Microencapsulation of Probiotic Strains for Swine Feeding

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Probiotics are live microorganisms which have health-promoting attributes. These bacteria must overcome biological barriers, including acid in the stomach and bile in the intestine to exert beneficial effects. The encapsulation consists in a provision of an outer layer to protect the core material from damage. Microencapsulating in calcium alginate, nowadays, is being used to bacteria immobilization owing to its easy handling, nontoxic nature, and low cost. The aims of this study were to improve the microencapsulating method for probiotic bacteria and to investigate whether the material used as coating, afford an increase on strain survival under simulated gastrointestinal conditions. Lactic acid bacteria used in this work were isolated from feces of young and healthy pigs and they were selected because of their probiotic properties. Our results showed that the optimal encapsulation process was achieved using 1 : 1 (v/v) 20% non fat milk cell suspension mixed with 1.8% sodium alginate solution. Alginate capsules hardening was carried out using 0.1 M calcium chloride solution for 30 min. This microencapsulating technique could protect the probiotic bacteria against gastric environment, allowing viable cells get to the intestinal tract. So it could be a useful way to deliver these beneficial bacteria to host.

Key words probiotic; microencapsulating; simulated gastrointestinal condition

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.[1—3)]

These bacteria must overcome biological barriers, including acid in the stomach and bile in the intestine to exert beneficial effects and they must have some properties such as colonization, immunomodulation, lowering cholesterol, improving lactose tolerance, cancer prevention.[4—6)]

Food or pharmaceutical preparations could contain probiotic strains but the number of viable cells in probiotic products has often been questionable. Some microbiological analyses of these products have confirmed that probiotic strains exhibit poor survival in traditional probiotic foods.[7)] Microbial behavior in food is largely governed by food characteristics (water availability, buffering capacity) and storage conditions.[8,9)]

Several reports have focused on the utilization of coacervation methods to coat probiotic strains with calcium alginate and have documented different degrees of success.[10,11)] Entrapment in calcium alginate beads has been frequently used for the immobilization of lactic acid bacteria because of its easy handling, nontoxic nature, and low cost.[12)] It was demonstrated that survival of bacteria entrapped in calcium alginate beads depends on several factors including alginate concentration and bacterial species.[13)]

Alginic acid is an anionic polysaccharide, which consists of α-L-guluronic acid (G) and β-D-mannuronic acid (M) subunits. Three blocks, the G-block (GB), the M-block (MB) and the MG-block are present in the alginate molecule, and these are closely associated with the structure of calcium-induced alginate gel (alg-Ca), which is formed in the presence of calcium. GB is particularly liable to form a Ca_2-chelate and alginate with a low m/g ratio generally forms the cured gel matrix.[14)]

Sodium alginate, in solution, gels in contact with calcium ions by cross-linking.[15)] Calcium-induced alginate gel beads are able to incorporate some compounds such as drugs or polysaccharides in the gel matrix.[16)] The beads have been used in various ways in the gastrointestinal tract, for example, for sustained release of drugs or to adsorb bile acid.[17)]

The application of these methodologies to improve probiotic survival, in food and during the gastrointestinal transit, is relatively new.[18)] A prerequisite for any effect of ingested bacteria is their successful implantation in the gastrointestinal tract, so bacteria must remain viable during gastric transit. This concept justifies the consumption of probiotic lactic acid bacteria included in foods or protected by encapsulation.[19,20)]

The aims of this study were to improve the microencapsulating method for probiotic bacteria by optimizing the encapsulation parameters and to investigate whether the material used as coating polymer afforded an increase on probiotic strains survival under simulated gastrointestinal conditions.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions Lactic acid bacteria (LAB) were isolated from pig feces at the Technological Ecophisiology Laboratory of CERELA, and the Public Health's Laboratory of Tucumán National University, Argentine.

LAB strains were selected by their probiotic properties: resistance to gastrointestinal conditions (LAB strains were kept at a simulated gastric medium, pH 1.2, during 2 h and then they were in contact with an artificial intestinal medium pH 7.2, for 30 min. Cell counts were determined by plating on Man–Rogosa–Sharpe (MRS) agar after incubation at 37 °C during 48 h), and inhibition of specific pathogen microorganisms such as *Salmonella cholerasuis*, *Salmonella typhimurium*, *Yersinia* sp. and *Proteus* sp. (Agar diffusion; broth dilution method and electron microscopy, were used to

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characterize the antimicrobial activity.)

LAB strains were kept at −20 °C in MRS broth[21] containing 30% glycerol (v/v). LAB were activated and grown in MRS medium.

Overnight cultures were harvested by centrifugation, washed and resuspended in phosphate buffer (PBS), (pH 7, 0.1 M), to a final concentration of 10^9 CFU/ml.

Microencapsulation The calcium alginate microencapsulating method, outlined by Chandramouli V. *et al.* (2004), was modified.

The materials used to obtain alginate capsules were: 1.8% sodium alginate sterile solution, a probiotic bacteria suspension in non fat milk (initial cell load 9.48±0.03) and 0.1 M calcium chloride solution (hardening solution).

The alginate mixture was prepared by mixing 1.8% (w/v) sodium alginate solution and non fat milk viable bacteria suspension.

Different percentages of non fat milk (10, 20%) and 1% EDTA were tested to optimize the encapsulation process. Capsules were prepared aseptically by dropping the alginate mixture, in 500 ml of 0.1 M calcium chloride sterile solution under gentle stirring. Calcium ions cross-linked sodium alginate to form calcium alginate microparticles.

The microcapsules obtained were hardened in 0.1 M $CaCl_2$ solution. The calcium alginate beads were rinsed twice with distilled water.[22]

Bacterial cell suspension in PBS 10% (w/v) was used as a control solution.

Efficacy of Cellular Release from Capsules To determine the complete release of encapsulated bacteria, samples were taken at different time intervals.

To evaluate the number of released viable bacteria, 5 capsules were resuspended in PBS (0.5 ml), using gentle shaking at room temperature for their dissolution. Cell counts were determined by plating on MRS agar, after incubation at 37 °C during 48 h.

Survival of Encapsulated Bacteria in Simulated Gastrointestinal Conditions The number of viable of bacteria in capsule *versus* viable bacteria in PBS (control) in gastric and intestinal conditions was compared. Control samples were 500 μl of cell suspensions in PBS (10^9 CFU/ml).

Counts of viable bacteria in capsules and control cell suspensions were determinate by spreading on MRS agar and incubation at 37 °C for 48 h.

(a) Gastric conditions: 20 capsules were placed in 2 ml of an acid solution (pH 1.2), that simulates gastric conditions (NaCl 2 g/l, pepsin 3.2 g/l, and HCl 7 ml).[23] Samples of 5 capsules were taken each 30 min during 2 h. The same procedure was carried out keeping in contact 2 ml of control cell suspension with the acid solution removing 500 μl of this suspension each 30 min.

(b) Intestinal conditions: 20 capsules were placed in 2 ml of a simulated intestinal medium (pH 7.2) containing KH_2PO_4 6.8 g/l, 0.2 N NaOH 250 ml, and pancreatin 10 g/l.[23] Samples of 5 capsules were taken each 15 min during 45 min. The same procedure was carried out keeping in contact 2 ml of control cell suspension with the intestinal medium taking 500 μl of this suspension each 15 min.

(c) In other experience, 5 capsules were placed in the simulated gastric solution during 2 h and then they were transferred to the simulated intestinal solution, until their complete dissolution. As a control sample, 500 μl of control cell suspension were placed in simulated gastric and intestinal solution as it had been described above.

Morphology Analysis Microcapsules were examined by Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM).

(a) SEM (JEOL JBM 6400): Microcapsules were mounted on metal grids using double-sided tape and coated with gold under vacuum.

(b) TEM (Zeiss EM 109 50 kV): Samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). The post-fixing was done with OsO_4 1% in the same buffer. The samples were dehydrated with increasing concentrations of acetone–alcohol. They were included on resin and then they were cut.

Statistical Analysis All experiments were carried out in duplicated. Data were represented as mean±S.D. and were submitted to one-way ANOVA analysis of variance using Minitab statistical software (version 1.4; Minitab, State College, Pennsylvania, U.S.A.).

RESULTS AND DISCUSSION

Table 1 shows the number of viable bacteria in capsules after testing different parameters (10% and 20% non fat milk) of the encapsulating process.

Skimmed milk and other chemicals compounds such as dimethylsulfoxide, glycerol, peptone, yeast extract, saccharose, glucose, methanol, polyvinylpyrrolidone, sorbitol, and malt extract have been used widely and with satisfactory results as protective additives. These substances can be added during growth of the microorganisms (viruses, bacteria, fungi, algae, and protozoa), or prior to freezing or drying. Skimmed milk at a concentration of 1—10% has often been used for the cryopreservation, but even more frequently in the freeze-drying of many microorganisms: *Leptospira interrogans*, mycoplasmas, *Pasteurella multocida*, and lactic acid bacteria.[24]

The level of cell viability after freeze drying varies according to numerous factors, including the strain of microorganism and also the efficacy of the protective agent used. Protective additives can be generally classed into two categories: amorphous glass forming, and eutectic crystallizing salts. Milk exerts its protective effect by raising the glass transition temperatures of the samples. The formation of a glassy state induces sufficient viscosity within and around a cell to arrest molecular mobility to a minimum. The inert amorphous glass is also able to retain waste products released by the cells within the glass structure prior to freezing, meaning that they are not left to concentrate and initiate irreversible electro-chemical changes on the plasma membrane during stor-

Table 1. Evaluation of the Use of Non Fat Milk to Optimize Encapsulation Process

		Log CFU/ml
Control (PBS)		4.55^{a}±0.49
Non fat milk	10%	6.26^{b}±0.06
	20%	7.34^{c}±0.38
20% non fat milk: 1.8% alginate (v/v)	1 : 1	7.59*±0.01
	1 : 2	$3.56^{\dagger}$±0.48

Mean±S.D. (n=3). Means with different superscripts differ significantly (p<0.05); initial cell load: 9.48±0.03.

age.[25)]

The use of non fat milk allowed a preservation significantly ($p<0.05$) higher of viable cells than control. The use of 20% non fat milk suspension protected bacteria better ($p<0.05$) than the use of 10% non fat milk.

Several authors have worked with different concentrations of sodium alginate (from 0.5 to 4%) as a way to find the optimal encapsulation process.[26—28)] These publications have led to different conclusions regarding the use of calcium alginate as a good matrix for bacterial cell's encapsulation.

In agreement with recent published works, uniform capsules were obtained using 1.8% alginate solution. Alginate concentrations beyond 2% (w/v) avoid uniform spherical capsule formation because of the viscosity increasing.[9)]

Sodium alginate solution 1.8% was tested at two different ratios (v/v), mixed with 20% non fat milk cell suspension. The encapsulation process using 1 : 2 non fat milk : alginate (v/v) was unsuitable because capsules could not been dissolved. These results showed that the use of a higher concentration of sodium alginate affected the dissolution of capsules significantly (Table 1).

The results that we have just obtained show that the mixture 1 : 1 (v/v) 20% non fat milk: 1.8% alginate, was the best one to use in the encapsulation process.

On the other hand, we thought that the calcium of milk could linked with alginate, becoming calcium alginate, increasing mixture viscosity and therefore affecting the encapsulating process. For this reason, we used an entrapping agent (EDTA) during the encapsulation process.

EDTA (1%) was added to non fat milk cell suspension and it was tested probiotic viability and viscosity of the new mixture. The entrapping agent did not affect cell viability and the viscosity of the suspension was not significant different (data not shown). Therefore the addition of EDTA was not necessary.

Our results showed that the optimal encapsulation process, for assayed probiotic bacteria, was achieved using 20% non fat milk cell suspension, mixed with 1.8% sodium alginate, at the ratio 1 : 1 (v/v) and 0.1 M calcium chloride as hardening solution during 30 min.

The entrapment ratio of bacteria (number of bacteria entrapped in capsule to that of bacteria used for encapsulation) was 0.86.

The counts of released viable bacteria from calcium alginate capsules are shown in Fig. 1. The complete release of the encapsulated bacteria took place after 4 h in contact with buffer. The number of released viable bacteria was similar during the following 8 h and the viable cells began to decrease at 16 h. Finally a significant reduction of cellular viability was observed at 24 h in contact with PBS, but this decrease was due to bacterial dead. The calcium alginate capsules were able to release microorganisms in a progress way and to protected them from the environmental during 16 h.

Then, the survival of viable bacteria in capsule, under simulated-gastrointestinal conditions, was determined. It was compared the survival and viability of free-probiotic bacteria (control) versus probiotic bacteria in capsule.

The viability of bacteria in capsule showed a significant declination only at 120 min in contact with a simulated gastric juice (pH 1.2), so calcium alginate matrix protected probiotic bacteria against an unfavorable environment.

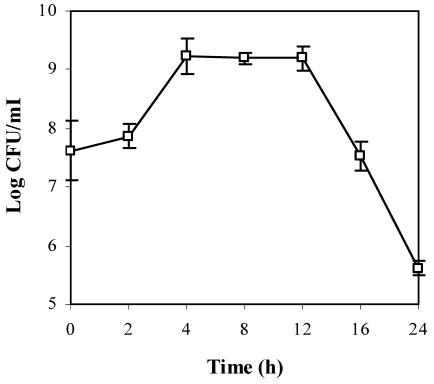

Fig. 1. Efficacy of Cell Release from Calcium Alginate Capsules
Bars indicate deviation from the mean.

Table 2. Survival of Bacteria in Capsules under Simulated Gastrointestinal Conditions

Time (min)	Control (Log CFU/ml)	Capsules (Log CFU/ml)
Simulated gastric juice (pH 1.2)		
30	$5.07^{a}\pm0.78$	$8.48^{b}\pm0.13$
60	$4.57^{a}\pm0.04$	$8.28^{b}\pm0.16$
90	$4.39^{a}\pm0.01$	$8.2^{b}\pm0.08$
120	$4.82^{a}\pm0.01$	$7.26^{c}\pm0.08$
Simulated intestinal juice (pH 7.2)		
15	$6.00^{a}\pm0.03$	$8.29^{c}\pm0.11$
30	$5.93^{a}\pm0.02$	$8.07^{c}\pm0.14$
45	$4.78^{b}\pm0.19$	$8.04^{c}\pm0.24$
Sequential exposure (pH 1.2 and pH 7.2)		
45	$3.75^{a}\pm0.25$	$7.63^{b}\pm0.03$

Mean±S.D. ($n=3$). Means with different superscripts letters differ significantly ($p<0.05$); initial cell load 9.5±0.02.

In other experience alginate capsules were exposed to simulated intestinal juice (pH 7.2). It was not observed a significantly decrease on the number of viable bacteria in capsule within 45 min in contact with the medium.

The viability of the encapsulated bacteria under simulated gastric and intestinal conditions was significantly better than control (Table 2).

The survival of probiotic bacteria in capsule after a sequential exposure in simulated gastric juice (pH 1.2) and simulated intestinal juice (pH 7.2), was also evaluated. It was found that the cell count was reduced only two log cycles, obtaining 7.63 CFU/ml after the sequential contact (Table 2).

Our results suggested that microencapsulating technique could protect probiotic bacteria against gastric environment, allowing cells get viable to the intestinal tract.

The results, here presented, support the data obtained by others authors, whose demonstrated that the probiotic microencapsulating improved bacteria survival.[29,30)] So calcium alginate encapsulation could be a good way to administrate these beneficial microorganisms orally.

Probiotic bacteria in calcium alginate beds were analyzed by Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM).

TEM shows the probiotic bacteria inside the capsules. Cells are surrounded by granules from milk suspension used in the encapsulation process (Figs. 2A, B).

It can be observed the viability and integrity of bacteria in capsule. TEM shows the excellent conservation of bacterial

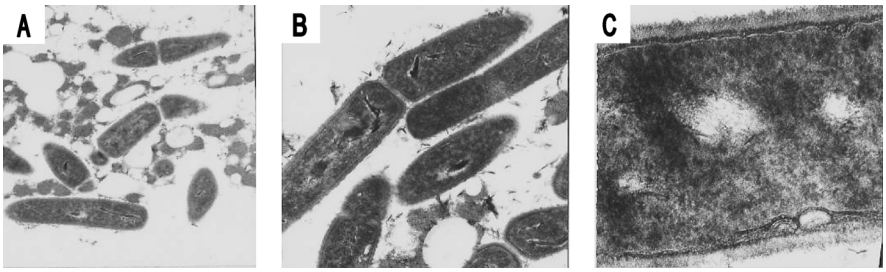

Fig. 2. Transmission Electron Microscope (TEM): (A and B) Viable Bacteria in Capsule Surrounded by Granules of Matrix (Magnificence 18700× and 33300× Respectively), (C) Viable Bacteria in Capsule, Its Structure Stayed Regular after the Encapsulating Process (Magnificence 140600×)

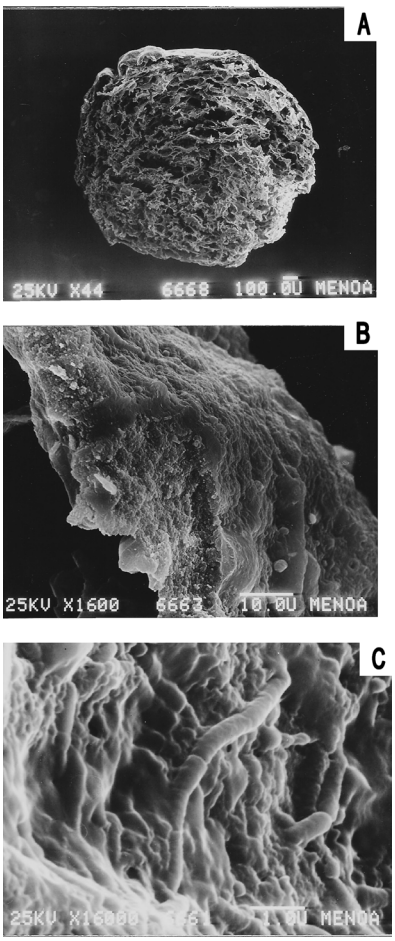

Fig. 3. Scanning Electron Microscope (SEM): (A) Alginate Capsule's Morphology (Magnificence 44×), (B) Bacteria Linked on Capsule's Surface (Magnificence 1600×), (C) Probiotic Viable Bacteria in Calcium Alginate Matrix (Magnificence 16000×)

structures, there was not any kind of cell injury during encapsulation process (Fig. 2C).

The shape and morphology of the alginate capsules was analyzed by SEM. Capsules are uniform and spherical, 1.400 μm diameter aprox., and they present a great number of pores inside. (Fig. 3A). It can be observed that there are bacteria linked on the capsules surface. (Fig. 3B).

Figure 3C shows probiotic bacteria fixed to alginate matrix.

In the present work data have showed that living cells were successfully encapsulated into alginate microcapsules and they could preserve their viability.

Calcium alginate acted as a barrier against gastric juice and intestinal conditions.

Capsules were completely dissolved under simulated intestinal conditions, releasing living cells into the intestinal tract.

All these results 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.

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