules (3-5, 9-11, 15-17) or did not treated with capsules (1 and 2, 7 and 8, 13 and 14). 6, 12 and 18 represents control health calves. 1, 3, 7, 9, 13 and 15 represents 1 day after exhibiting diarrhea signs, 4, 10 and 16 represents 2 day after exhibiting diarrhea signs, 2, 5, 8, 11, 14 and 17 represents 3 day after exhibiting diarrhea signs

intestinal microbial community of the calves suffering from diarrhea was different from that of control healthy calves as shown in Fig. 3. The numbers of the band formed by ERIC-PCR with faecal samples of diarrhea calf were significantly less compared with that of healthy calf.

However, the band numbers of faecal samples of diarrhea calf treated with probiotic capsules increased gradually until which similar to that of control healthy calf at 3 days post administration. Furthermore, there are fthe major bands (about 300, 450, 550 and 900 bp) were detected in each faecal sample of healthy calf and two major bands (about 300 and 1100 bp) were observed in each faecal sample of diarrhea calf. It is interested to note that three major bands with sizes of 300, 450 and 550 bp observed in faecal sample of diarrhea calf at 3 days post inoculated with capsules were consistent with that of health calf (Fig. 3).

DISCUSSION

The encapsulation consists in a provision of an outer layer to protect the core material from damage. Microencapsulating in calcium alginate, now a days is being used to bacteria immobilization owing to its easy handing, nontoxic nature and low cost (Urbanska *et al*., 2007). In this study, the conventional encapsulation method with sodium alginate in calcium chloride has been used to encapsulate *Lactobacillus brevis* to protect this organism from the harsh gastrointestinal conditions. It was found in this study that the cell count of survival bacteria in capsule was reduced only one log cycles obtaining 10^8 cfu g^{-1} after the contact with simulated gastric juice (pH 1.2). Importantly, there was no significant difference of the number of survival bacteria in capsule after exposure in simulated intestinal juice (pH 7.2). The results suggested that microencapsulating technique could protect probiotic bacteria against unfavorable environment allowing cells get viable to the intestinal tract.

These results is consistent with previous reports of calcium alginate encapsulation could be a good way to administrate these beneficial microorganisms orally as the probiotic microencapsulating improved bacteria survival (Adhikari *et al*., 2000). The calcium alginate capsules were able to release microorganisms in a progress way and to protected them from the environmental damage (Lyer *et al*., 2005). The results presented in this study showed that capsules were completely dissolved under simulated intestinal conditions at 60 min post contact, releasing living cells into the intestinal tract. However, there was no or less release of the encapsulated bacteria in simulated stomach conditions. These combined data showed that the calcium alginate encapsulation is a good alternative to protect probiotic bacteria, so it could be a useful way to deliver these beneficial microorganisms to host.

CONCLUSION

Neonatal diarrhea is one of the main causes of calf death worldwide and also of financial loss in the cattle industry. The crowding of animals originating from different locations is one of the most common factors leading to the spreading of pathogens resulting in changes of the intestinal microflora and subsequently, the appearance of diseases (Von Buenau *et al*., 2005). Disorders of the intestinal tract were frequently treated with viable nonpathogenic bacteria to change or replace the intestinal microflora (Delia *et al*., 2007; Guarino *et al*., 2009). Today, probiotic treatment is increasingly becoming the focus of clinical interest (Mullowney and Patterson, 1985; Abe *et al*., 1995; Ewaschuk *et al*., 2004). In the studies presented here, the effect of the probiotic microencapsulating on therapy of neonatal calf under field conditions was investigated using ERIC-PCR methods. The analysis of ERIC-PCR fingerprints showed that the administration of microencapsulation of *Lactobacillus brevis* had a strong beneficial effect on the replacement of the intestinal microflora of diarrhea calves. ERIC-PCR profiles of fecal samples from three diarrhea calves were distinguished from that of control health calves. However, ERIC-PCR profiles of diarrhea calves treated with probiotic capsules changed until similar to that of health calves. These findings seem to suggest that administration of the probiotic capsules may have some positive effect on the treatment of neonatal calf diarrhea.

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