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ORIGINAL ARTICLE

Whey protein coating bead improves the survival of the probiotic *Lactobacillus rhamnosus* CRL 1505 to low pH

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Abstract

Aims: To evaluate the efficacy of a novel microencapsulation procedure using whey protein and pectin to improve the survival rate of *Lactobacillus rhamnosus* CRL 1505 to low pH and bile.

Methods and Results: *Lactobacillus rhamnosus* CRL 1505 was encapsulated by ionotropic gelation using pectin (PE) and pectin–whey protein (PE–WP). Both types of beads ($MC_{PE/WP}$ and $MC_{PE–WP/WP}$) were covered with a layer of whey protein by complex coacervation. The noncapsulated lactobacilli were not sensitive to bile salts but to acid. Both microparticles protected *Lact. rhamnosus* CRL 1505 at pH 2·0, but only $MC_{PE/WP}$ was effective at pH 1·2.

Conclusions: The combination of ionotropic gelation and complex coacervation techniques is efficient to obtain microcapsules of pectin covered with whey proteins. The $MC_{PE/WP}$ beads were more stable than the $MC_{PE–WP/WP}$ beads in simulated gastric conditions, thus offering better protection to *Lact. rhamnosus* CRL 1505 at low pH.

Significance and Impact of the Study: Pectin beads with a whey protein layer ($MC_{PE/WP}$) could be used as probiotic carrier in functional foods of low pH (e.g. apple juice), thus protecting *Lact. rhamnosus* CRL 1505 against the stressful conditions of the gastric tract.

Introduction

Functional foods containing probiotic bacteria (mainly lactic acid bacteria) are increasingly popular in the marketplace because of the health benefits ascribed to probiotics when consumed in proper amounts (Sanders and Marco 2010). For exerting these effects, probiotics have to overcome the low pH and bile, the main natural barrier of the gastrointestinal tract, thus arriving alive to the gut. The microencapsulation has been suggested as a useful tool for improving the tolerance of probiotic bacteria; however, controversial results make difficult the selection of the best encapsulation procedure (Liserre *et al.* 2007).

Among the encapsulating agents (cellulose, acetate, phthalate, vegetable gum, κ-carrageenan, etc.), alginate is the most commonly used despite its instability in the presence of calcium chelators or at very acid conditions (Ivanova *et al.* 2000). In contrast, pectin is less sensitive to chemical agents and more resistant to the gastric and intestinal environments (Berger and Ruhlemann 1988; Parkar *et al.* 2010; Voo *et al.* 2011). Another suitable encapsulating agent is the whey protein, an important by-product of cheese industry that causes high environmental contamination. Different strategies for producing microcapsules with whey protein include spray drying (Picot and Lacroix 2004), cold-induced gelation (Barbut and Foegeding 1993), complex coacervation (Oliveira *et al.* 2007) and a combination of methods (Lambert *et al.* 2008). Results obtained are controversial.

Lactobacillus rhamnosus CRL 1505, a probiotic lactobacillus, is effective in preventing respiratory and intestinal infections in preclinical and clinical studies (Salva *et al.* 2011). Since 2008, the probiotic CRL 1505 is successfully being administered as probiotic yogurt (Yogurito®, Cerros Tucumanos, Tucumán, Argentina) to 100 000 scholar children of high-vulnerable populations in the framework of the social feeding programmes of the Tucumán government, in Argentina.

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The aim of this work was to evaluate the efficacy of a novel microencapsulation procedure to enhance the resistance of *Lact. rhamnosus* CRL 1505 to low pH and bile salts under simulated gastrointestinal conditions. Whey protein and pectin were used as encapsulating agents, and a combination of ionotropic gelation and complex coacervation techniques was evaluated for obtaining the beads.

Materials and Methods

Microencapsulation of *Lactobacillus rhamnosus* CRL 1505

Lactobacillus rhamnosus CRL 1505 (culture collection CERELA, Tucumán, Argentina) was grown in MRS broth (Oxoid, Basingstoke, UK) at 37°C. The 18-h-old cells were harvested and washed by centrifugation (8000 ***g***, 15 min, 4°C) with sterile saline solution. *Lactobacillus rhamnosus* CRL 1505 (washed cell pellet) was microencapsulated using the following: (i) a solution of pectin (PE) (2%, w/v; GENU®, CP Kelco, Limeira, São Paulo, Brazil) adjusted to pH 4·0 with HCl and (ii) a solution of pectin (4%, w/v) and whey protein (2% w/v; NZMP, Auckland, New Zealand) (ratio, 1 : 1) adjusted to pH 7·0 with 2·5 N NaOH. Unsalted commercial butter (melted at 50°C) was added to both formulations to a final concentration of 2% (w/v). The mixtures were homogenized (ultra-turrax homogenizer T-18; Ika, Werke Staufen, Germany) at 1000 ***g*** for 3 min to obtain an emulsion (oil-in-water) atomized in 300 ml calcium chloride (2% w/v, pH 4·0) under stirring (230 ***g***). After 30 min (complete gelation), the microcapsules were washed (sterile distilled water, pH 4·0) and sieved (Ø = 25 μm). The microcapsules were covered with a whey protein (WP) layer (complex coacervation) by dispersing the microparticles in a WP solution (8% w/v, pH 4·0) under magnetic stirring (230 ***g***, 30 min). The microcapsules obtained were named as $MC_{PE/WP}$ (pectin core with a WP overcoating) and $MC_{PE–WP/WP}$ (pectin–whey core with a WP overcoating). Both microcapsules were put into sterile Petri plates, frozen at −20°C for 24 h, freeze-dried in a chamber freeze dryer (501; Edwards, London, UK) (initial temperature, −40°C; pressure, 0·1 mm; final temperature, 25°C; total cycle time, 48 h) and stored under refrigeration. The freeze-dried microcapsules were rehydrated with citrate (2%, w/v) solution for 15 min at 25°C to release the lactobacilli cells. Samples of microcapsules before freeze drying (wet beads) and after rehydration were pour plated (MRS agar; Britania, Buenos Aires, Argentina), and plates were incubated (37°C, 48 h) for colony counting. Results were expressed as $\log_{10}$ CFU ml^{-1}. Noncapsulated lactobacilli are referred as free lactobacilli.

Microparticle characterization

The surface and inner morphology of the wet and freeze-dried (rehydrated with sterile distilled water, pH 4·0 for 15 min) microcapsules was determined by SEM (scanning electron microscopy LEO 440i, Cambridge, UK). The microcapsules were fixed in stubs using double-faced copper adhesive tape (SC7620; Polaron, Ringmer, UK). The diameter (μm) of 300 microcapsules (wet and rehydrated) was determined (Scion Image program) from the photographs taken with an optical microscope digital camera (Global Lab Image program).

Survival of free and encapsulated lactobacilli in simulated gastric juice (SGJ)

SGJ adjusted to pH 1·2 or pH 2·0 (FAO/WHO, 2001) contains (g l^{-1} dist. water) 1·1 KCl, 2·0 NaCl, 0·1 $CaCl_2$, 0·4 KH_2PO_4 and 3·5 mucin. Pepsin (final conc. 0·2 g l^{-1}) was added after SGJ sterilization (Bermejo *et al.* 2002). Fresh cell pellets (18-h-old cultures) or dried/rehydrated microcapsules were added to SGJ and incubated for 60 and 120 min at 37°C under agitation. During simulated gastric system, samples were collected before the addition of SGJ (time zero) and after 60- and 120-min incubation. The samples were neutralized at pH 7·0 (0·1 mol l^{-1} $NaHCO_3$) for cell viability determination. To evaluate the number of microencapsulated lactobacilli, the cells were first released from the capsules using sodium citrate (2%, w/v). *Lactobacillus rhamnosus* CRL 1505 viability was determined by the plate dilution method (plating in mass) in MRS agar. The plates were incubated aerobically at 37°C for 48 h.

For bile assay, free lactobacilli and rehydrated microencapsules were incubated in bile solution (0·5 and 1%, w/v; pH 7·0) under agitation for 5 h at 37°C, and the cell viability was determined. The samples were collected before the addition of bile solution (time zero) and after 60- and 120-min incubation for cell viability determination.

Statistical analysis

Results of three independent assays are presented as mean values ± standard deviation (SD). Data were analysed by ANOVA and Tukey's test. Statistical analysis was carried out with the STATISTICA 5.5 program (Statsoft, Tulsa, OK, USA). Results were considered significantly different at $P < 0{\cdot}05$.

Results

Coated beads ($MC_{PE–WP/WP}$ and $MC_{PE/WP}$) were obtained by a combination of ionotropic gelation (pectin and calcium ion) and complex coacervation (whey

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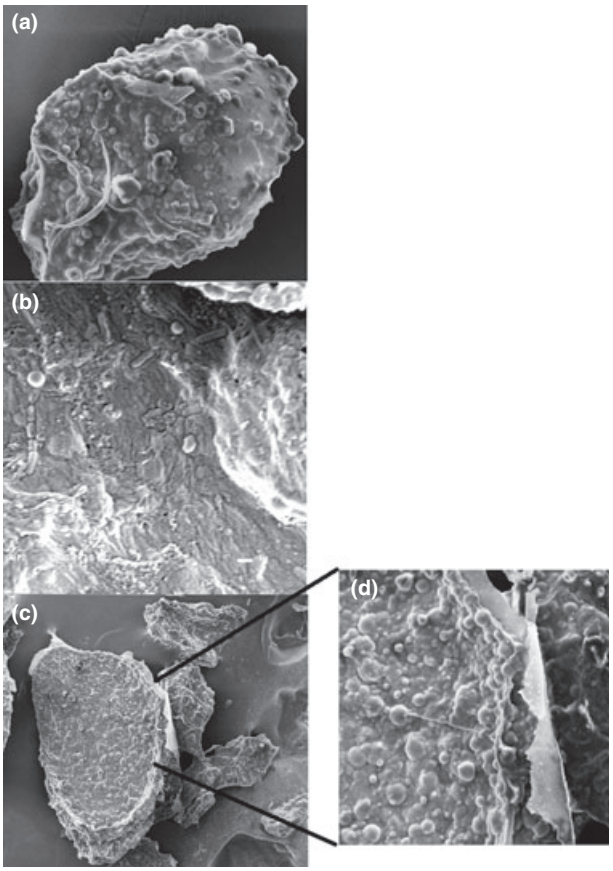

Figure 1 Scanning electron microscopy micrographs. Dry microparticles obtained from pectin/butter gelled microcapsules coated with whey protein (MCPE/WP). (a) Whole microparticle. (b and c) Arrows show the whey protein layer coating the particle (a and c: bars = 10 μm; b: bar = 30 μm). (d) Broken particle with microbial cells inside.

protein coating). Both microcapsules had similar ($P \geq 0{\cdot}05$) size (185 ± 20 μm) with a round, flattened shape (not completely smooth) without visible cracks or pores on the surface. The microcapsules remained stable after lyophilization without changes in size, morphology or overcoating. As an example of beads morphology, Fig. 1a shows freeze-dried $MC_{PE/WP}$ beads with the lactobacilli inside (Fig. 1b). The WP overcoating is presented in Fig. 1c,d (amplified).

The viability of *Lact. rhamnosus* CRL 1505 in beads $MC_{PE/WP}$ and $MC_{PE–WP/WP}$ was high ($8{\cdot}4 \pm 0{\cdot}2$ and $9{\cdot}2 \pm 0{\cdot}1$ $\log_{10}$ CFU g^{-1}, respectively) (data not shown). Figure 2 shows the survival rate of free and encapsulated *Lact. rhamnosus* CRL 1505 in SGJ at pH 1·2 and 2·0. The free lactobacilli displayed a great sensitivity to acid; no viable cells were detected after 60-min incubation at pH 1·2 (Fig. 2a), while at pH 2·0, 50 and 75% viability loss was obtained after 60- and 120-min incubation, respectively (Fig. 2b). In contrast, both $MC_{PE–WP/WP}$ and $MC_{PE/WP}$ microcapsules were able to protect (*c.* 100% cell survival) the lactobacilli at pH 2·0 (Fig. 2b). At pH 1·2, the $MC_{PE/WP}$ beads were more efficient (95% cell survival) than $MC_{PE–WP/WP}$ (36% survival) after 120-min incubation (Fig. 2a).

The bile tolerance assays revealed that *Lact. rhamnosus* CRL 1505 was not sensitive to bile at the concentrations used (0·5 or 1%) because 100% survival was obtained after 5-h incubation (data not shown).

Discussion

The process of microencapsulation proposed is very simple and has advantages compared to traditional techniques: among others, the low temperature (25°C) used (a key factor in case of probiotic bacteria or thermo-sensitive micro-organisms), the low cost of the encapsulating agents (pectin and whey protein) or the potential prebiotic effect of pectin (Hasselwander 2008). Besides, organic solvents – difficult to remove – are not required in the process (Heelan and Corrigan 1998).

The effectivity of coating is based on the electrostatic complexes formed by the whey protein and the pectin, which occurs at a pH below the isoelectric point (IEP) of the whey protein (pH 5·2–5·4) (Guérin *et al.* 2003; San-

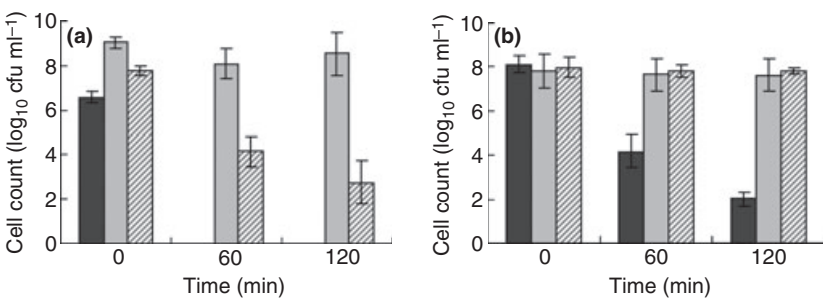

Figure 2 Viability of free (■) and $MC_{PE/WP}$ (▥) and $MC_{PE–WP/WP}$ (▨) microencapsulated *Lactobacillus rhamnosus* CRL 1505 in simulated gastric juice at pH 1·2 (a) and at pH 2·0 (b).

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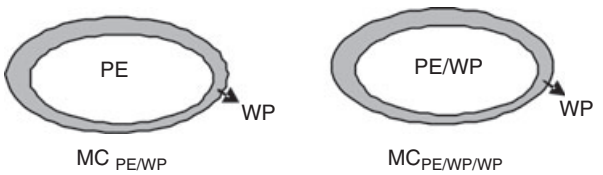

Figure 3 Two types the microparticles ($MC_{PE–WP/WP}$ and $MC_{PE/WP}$).

tipanichwong *et al.* 2008; Gentès *et al.* 2010). The attraction between these two macromolecules involves mainly the $-NH_3$ groups (with positives charges) of the whey protein and the carboxyl groups (with negative charges) of pectin. In the present study, observations by SEM evinced the presence of a continuous layer of whey protein covering the microparticles (obtained by complex coacervation at pH 4·0), thus indicating the effectivity of the procedure.

One of the main barriers for oral probiotic bacteria is the stomach low pH, which is related to the high hydrochloric acid concentration of the gastric acid. Microencapsulation may be a useful tool for improving the acid tolerance of probiotics, but results may be affected by the encapsulating agent used. In this study, the presence of whey protein and pectin inside the capsule was the only difference between the microparticles $MC_{PE–WP/WP}$ and $MC_{PE/WP}$ (Fig. 3); however, the former type of bead was less effective in protecting *Lact. rhamnosus* CRL 1505 in SGJ at pH 1·2. These results would be related to the electrostatic interactions occurring between the carboxyl groups of PE and the $-NH_3$ groups of WP inside the capsule (Girard *et al.* 2004). Once the complex PE–WP (core of $MC_{PE–WP/WP}$) is formed and the charges neutralized, it may aggregate to form interpolymer complexes, thus remaining few carboxyl groups free to interact with the WP overcoating. As a result, the WP coating in $MC_{PE–WP/WP}$ might be slightly attached to the bead surface, and this kind of microcapsules would behave as noncoating microparticles. This fact would explain the low protection offered by $MC_{PE–WP/WP}$ to the lactobacilli in SGJ at pH 1·2.

In contrast, all carboxyl groups of PE in $MC_{PE/WP}$ microparticles are exposed on the surface and may interact with the WP overcoating. This type of bead was effective in protecting *Lact. rhamnosus* CRL 1505, and the survival rate obtained (7 $\log_{10}$ CFU ml^{-1}) was one log unit higher than the amount of probiotic required for having health benefits in the host (Salva *et al.* 2010, 2011).

Lactobacillus rhamnosus CRL 1505 was naturally tolerant to bile at the concentrations assayed (0·5 and 1%) in contrast to the deleterious effect reported for free probiotic lactic acid bacteria (Guérin *et al.* 2003; Ding and Shah 2009).

In conclusion, pectin microcapsules with a whey protein overcoating ($MC_{PE/WP}$) could be used as probiotic carrier in functional foods, thus protecting *Lact. rhamnosus* CRL 1505 against the stressful conditions of the gastric tract.

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