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Properties of *Lactobacillus reuteri* chitosan-calcium-alginate encapsulation under simulated gastrointestinal conditions

Hui-Ying Huang,[1¶] Yi-Ju Tang,[1¶] V. An-Erl King,[2] Jen-Wei Chou,[3] Jen-Horng Tsen[1*]

[1]Department of Nutrition, China Medical University, Taichung, Taiwan, ROC. [2]Department of Food Science and Biotechnology, National Chung-Hsing University, Taichung, Taiwan, ROC. [3]Division of Gastroenterology and Hepatology, Department of Internal Medicine, China Medical University Hospital, College of Medicine, China Medical University, Taichung, Taiwan, ROC

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Summary. The protective effects of encapsulation on the survival of *Lactobacillus reuteri* and the retention of the bacterium's probiotic properties under simulated gastrointestinal conditions were investigated. Viable counts and the remaining probiotic properties of calcium (Ca)-alginate encapsulated (A group), chitosan-Ca-alginate encapsulated (CA group), and unencapsulated, free *L. reuteri* (F group) were determined. Encapsulation improved the survival of *L. reuteri* subjected to simulated gastrointestinal conditions, with the greatest protective effect achieved in the CA group. The degree of cell membrane injury increased with increasing bile salt concentrations at constant pH, but the extent of injury was less in the encapsulated than in the free cells. Adherence rates were, in descending order: CA (0.524%) > A (0.360%) > F (0.275%). *Lactobacillus reuteri* cells retained their antagonistic activity toward *Listeria monocytogenes* even after incubation of the lactobacilli under simulated gastrointestinal conditions. Displacement of the pathogen by cells released from either of the encapsulation matrices was higher than that by free cells. The safety of *L. reuteri* was demonstrated in an in vitro invasion assay. [**Int Microbiol** 2015; 18(1):61-69]

Keywords: *Lactobacillus reuteri* · *Listeria monocytogenes* · chitosan–calcium-alginate encapsulation · probiotic properties · simulated gastrointestinal conditions

Introduction

Most probiotics belong to strains of lactic acid bacteria (LAB) and their positive effects on human health are well established [40]. These benefits of LAB include: balancing gut microbiota [38], inhibiting infection by pathogens [11], lowering blood cholesterol [20], and reducing the risk of colon cancer [19]. For probiotics to exert these and other beneficial effects, they must pass safely through the gastrointestinal tract and then adhere to and colonize the intestinal canal [42]. However, the survival of many microbes exposed to gastric acid and bile salts is poor.

Using encapsulation to immobilize LAB cells can improve their survival under adverse conditions [45]. Encapsulation offers many advantages for the encapsulated cells, including the maintenance of stability, activity, and high volumetric productivity, the improvement of process control, protection against damage, and a reduced susceptibility to contamination [25,41]. Several encapsulation techniques to improve the survival of microorganisms in dairy products [35] and artificial gastrointestinal juice [10] have been tested, with

*Corresponding author: J.-H.Tsen
Department of Nutrition
China Medical University
91, Hsueh-Shih Road
Taichung, 404 Taiwan, ROC
E-mail: jhtsen@mail.cmu.edu.tw

¶These authors contributed equally to the work.

alginate encapsulation as one of the most successful [18]. The encapsulation of LAB in Ca-alginate beads improved the survival of the cells under harsh conditions [25,31]. Chitosan has also been used as the encapsulation material, especially for drug delivery in the gastrointestinal tract, based on its absorption-enhancing, controlled-release, and bioadhesive properties [8]. Chitosan is obtained from chitin via *N*-deacetylation and the immersion of Ca-alginate beads in chitosan solution results in the formation of an outer, protective membrane [21]. Many studies have shown that encapsulation of LAB in a chitosan-alginate complex is effective in reducing the decline of viable cells exposed to simulated gastric and intestinal juice and improves their survival during refrigerated storage [2,22,24].

In addition to tolerating gastric acid and bile salts, probiotics must be able to adhere to gut surfaces, colonize the gut together with the resident microbiota, and compete with pathogens [11]. The probiotic traits of LAB with respect to gastric acid and bile tolerance, adhesiveness, and competition with pathogens have been investigated [13,31,49] but these studies often failed to examine the influence of sequential acid and bile salt exposure, as occurs under physiological conditions in the human gastrointestinal tract [25]. Moreover, few studies have considered the remaining probiotic properties of LAB cells after their exposure to simulated gastrointestinal conditions. In a previous work [16], we found that the exposure of LAB strains to low pH followed by high concentrations of bile salts led to a decline in cell survival. In addition, there was a loss of adhesiveness of viable LAB, perhaps due to the cell injury caused by the harsh conditions. LAB strains that survived sequential incubations at pH 4 and 0.1% bile salt had higher adherence rates but a slightly lower rate of pathogen displacement than an unexposed LAB strain. In this study, *Lactobacillus reuteri* was encapsulated in Ca-alginate and chitosan-Ca-alginate and the protective effects of encapsulation on the survival of cells exposed to sequential acid and bile treatments was evaluated and compared. In addition, we assessed the adhesiveness, safety, and displacement of *Listeria monocytogenes* by free and encapsulated bacteria subjected to simulated gastrointestinal conditions.

Materials and methods

Bacterial strains and culture conditions. *Lactobacillus reuteri* BCRC 14625 and *Listeria monocytogenes* BCRC 14847 were purchased from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute at Hsinchu, Taiwan. *Lactobacillus reuteri* was grown in de Man, Rogosa and Sharpe (MRS) broth or on MRS agar medium (Difco). *Listeria monocytogenes* was cultured with Bacto brain heart infusion (BHI; Difco). Both strains were incubated at 37ºC and propagated under microaerophilic conditions. The stock cultures were preserved at –80ºC in 20% glycerol.

Encapsulation of *Lactobacillus reuteri*. Bacteria grown at 37ºC for 24 h were centrifuged at 4ºC, 8000 ×*g* for 10 min. The harvested cell pellets were washed and then resuspended in phosphate-buffered saline (PBS). The bacterial suspension was mixed with an equal volume of 2% (w/v) sodium alginate (Na-alginate) solution to obtain a cell suspension containing 1.0–9.9 × 10^8 colony-forming units (CFU)/ml. The solution was transferred dropwise into 0.1 M $CaCl_2$ through the tube of a peristaltic pump, resulting in the formation of calcium alginate (Ca-alginate) beads containing *L. reuteri* cells (A group). A suspension of LAB mixed with an equal volume of H_2O served as the control free cell group (F group). The chitosan-Ca-alginate beads used to encapsulate *L. reuteri* cells were prepared as previously described [23]. Briefly, 0.4 g of chitosan (Sigma) was dissolved in 90 ml of distilled water (DW) previously acidified with 0.4 ml of glacial acetic acid. The final concentration of chitosan solution was first adjusted to 0.4% (w/v) and then to pH 5.7–6.0 with 1 N NaOH. The chitosan solution was then filtered and the volume was increased to 100 ml with DW. Ca-alginate beads containing *L. reuteri* prepared as described above were then immersed in the 0.4% chitosan solution and shaken at 100 rpm for 1 min to produce chitosan-coated-Ca-alginate beads (CA group). The interactions of the three groups with the pathogen *List. monocytogenes* were assessed in wells containing Caco-2 cells and pre-adhered, FITC-labeled *List. monocytogenes*. The adherence rate of the pathogen under LAB-free conditions was designated as 100%.

Microscopic observation of bead structure. The microstructures of the beads and the encapsulated LAB were examined using scanning electron microscopy [34]. The Ca-alginate and chitosan-Ca-alginate beads were fixed with 2.5% glutaraldehyde at 4ºC for 16 h, washed with PBS, and dehydrated in an ethanol gradient (30, 50, 70, 80, 85, 90, 95, and 100% ethanol, each for 15 min). The beads were then dried by critical point drying, coated with gold, and observed in a JEOL, JSM-7401F (Japan) scanning electron microscope.

Tolerance of simulated digestive juice. The initial bacterial counts were obtained as follows: for the F group, a previously prepared *L. reuteri* suspension was mixed with an equal volume of aseptic water. For the A and CA groups, the *L. reuteri* suspension was mixed with an equal volume of Na-alginate solution. The number of cells in 1 ml of each of the three suspensions was counted after 24–48 h of incubation at 37ºC using the standard plate count (SPC) method. The data were expressed as the log CFU/ml. The initial LAB counts were expected to be the same in the free and encapsulated samples. One ml of free *L. reuteri* cells for the F group and gel beads prepared from 1 ml of an equal volume LAB cells and Na-alginate solution for the A and CA groups (~40 gel beads for each encapsulation group) were added to 10 ml of 0.1% peptone water adjusted to pH 2–3 [11]. The samples were shaken at 150 rpm at 37°C for 3 h. The acid-treated free cells were centrifuged (25°C, 8000 ×*g*, 10 min) and the pellets then dissolved in 1 ml of aseptic water. Acid-treated beads of the A and CA groups were collected and drained dry. The dried beads and 1 ml of acid-treated free cells were then transferred to 0.1%, 0.5%, or 1% oxgall bile (Sigma) and shaken as described for the acid treatment. After bile treatment, the free cells and gel beads were treated as previously described and then transferred to 10 ml of simulated colon fluid (0.1 M KH_2PO_4, pH 7.4 ± 0.2), with shaking for 1 h. Under these conditions, the gel beads depolymerized, releasing encapsulated *L. reuteri*. Both the free and the released cells were centrifuged, re-suspended in 1 ml of aseptic water, and the number of viable cells was determined.

β-Galactosidase activity of *Lactobacillus reuteri* after simulated digestive juice treatment. The β-galactosidase activity of *L. reuteri* after simulated digestive juice treatment was assayed as described previously [32]. Both free and encapsulated LAB cells were treated with acid solutions of pH 2 or pH 3, then with 0.1%, 0.5% or 1% bile salt solutions, each for 3 h, and then with simulated colon fluid for1 h. The free cells and the cells released from the beads were collected by centrifugation, washed, and re-dissolved in 1 ml of aseptic water. The cell suspensions were then mixed with 4 ml of 0.005 M *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) and incubated at 37ºC for 10 min to allow color formation. After the reaction was stopped by the addition of 2 ml of 0.1 M sodium carbonate, the samples were centrifuged at 8000 ×*g* at 1°C for 10 min to precipitate and remove the cells. The absorbance of the supernatant was measured at 420 nm with a spectrophotometer (Hitachi, U-2000, Japan). The amount of *o*-nitrophenol (ONP) formed in the sample was calculated by comparison with a standard curve. β-galactosidase activity was expressed as micromoles of ONP formed per milliliter of cell suspension in 10 min.

Cell culture. The epithelial-like Caco-2 cell line BCRC 60182, originally isolated from a human colon adenocarcinoma [36], was purchased from Bioresource Collection and Research Center of the Food Industry Research and Development Institute at Hsinchu, Taiwan. The cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Biochrom AG, Germany) supplemented with 10% (v/v) heat-inactivated (56ºC, 30 min) fetal bovine serum (FBS; Biological, Israel) and 1% (v/v) penicillin-streptomycin (stock solution 100 unit/ml; BioSource, USA) and incubated at 37ºC in a CO_2 incubator (NUAIRE, NU-5500, USA) with a 5% CO_2/95% air atmosphere. Subcultured cells were seeded at a concentration of 3×10^5 cells per well in a six-well tissue culture plate. The culture medium was replaced every other day. Mature monolayers, obtained after 15 days of incubation, were used for the following assays [4].

Adhesion assay. At least 1.5 h before the adhesion assay, the DMEM medium was aspirated from the wells and the Caco-2 cell monolayer was fixed with 0.25% glutaraldehyde for 15 min followed by three washes with PBS. Fresh DMEM not containing FBS or antibiotics was added to each well [28]. After exposure to the above-described simulated gastrointestinal conditions (pH 3, 0.1% bile salt, and simulated colon fluid), 1 ml of *L. reuteri* cells released from the gel beads or the free *L. reuteri* cell suspension was added to the respective wells and incubated at 37ºC for 2 h in a 5% CO_2/95% air atmosphere. The monolayers were then washed five times with PBS to remove non-adherent LAB cells [14] and lysed with 1 ml of a 1% Triton X-100 solution (Sigma) for 5 min to release adherent bacteria. The number of adherent LAB released from the surface of the Caco-2 cells was determined by the SPC method using plates incubated for 24-48 h at 37°C [17]. The adherence rate (%) was calculated as:

$$\text{Adherence rate (\%)} = \frac{\text{No. of adherent LAB (CFU/ml)}}{\text{No. of LAB added to each well (CFU/ml)}} \times 100\%$$

Antagonistic activity of *Lactobacillus reuteri* against *Listeria monocytogenes*. A suspension of *List. monocytogenes* cells prepared from a culture was centrifuged (25ºC, 8000 ×*g*, 10 min) to pellet the cells, which were then washed three times and resuspended in PBS. The suspension was mixed with an equal volume of 2 mg of fluorescein isothiocyanate (FITC; Sigma)/ml for 20 min to stain the bacterial cells. Excess fluorochrome was removed by centrifugation of the FITC-labeled bacterial suspension, discarding the supernatant and washing the cell pellet three times with PBS. To test the displacement effect of *L. reuteri*, 1 ml of FITC-labeled *List. monocytogenes* suspension was added to a well with a fixed Caco-2 cell monolayer and incubated for 2 h. One ml of gel-released or free LAB cells treated as described for the adhesion assay was added to a well with pre-adherent *List. monocytogenes* cells. After 2 h of incubation at 37°C, the incubation medium was discarded and the Caco-2 cell monolayer was washed five times with PBS to remove both non-adherent LAB and pathogen cells. The adherence of FITC-labeled *List. monocytogenes* cells to the Caco-2 cell monolayer was assessed with a spectrofluorometer (Bio-Tek, FLX-800, USA) by recording the fluorescence intensity at 485 nm excitation and 528 nm emission. The antagonistic activity of *L. reuteri* was expressed as the adherence rate (%) of the pathogen (non-displaced *List. monocytogenes* cells) as determined by its fluorescence intensity; that is, the greater the antagonism of *L. reuteri*, the greater the displacement of *List. monocytogenes*. The "LAB-free" control consisted of FITC-labeled *List. monocytogenes* adhered to the Caco-2 cells and not exposed to competitive LAB. The adherence rate under these conditions was defined as 100%.

Invasion assay. The in vitro safety of *L. reuteri* was evaluated in an invasion assay as previously reported [44]. The Caco-2 cell monolayer was washed twice with PBS and re-incubated for 1.5 h in fresh DMEM medium without penicillin and streptomycin. Gel-released and free LAB cells, both pre-treated with simulated gastrointestinal conditions, were suspended in antibiotic-free DMEM medium. One ml of LAB suspension was loaded into a well containing a Caco-2 cell monolayer and incubated for 2 h at 37ºC in a 5% CO_2/95% air. The medium was then aspirated and Caco-2 cells were washed three times with PBS to remove non-adherent LAB. DMEM containing 10 mg tetracycline/ml was added to the well and incubated for another 2 h to kill the adherent LAB. The Caco-2 cells were washed five times with PBS and lysed with 1% Triton X-100. Viable counts of LAB released from the Caco-2 cells were determined. The safety of *L. reuteri* was expressed as the invasion rate and calculated as follows:

$$\text{Invasion rate (\%)} = \frac{\text{No. of LAB released from Caco-2 cells (CFU/ml)}}{\text{No. of LAB added to each well (CFU/ml)}} \times 100\%$$

Statistical analysis. Statistical differences between samples were determined using Statistical Analysis System software (version 9.2, 2008). Duncan's new multiple range test, Dunnett's test of the GLM, and Student's t-test were used to determine significance, defined as a $P < 0.05$.

Results

Microscopy of the gel beads and encapsulated cells. Figure 1 shows the scanning electron micrographs of the internal and external structures of the Ca-alginate (Fig. 1A, B), the chitosan-Ca-alginate (Fig. 1C,D) gel beads and the respective encapsulated *L. reuteri* cells. Rod-shaped *L. reuteri* spread throughout the gel beads of both encapsulation matrices but the density of bacteria was lower on the surface of the chitosan-Ca-alginate beads than on the surface of the Ca-alginate beads. By more effectively encapsulating LAB cells, chitosan-Ca-alginate may better protect cells from unfavorable environmental conditions.

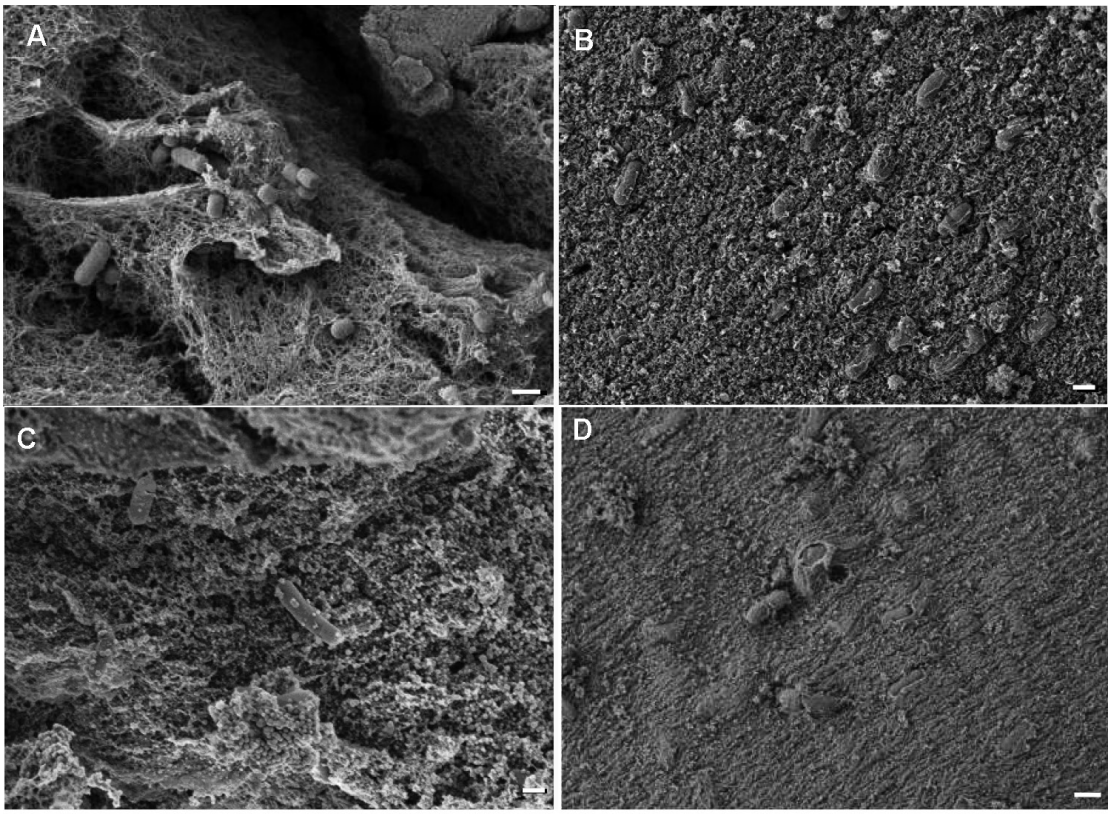

Fig. 1. Scanning electron micrograph of gel beads containing encapsulated *Lactobacillus reuteri* cells (5000× magnification). (**A**) Internal and (**B**) external structure of a Ca-alginate gel bead. (**C**) Internal and (**D**) external structure of a chitosan-Ca-alginate gel bead. Scale bar 1 μm.

Protection of *Lactobacillus reuteri* by encapsulation against simulated digestive juice. Table 1 shows the viable counts (log CFU/ml) of encapsulated and free *L. reuteri* after treatment first with an acid solution of pH 2 or 3, then with different concentrations of bile salt for 3 h, and then with simulated colon fluid for 1 h. LAB survival increased with increasing pH and lower bile salt concentrations. No viable counts were found for groups F and A group at pH 2 in combination with any of the bile salt concentrations. By contrast, under the same treatment conditions, a portion of the cells in the CA group survived. The viable counts of the CA group were the highest among the free and encapsulated groups. These results demonstrated that encapsulation improves the survival of *L. reuteri* after acid and bile salt treatments, with CA having a significantly better protective effect ($P < 0.05$).

Table 1. Viable counts (log CFU/ml*) of encapsulated and free *Lactobacillus reuteri* pre-treated with a pH 2 or 3 solution for 3 h, then with 0.1, 0.5, or 1% bile salt for 3 h, and finally with simulated colonic fluid for 1 h

Cell treatment	Initial bacteria count (log CFU/ml)	pH 2			pH 3		
		0.1% bile	0.5% bile	1% bile	0.1% bile	0.5% bile	1% bile
Free cells (control)	8.83 ± 0.07	ND^{bx}	ND^{bx}	ND^{bx}	3.14 ± 0.02^{cx}	1.22 ± 0.07^{cy}	1.00 ± 0.00^{cy}
Alginate-coated	8.83 ± 0.07	ND^{bx}	ND^{bx}	ND^{bx}	5.70 ± 0.08^{bx}	3.72 ± 0.05^{by}	3.15 ± 0.02^{by}
Chitosan-Ca-alginate-coated	8.83 ± 0.07	2.62 ± 0.07^{ax}	2.07 ± 0.07^{ay}	1.40 ± 0.07^{az}	5.96 ± 0.067^{ax}	3.99 ± 0.09^{ay}	3.37 ± 0.08^{ay}

*One ml of an equal volume of LAB and aseptic water (free LAB) or LAB and Na-alginate (encapsulated LAB).

$^{a-c}$Different letters within the same column differ significantly ($P < 0.05$; n = 3).

$^{x-z}$Different letters within a row of the same pH values differ significantly ($P < 0.05$; n = 3).

Table 2. β-Galactosidase activity (μmol/10 min/ml) of encapsulated and free *Lactobacillus reuteri* pre-treated with a pH 2 or 3 solution for 3 h, then with 0.1, 0.5, or 1% bile salt for 3 h, and finally with simulated colonic fluid for 1 h

Cell treatment	Blank*	pH 2			pH 3		
		0.1% bile	0.5% bile	1% bile	0.1% bile	0.5% bile	1% bile
Free cells (control)	0.066^{x}	0.008^{bx}	0.013^{bx}	0.052^{bx}	1781.769^{ax}	194176.592^{ay}	338656.801^{az}
Alginate-coated	0.066^{x}	0.023^{bx}	0.095^{bx}	0.160^{bx}	16.850^{bx}	1906.229^{by}	7585.133^{bz}
Chitosan-Ca-alginate-coated	0.066^{x}	558.554^{ax}	6780.474^{ay}	58144.768^{az}	9.668^{bx}	964.351^{bx}	4530.215^{bxy}

*Blank: β-galactosidase activity of cells not exposed to acid, bile salt and simulated colonic fluid.

$^{a-b}$Different letters within the same column differ significantly ($P < 0.05$; n = 3).

$^{x-z}$Different letters within the same row differ significantly ($P < 0.05$; n = 3).

β-Galactosidase activity of encapsulated *Lactobacillus reuteri* exposed to simulated digestive juice. Table 2 shows the β-galactosidase activity (μmol/10 min/ml) of encapsulated and free *L. reuteri* after exposure of the cells to simulated gastrointestinal conditions. As evidenced by the activity values, injury of the *L. reuteri* cell membrane increased with increasing bile salt concentrations at the same pH value. At pH 3, encapsulated cells of the A and CA groups had significantly lower β-galactosidase activity than did the free cells.

Adhesion of encapsulated *Lactobacillus reuteri* after simulated digestive juice treatment. The adherence rates of encapsulated and free *L. reuteri* after treatment with simulated gastrointestinal conditions are shown in Table 3. *L. reuteri* cells sequentially exposed to pH 3, 0.1% bile salt, and simulated colonic fluid retained their adhesion ability, with the adhesion of the CA group (0.524%) > A group (0.360%) > F group (0.275%). Thus, the encapsulated cells were more adhesive than the free cells.

Antagonistic activity toward *Listeria monocytogenes*. Figure 2 shows the displacement effect of *L. reuteri* on FITC-labeled *List. monocytogenes* previously adhered to Caco-2 cells. The corresponding fluorescence microscopy photographs are shown in Fig. 3. After treatment with simulated gastrointestinal conditions, all three groups of *L. reuteri* were able to displace *List. monocytogenes*. The adherence rate (%) of the LAB-free group in the absence of *L. reuteri* cells was designated as 100%. After the addition of LAB cells of the F, A, and CA groups to pre-adhered *List. monocytogenes*, adherence of the pathogen was reduced to 93.61%, 69.63%, and 67.02%, respectively. Thus, after sequential acid and bile treatments, the antagonism of *List. monocytogenes* adhered to Caco-2 cells was significantly greater by the encapsulated cells than by free *L. reuteri* ($P < 0.05$). The fluorescence images in Fig. 3 qualitatively confirm these results, as the density of *List. monocytogenes* luminescence was progressively reduced from the F group to the A group and the CA group (Fig. 3).

Safety of *Lactobacillus reuteri* after simulated digestive juice treatment. The invasion rates of encapsulated and free *L. reuteri* after exposure of the cells to simulated gastrointestinal conditions were zero in all cases. The lack of invasiveness means that, after acid and bile salt treatments, LAB are recognized as safe in vitro.

Discussion

To achieve the health benefits of dietary probiotics, the food product should contain at least 10^7 live organisms per gram or milliliter [33,43]. Encapsulation improves the survival of pro-

Table 3. Adherence rates (%) of encapsulated and free *Lactobacillus reuteri* cells after their exposure to simulated gastrointestinal conditions

Cell treatment	Adherence rate (%)
Free cells	0.275^{a}
Alginate-coated	0.360^{b}
Chitosan-Ca-alginate-coated	0.524^{c}

$^{a-c}$Means followed by different superscript lowercase letters differ significantly from each other ($P < 0.05$: n = 3).

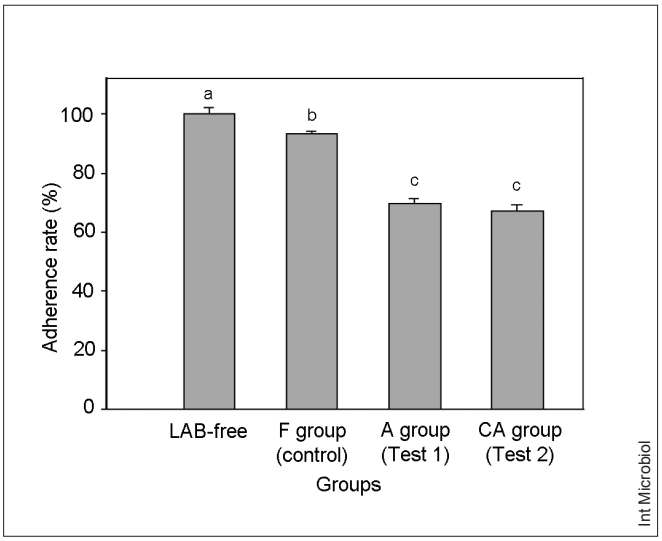

Fig. 2. Displacement by *Lactobacillus reuteri* of pre-adhered and FITC-labeled *Listeria monocytogenes* from Caco-2 cells. [a-c] Groups with different letters differ significantly from each other ($P < 0.05$). LAB-free group: pathogen adhesion in the absence of *L. reuteri*; F group: non-encapsulated, free *L. reuteri* cells; A group: Ca-alginate encapsulated *L. reuteri* cells; CA group: chitosan-Ca-alginate encapsulated *L. reuteri* cells.

biotic microorganisms during both food processing and passage through the gastrointestinal tract [35,13]. The protective effects of the Ca-alginate encapsulation of probiotics against the action of simulated gastric acid and bile salt have been reported previously [13,25]. However, bacterial viability varies depending on the encapsulation method, wall material, and probiotic strains. Ca-alginate beads with and without chitosan coating have been used to encapsulate *Lactobacillus acidophilus* 547, *Bifidobacterium bifidum* ATCC 1994, and *L. casei* 01, and the survival advantage conferred by the encapsulation of probiotics when subjected to simulated gastrointestinal conditions have been investigated [22]. The results of those studies were similar to those of the present study of *L. reuteri*, as they also demonstrated the superiority of chitosan-coated alginate beads in protecting against the action of acid and bile salt.

The acid and bile tolerance properties of LAB have been investigated in several in vitro studies [13,31,49]. In most cases, acid and bile tolerance were examined and reported separately; however, these conditions do not resemble those of the human gastrointestinal tract [2,7], where ingested LAB first pass through the highly acidic environment of the stomach, are then transported along the small intestine, and finally colonize the colon [30,37]. Thus, simulated gastrointestinal conditions in vitro studies should be based on the sequential exposure of LAB to acid and bile [25]. In this study, *L. reuteri* encapsulated using Ca-alginate and chitosan-Ca-alginate were treated sequentially with acid and bile salt before being released into simulated colonic fluid. In agreement with previous reports [22,25], the results showed that the viability of encapsulated *L. reuteri*, especially those in the CA group, was significantly greater than that of the free cells (Table 1). Thus, encapsulation can protect probiotics from the detrimental effects of the harsh conditions of the gastrointestinal tract.

Because the *L. reuteri* tolerance tests were conducted immediately after the beads had been prepared, most of the LAB cells were still encapsulated inside the Ca-alginate or chitosan-Ca-alginate matrix. During their brief, sequential acid and bile salt treatments, there was little release of the encapsulated LAB cells, as confirmed by the fact that no live *L. reuteri* cells were detected in the acid or bile salt solutions even under the mildest conditions (data not shown).

Injury to the bacterial cells was monitored based on the action of β-galactosidase, located on the inner membrane of LAB. ONPG (*o*-nitrophenyl-β-galactoside) is a colorless substrate analog for the detection of β-galactosidase activity. When the cell membrane is injured, causing permeability to rise, ONPG enters the cell and reacts with β-galactosidase to produce the yellow-colored *o*-nitrophenol (ONP), which can be quantified by colorimetric assay. In a previous study, the simulated intestinal juice of oxgall increased cellular permeability and therefore β-galactosidase activity [32]. In this study, β-galactosidase activity increased with increasing bile salt concentrations whether at pH 2 or pH 3. Encapsulated *L. reuteri* and especially in the CA group had the lowest β-galactosidase activity at pH 3.

Since the first step in microbial colonization, and therefore the initiation of health benefits, is adherence to the intestinal surfaces, measurements of adhesiveness are essential in probiotic screening [15]. The adhesiveness of probiotics as well as pathogens reflects the characteristic of the bacterial cell surface [1] and involves receptors on the surface of the host

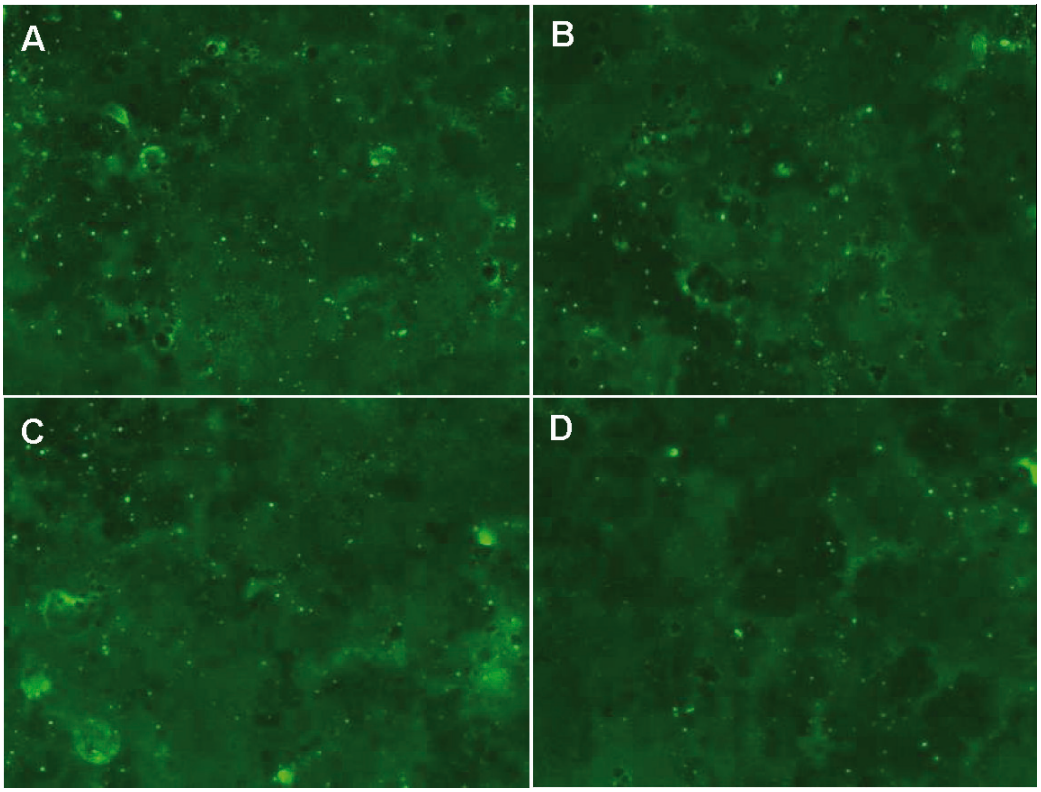

Fig. 3. Fluorescence microscopy photographs showing the displacement of pre-adhered and FITC-labeled *Listeria monocytogenes* by *Lactobacillus reuteri.* (**A**) LAB-free group; (**B**) free *L. reuteri* cells (F group); (**C**) Ca-alginate group (A group); (**D**) chitosan-Ca-alginate group (CA group).

intestinal cells [29]. In the case of LAB, adherence to human intestinal mucosa requires extracellular glycoprotein or protein structures on the bacterial cell surface [39,46]. According to the adherence rates in Table 3, both released and free *L. reuteri* subjected to simulated gastrointestinal conditions retained their ability to adhere to Caco-2 cells, with the released encapsulated *L. reuteri* displaying greater adhesiveness than free cells. This result suggests that encapsulation should protect cell surface factors such as proteins or carbohydrates from the injury caused by acid and bile salt and thus would maintain bacterial adhesiveness.

Inhibition of the adhesion of pathogens to intestinal epithelial cells by LAB has been demonstrated in vivo [3,26] and may be related to competition for specific adhesion receptors on the surface of gut cells [27]. Another study [9] has found that reuterin, produced by *L. reuteri*, inhibits both Gram-positive and Gram-negative bacteria. The inhibition of *Helicobacter pylori* by *L. reuteri* was proposed to involve bacteriostatic activity, the production of inhibitory compounds such as lactate and bactericidal substances, competition for nutrients, immunostimulation of mucosal IgA production, and/or the adherent capacity of *L. reuteri* to epithelial cells [12]. In this study, pathogen displacement from the intestinal surface was modeled using Caco-2 cells incubated first with *List. monocytogenes* and then with LAB previously exposed to simulated gastrointestinal conditions. Pathogen adherence was significantly reduced by cells in both the free and encapsulated groups, with the latter having a more potent effect.

In a previous study, we investigated the adhesion inhibition and displacement abilities of three LAB strains (*Lactobacillus acidophilus* BCRC 10695*, L. paracasei* BCRC 14023*, Bifidobacterium bifidum* BCRC 14615) using the pathogen *Clostridium perfringens* BCRC 13019 [16]. The adhesion inhibition test measured the ability of LAB cells preadhered to Caco-2 cells to inhibit the adhesion of *C. perfringens.* The displacement assay examined the displacement effect of LAB cells pretreated or not with acid and bile salt. Because the experimental design used in the present work sought to mimic the conditions of the gastrointestinal tract, only the displacement assay was performed and the antagonistic ability of encapsulated and free *L. reuteri* cells after sequential acid and bile salt treatments was compared. The re-

sults confirmed the protective effect of encapsulation based on the lower adherence rates of *List. monocytogenes* achieved with A and CA group cells than with F group cells (Fig. 2).

The invasion assay was used to determine the infectivity and pathogenicity of the tested LAB, as previously described [44]. LAB are generally recognized as safe for multiple uses. The health benefits of *L. reuteri* based on their colonization of the gastrointestinal tract and immunomodulation have been reported [47] and their daily ingestion is safe, with no negative side effects [6,48]. The safety of *L. reuteri* BCRC strain 14625 used in this research had been previously demonstrated [5] and was confirmed in this study by the absence of invasion by free or released cells exposed to acid (pH 3) and then bile salt (0.1%).

In summary, this study investigated the protective effects of Ca-alginate and chitosan-Ca-alginate encapsulation of *L. reuteri*, and the remaining probiotic properties of the released bacteria after their sequential exposure to simulated gastrointestinal conditions. The results showed that, compared to free (non-encapsulated) cells, encapsulation, especially using chitosan-Ca-alginate gel beads, improves the survival of acid- and bile-salt-treated *L. reuteri*, reduces injury to the cell membrane, and preserves the probiotic properties of adhesiveness and pathogen antagonism. Our method of challenging gel-encapsulated lactic acid bacteria with sequential simulated acid and bile salt treatment is a suitable approach to investigating their potential probiotic properties [16].

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Competing interests. None declared.

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