Research Article

Received: 27 July 2021 Revised: 29 October 2021 Accepted article published: 13 November 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.11638

Reevaluating a non-conventional procedure to microencapsulate beneficial lactobacilli: assessments on yield and bacterial viability under simulated technological and physiological conditions

María J García,[a,b,c] Francesca Ruíz,[a,b*] Paula Asurmendi,[a,b,c] Liliana Pascual[a,b] and Lucila Barberis[a,b]

Abstract

BACKGROUND: Maintaining viability of beneficial microorganisms applied to foods still constitutes an industrial challenge. Many microencapsulation methodologies have been studied to protect probiotic microorganisms and ensure their resistance from manufacturing through to consumption. However, in many Latin-American countries such as Argentina there are still no marketed food products containing microencapsulated beneficial bacteria. The objectives of this work were: (i) to obtain microcapsules containing *Lactobacillus fermentum* L23 and *L. rhamnosus* L60 in a milk protein matrix; and (ii) to evaluate the viability of microencapsulated lactobacilli exposed to long-term refrigerated storage, mid–high temperatures and simulated gastrointestinal conditions.

RESULTS: The method of emulsification/rennet-catalyzed gelation of milk proteins used in this study led to high encapsulation yields for both strains (98.2–99%). Microencapsulated lactobacilli remained viable for 120 days at 4 °C, while free lactobacilli gradually lost their viability under the same conditions. Microencapsulation increased the resistance of lactobacilli to mid–high temperatures, since they showed survival rates of 95–99.3% at 50 °C, and of 72.5–74.4% at 65 °C. Under simulated gastric conditions, the microencapsulated lactobacilli counts were higher than 8.5 log CFU mL^{-1} and showed survival rates between 96.61% and 97.74%. Furthermore, in the presence of bile (0.5–2% w/v) the survival of microencapsulated strains was higher than 96%.

CONCLUSION: The microencapsulation process together with the matrix of milk proteins used in this study protected beneficial *Lactobacillus* strains against these first simulated technological and physiological conditions. These findings suggest that this microencapsulation method could contribute to secure optimal amounts of living lactobacilli cells able to reach the intestine.

Keywords: microencapsulation; milk proteins; lactobacilli; simulated technological; gastrointestinal conditions

INTRODUCTION

In recent years, there has been increasing interest in the study of functional foods, which contribute not only to the quality of life of consumers but also to the prevention of diseases. Within this group of foods, products added with probiotic microorganisms are the most relevant due to the diverse beneficial effects that they provide to consumer health. Consequently, the search and selection of probiotic microorganisms and their incorporation into certain foods remains as one of the main areas of development in the food industry.[1,2] Recent global market studies point out that the production of food added with probiotic bacteria has expanded specially in the last decade, introducing several products into the functional food trade. In the market, probiotic products mainly contain certain pre-selected strains of *Lactobacillus* spp., *Streptococcus* spp. and *Bifiobacterium* spp.[3] Among the carrier foods, dairy products such as yoghurts and other fermented milks are the most used for probiotic delivery. Other dairy probiotic products are cheeses, ice cream, milk and cream. Moreover,

* *Correspondence to: F Ruíz, Área de Bacteriología, Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36 Km. 601, 5800 Río Cuarto, Córdoba. Argentina. E-mail: fruiz@exa.unrc.edu.ar*

a *Área de Bacteriología, Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto (UNRC), Río Cuarto, Córdoba, Argentina*

b *Instituto de Biotecnología Ambiental y Salud (INBIAS), UNRC-CONICET, Río Cuarto, Córdoba, Argentina*

c *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Centro Científico Tecnológico-Córdoba (CCT-Córdoba), Córdoba, Argentina*

several types of probiotic non-dairy products have been developed, such as fruit and vegetable juices, fermented cereal beverages, soybean products and meats.[4-6] Investigations on the compositional quality of commercial probiotic supplements marketed worldwide, including functional foods, indicated that their content does not always comply with the corresponding label information in terms of viability.[7] In fact, the survival rate of these microorganisms in fermented milks could be very low.[8] By definition, probiotics are 'live microorganisms that when administered in adequate amounts confer a health benefit on the host'.[9] Therefore, maintaining the viability of probiotic microorganisms is one of the most important and basic requirements for the functionality of these beneficial bacteria, and it still constitutes an industrial challenge.[6] The technological conditions of food processing, which often include changes in pH, high temperatures, presence of oxidizing agents and long storage periods, may reduce the viability of probiotic bacteria.[10,11] Furthermore, other reductions in probiotic viability take place once they are consumed due to physiological conditions such as the acid pH of the stomach and the presence of bile salts in the upper parts of the small intestine, which are unfavorable to them.[12,13] In this context, probiotic microorganisms should be protected to survive and reach optimal quantities in the intestine, and consequently to exert their benefits on host health.[6]

Currently, there is special interest in enhancing survival of probiotics used in the food industry, and microencapsulation is a promising technique for achieving this goal. Several studies have demonstrated that this process improves the resistance of probiotic microorganisms to adverse technological and physiological conditions.[14] Microencapsulation is the process through which microorganisms are introduced into a matrix, creating a physical barrier between the bacteria and the external environment.[10,15] Probiotic microencapsulation effectiveness depends on two basic factors: the encapsulating material and the selection of the more adequate process to obtain these microparticles.[16] Nowadays, several food-grade biopolymers have been used as encapsulating matrices. Among them, alginate, chitosan, starch, carrageenan, pectin and milk proteins are the most frequently used in different studies.[14,17-19] In particular, milk proteins used as encapsulating matrix rely mainly on certain physicochemical properties, such us low viscosity, high solubility, high emulsifying capacity and capacity to form high-density gels. Furthermore, the buffering capacity of milk proteins could contribute to conferring protection to probiotics under the acid conditions found in the food in which they will be added, and then against the stomach acid in the host.[18,20] All of these characteristics make them one of the most technologically attractive encapsulating materials.[15,20,21] However, the use of skim milk concentrates as encapsulating matrix continues to be rarely exploited, compared with other materials such as whey proteins or sodium caseinate.[17,22-25] The techniques most commonly used for microencapsulation of probiotic microorganisms are spray drying, extrusion, emulsification and coacervation.[26] Among these techniques, emulsification would represent one of the most suitable methodologies to obtain microparticles in a relatively short time and at low cost.[14]

Our research group has extensively studied and characterized two human *Lactobacillus* strains – *L. fermentum* L23 and *L. rhamnosus* L60 – as probiotics through several *in vitro* and *in vivo* experiences.[27-30] One of the most important properties demonstrated for these *Lactobacillus* strains is their strong antimicrobial activity. In previous studies, we have already proved that bacteriocins produced by these lactobacilli strains (L23 and L60) were the main biometabolites responsible for inhibition of pathogenic microorganisms. The antimicrobial effect of both bacteriocins has been shown against a wide range of microorganisms, including Gram-positive bacteria, Gram-negative bacteria and fungi.[27-34]

Unfortunately, although several investigations on probiotic microencapsulation have been carried out worldwide, only a few food products containing microencapsulated beneficial bacteria have been developed to date.[35,36] In fact, most of these products have been marketed in countries such as Germany, Austria, Switzerland, the USA, Canada, and Mexico. However, to the extent of our knowledge, in Argentina as well as in many other Latin-American countries, there are still no marketed microencapsulated probiotics for food products. Furthermore, addition of microencapsulated probiotics to food products may not be necessarily declared in the label information, as recommended by FAO/WHO,[37] thus remaining unnoticed. Consequently, this continues to be a scientific challenge for biotechnological research applied to the food industry.

Considering the difficulty in choosing the most suitable encapsulation method for the protection of viable bacteria, we have adapted an interesting, although less explored, method using skim milk concentrates as encapsulating material. This method combines the enzymatic properties of rennet, the advantages of the emulsification technique and the subsequent gelation of milk proteins to obtain water-insoluble microparticles. The use of highly concentrated aqueous milk protein solutions of low viscosity enables the formation of microcapsules with a high-density gel network creating a favorable micro-environment for retaining viability of probiotic microorganisms.[38]

Throughout this first study, we intend to contribute with a reproducible and economically feasible methodology to obtain microparticles containing specific lactobacilli strains with probiotic potential. The objectives of this work were: (i) to obtain microcapsules containing *L. fermentum* L23 and *L. rhamnosus* L60 in a milk protein matrix; and (ii) to evaluate the viability of microencapsulated lactobacilli exposed to long-term refrigerated storage, mid–high temperatures and simulated gastrointestinal conditions.

MATERIAL AND METHODS

Lactobacillus spp. and culture conditions

Lactobacillus strains *L. fermentum* L23 and *L. rhamnosus* L60 have been identified by standard biochemical tests: the API 50 CHL system (BioMèrieux, Inc., France) and 16S r-RNA analyses.[27,28] The bacterial sequencings of 16S r-RNA of *L. fermentum* L23 and *L. rhamnosus* L60 have been deposited in GenBank under the accession numbers GQ 455406 and EF 495247, respectively.[33] Lactobacilli were cultured in de Man Rogosa and Sharpe (MRS; Britania, Buenos Aires, Argentina) broth or agar for 18–24 h at 37 °C under a 5% CO_2 atmosphere. Strains were stored at −80 °C in MRS broth containing 30% (v/v) glycerol.

Microencapsulation of *L. fermentum* L23 and *L. rhamnosus* L60

Microcapsules containing *L. fermentum* L23 and *L. rhamnosus* L60 were produced by the process of emulsification and enzymatic gelation in a matrix of skim milk (SM) described by Heidebach *et al.*[38] Successive cultures of lactobacilli were carried out in MRS for 18 h at 37 °C under microaerobic conditions. The bacterial cells were concentrated by centrifugation (5730 × *g*, 20 min)

and washed twice with sterile phosphate-buffered saline (PBS, pH 7.1). Each bacterial concentrate of lactobacilli (2 mL) was added to 28 mL SM reconstituted at 35% (w/v) to obtain a bacterial concentration of 10^9–10^{10} CFU mL^{-1}. The lactobacilli suspensions in SM were refrigerated at 5 °C and, subsequently, 400 μL of a rennet solution (28 IMCU mL^{-1}) (CHR Hansen, Buenos Aires, Argentina) was added. To allow the cleavage of κ-casein by rennet enzyme (chymosin), the lactobacilli suspensions were kept at 5 °C for 60 min. After this period, 180 μL of a $CaCl_2$ solution (10% w/v) was added to each of these treated mixtures. The mixtures (15 g) were emulsified with 150 g vegetable oil by magnetic stirring for 5 min at 22 x g. Finally, the temperature was raised to 40 °C for 15 min applying continuous agitation to allow gelation of the proteins and the formation of microparticles. The oily residue was removed by centrifugation at 360 × *g* for 1 min. The sediment was washed twice with sterile double-distilled water. The supernatant was removed and the sediment containing the microcapsules was collected into sterile plastic vials and stored at 4 °C for 24 h before the following tests were performed. The microcapsules containing each *Lactobacillus* strain were checked by optical microscopy at 100× and 250× magnifications.[39] Suspensions of free non-encapsulated lactobacilli were used as controls. Those suspensions were obtained following the same procedure of microencapsulation without addition of rennet solution.

Encapsulation yield

The microspheres were mechanically disrupted to release the lactobacilli from the encapsulating matrix. One gram of microcapsules was resuspended in sterile double-distilled water and homogenized for 2 min at 5600 x g.[38] Disintegration of the microcapsules was verified by optical microscopy.[40] Subsequently, counts of lactobacilli (log CFU g^{-1}) were carried out on MRS agar plates and incubated for 24–48 h at 37 °C under microaerobic conditions. The encapsulation yield (EY) was calculated according to Eqn (1):

$$EY = (N/N_0) \times 100 \quad (1)$$

where N_0 represents the log of lactobacilli counts in SM before the encapsulation process and N represents the log of lactobacilli counts after obtaining the microcapsules.[40]

Determination of microencapsulated lactobacilli viability under long-term refrigerated storage

To evaluate the change in viability of microencapsulated lactobacilli during long-term refrigerated storage, an adaptation of the procedure previously described by Favarin *et al.*[41] was performed. Microencapsulated lactobacilli were stored at 4 °C for 120 days. At 0, 15, 30, 45, 60, 90 and 120 days, samples (0.1 g) were taken to determine bacterial counts over time. At each time, the microspheres were disrupted and lactobacilli counts were determined as previously described. The same procedure was performed with controls (free lactobacilli).

Determination of viability of microencapsulated lactobacilli exposed to mid–high temperatures

The effect of mid–high temperatures on microencapsulated lactobacilli was carried out as described by Dianawati *et al.*[25] The microcapsules containing *L. fermentum* L23 and *L. rhamnosus* L60 (0.5 g) were added to test tubes containing 4.5 mL prewarmed MRS broth and exposed to 50 and 65 °C in a water bath during 30 min. After heat treatment, the contents were quickly cooled at room temperature in an ice box. Lactobacilli counts at time 0 (t_0) and at the end of the experiment (t_{30}) for both temperatures were determined. The same procedure was performed with controls (free lactobacilli). The survival rate (%) of microencapsulated lactobacilli was calculated according to the Eqn (2):[24]

$$\text{Survival rate}\,(\%) = N/N_0 \times 100 \quad (2)$$

where N_0 represents the log of initial lactobacilli counts and N represents the log of lactobacilli counts obtained after 30 min exposure to each temperature.

Determination of microencapsulated lactobacilli viability in simulated gastrointestinal conditions

To evaluate the survival of microencapsulated lactobacilli exposed to simulated gastric conditions, the methodology suggested by Chen *et al.*[42] was performed. Two solutions of NaCl (0.2% w/v), adjusted to pH 2 and 2.5 with hydrochloric acid (5 mol L^{-1}), were used to simulate the stomach acid. The microcapsules containing each *Lactobacillus* strain (0.5 g) were added to 4.5 mL of those solutions and incubated at 37 °C for 120 min. At 0, 60 and 120 min, samples (1 mL) were taken and washed with sterile PBS (pH 7.1). From each sample, lactobacilli were released from the microcapsules and bacterial counts were performed. The survival rates of microencapsulated lactobacilli to both pH values were calculated as described before. For each *Lactobacillus* strain, these percentage values were compared with those obtained from controls (free lactobacilli), which were evaluated under the same conditions.

As another stressing condition that occurs in the upper parts of the small intestine, the effect of bile solutions at different concentrations was tested on microencapsulated lactobacilli according to the methodology described by Pan *et al.*[43] The microcapsules containing each *Lactobacillus* strain (0.5 g) were added io 4.5 mL of different solutions with 0.5, 1 and 2% w/v (pH 7) bile concentrations during 120 min at 37 °C. At 0, 60 and 120 min, the log of lactobacilli counts and their survival rate (%) were determined. Controls (free lactobacilli) were evaluated under the same conditions.

Statistical analyses

All tests were performed in triplicate and results were expressed as mean ± SD. Bacterial counts (CFU mL^{-1}) were log-transformed for each experiment. All data were analyzed by analysis of variance (ANOVA). To compare mean bacterial counts for each treatment through time, Tukey's test was used. Statistical differences were considered as significant at $P < 0.05$ using INFOSTAT software (version 2011, GrupoInfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

RESULTS AND DISCUSSION

Encapsulation yield

After using the method of emulsification/rennet-catalyzed gelation of milk proteins, uniform rounded microcapsules containing *L. fermentum* L23 and *L. rhamnosus* L60 by were observed by optical microscopy (Fig. 1). When comparing the concentrations of lactobacilli added to milk before and after the encapsulation process, we observed that the lactobacilli counts did not show significant differences ($P > 0.05$). In the microcapsules, the initial cell concentrations were 9.81 and 9.11 log CFU g^{-1} for L23 and L60, respectively. Consequently, the obtained EY values were 98.2

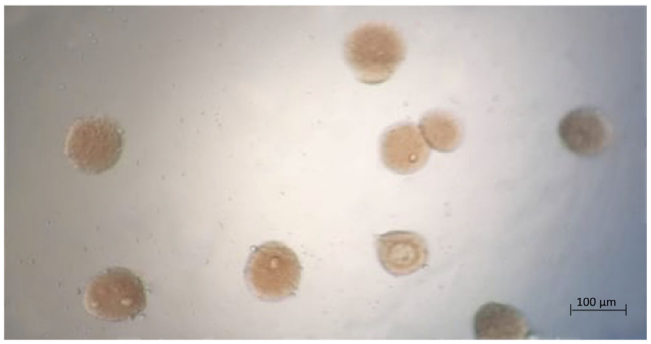

Figure 1. Optical microscopy image of microcapsules obtained by emulsification/rennet-catalyzed gelation of milk proteins (100× magnification).

and 99%, respectively. The results of this study coincide with those reported by Heidebach *et al.*,[38] who evaluated the microencapsulation of other bacterial strains with probiotic potential using a similar methodology, and also obtained high EY values. It is well known that this parameter relies on the types and concentration of polymer materials as encapsulation matrix, and the encapsulation method used.[44] High EY values (98%) have also been reported by Afzaal *et al.*[45] using a different microencapsulation method in a matrix of sodium alginate. It is noteworthy that the microencapsulation process used in this study allowed us to obtain EY values significantly higher than those from other recent works.[13,44,46-49] In those reports, the EY values ranged from ~50% to 94.53% when applying other encapsulation methods and different encapsulating materials.

These findings demonstrated that the viability of the beneficial strains was not affected by the encapsulation process. Furthermore, the high EY values obtained in this work would indicate that milk proteins used as encapsulating matrix provided optimal conditions to retain these microorganisms. Thus, these high EY values contribute to ensure a high initial concentration of these beneficial microorganisms inside the microcapsules.

Effect of long-term refrigerated storage on microencapsulated lactobacilli

During storage at 4 °C, counts of free L23 and L60 decreased from 9.84 to 5.15 and from 8.98 to 2.15 log CFU g^{-1} after 90 days, respectively. Moreover, both free lactobacilli strains were not detected in the bacterial counts at 120 days (Fig. 2(A, B)). In contrast, during the first 60 days under this refrigeration temperature, the log counts of microencapsulated lactobacilli remained at high values. In this period, the mean log counts ranged between 9.81 and 8.88 log CFU g^{-1} and between 9.11 and 8.56 log CFU g^{-1} for L23 and L60 strains, respectively. Subsequently, viability of microencapsulated L23 and L60 strains continued up to 120 days, showing viability percentage values of 73.60% (7.23 log CFU g^{-1}) and 60.37% (5.5 log CFU g^{-1}), respectively. These results demonstrated that the microencapsulation process used in this study protected both *Lactobacillus* strains, as their viability was significantly higher ($P < 0.05$) than that of free cells during a long period (120 days) at 4 °C.

At this point, these findings partially agreed with a previous work performed by Maciel *et al.*,[50] who used microcapsules of milk protein and reported high viability of other beneficial bacterial strains, although during a shorter time frame (90 days) than that tested in our study. Similar results have been reported by Tarifa *et al.*,[51] who observed that microencapsulated *Lactobacillus* spp. in pectin

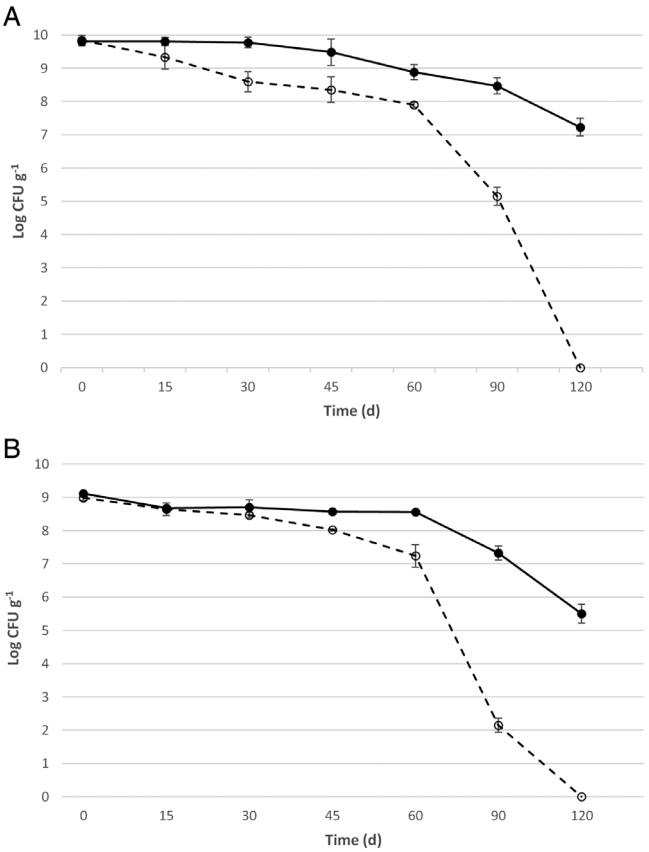

Figure 2. Viability of free and microencapsulated *Lactobacillus* strains during long-term refrigerated storage: (A) *L. fermentum* L23; (B) *L. rhamnosus* L60. Free lactobacilli (--○--); microencapsulated lactobacilli (—●—). Results are the mean of three replicates and error bars represent the standard deviation.

microgels remained above 7 log CFU g^{-1} after 42 days of storage. However, Riaz *et al.*[48] and Corbo *et al.*,[52] using other microencapsulation materials, observed that counts of *Bifidobacterium* spp. and *Lactobacillus* spp. decreased to 10^4–10^6 CFU g^{-1} from high initial concentrations after only 30–32 days of refrigerated storage. Thus, our results of long-term refrigerated storage are meaningful since one of the main requirements for the use of probiotic microorganisms in foods is that they must survive not only throughout the production process but also during the shelf life of the product, as most products containing probiotics require refrigerated temperatures for their conservation.

Effect of mid–high temperatures on microencapsulated lactobacilli

Microcapsules should resist different food processing conditions, which commonly include exposure to high temperatures. Different researches have studied the protection capacity of microcapsules on probiotics exposed to mid–high temperatures in the range of 40–70 °C.[22,24,25] In the present study, the encapsulated lactobacilli were exposed to relatively high temperatures (50 and 65 °C) for 30 min. The viability of microencapsulated lactobacilli after both heat treatments is shown in Fig. 3. From an initial mean count of 8.62 log CFU mL^{-1} of free L23 strain, the bacterial viability decreased to 7.74 and 3.87 log CFU mL^{-1}, respectively, after exposure to 50 and 65 °C for 30 min. By comparison, microencapsulated L23 strain had a significantly higher

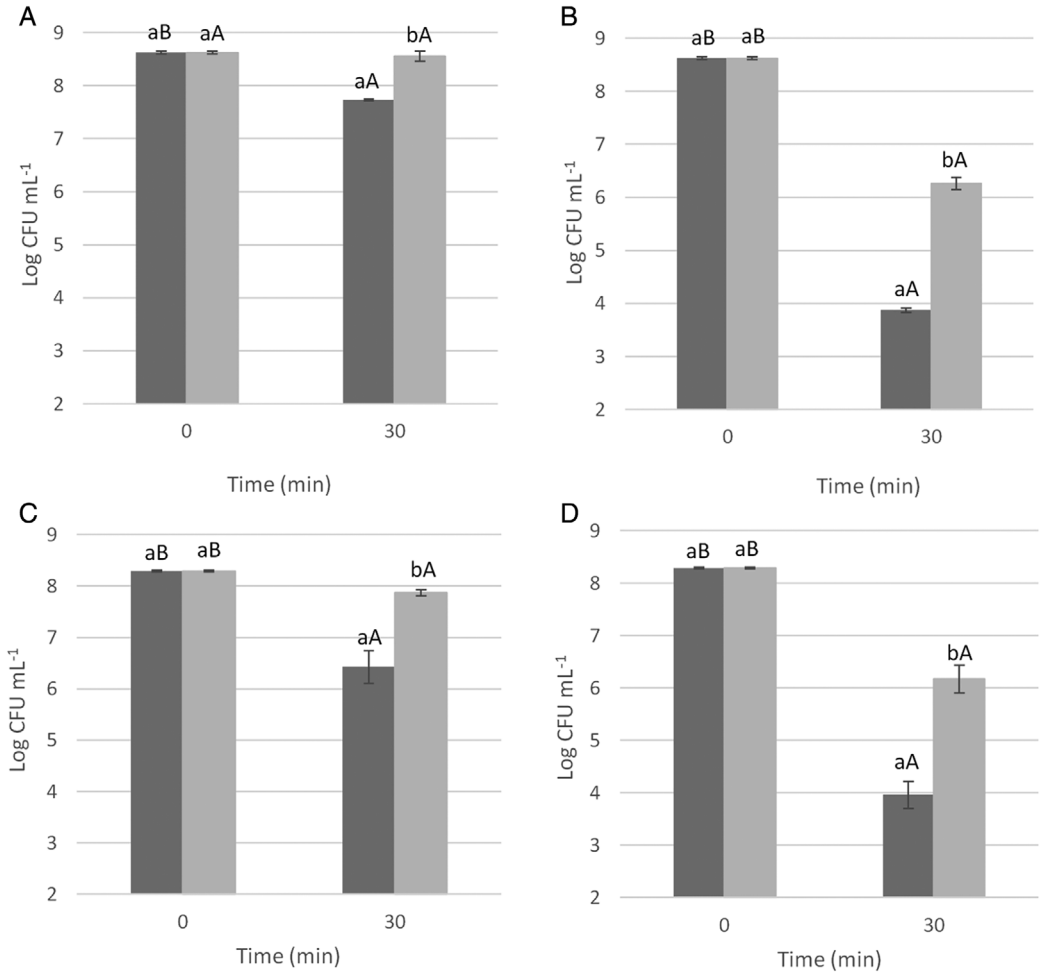

Figure 3. Survival of free and microencapsulated *Lactobacillus* spp. after exposure to mid–high temperatures: (A) *L. fermentum* L23, treatment at 50 °C; (B) *L. fermentum* L23, treatment at 65 °C; (C) *L. rhamnosus* L60, treatment at 50 °C; (D) *L. rhamnosus* L60, treatment at 65 °C. ■, free lactobacilli counts; ■, microencapsulated lactobacilli counts. Results are the mean of three replicates and error bars represent the standard deviation. Different lower-case letters indicate significant differences between different color bars; different upper-case letters indicate significant differences between the same color bars. All statistical studies were performed using Tukey's test ($P < 0.05$).

viability ($P < 0.05$), maintaining mean log count values of 8.56 and 6.26 log CFU mL^{-1} after treatment at 50 and 65 °C, respectively (Fig. 3(A,B)). For free L60 strain, lactobacilli counts decreased from an initial population of 8.29 to 6.43 and 3.96 log CFU mL^{-1} after both heat treatments. Microencapsulated L60 strain showed a higher resistance, obtaining mean log count values of 7.87 and 6.17 log CFU mL^{-1} after treatment at 50 and 65 °C, respectively (Fig. 3(C,D)).

The microencapsulation of both lactobacilli strains increased their resistance to treatment with these mid–high temperatures. Statistically significant differences between mean log counts values of microencapsulated lactobacilli after heat treatment were obtained compared with controls ($P < 0.05$). Survival rates (%) of microencapsulated L23 and L60 strains were 99.3% and 95%, respectively, at 50 °C. Although significant differences were found compared with the initial log counts ($P < 0.05$), microencapsulated L23 and L60 strains showed high survival rates, with values of 72.5% and 74.4%, respectively, after treatment at 65 °C.

These results do not coincide with Bosnea *et al.*,[22] who tested other beneficial strains microencapsulated with whey protein and gum arabic by complex coacervation and obtained survival levels up to 50% after treatment at 65 °C for 30 min. In contrast, our results showed that, applying the same conditions, the microcapsules were able to protect both *Lactobacillus* strains, reaching notably higher survival rates (72.5–74.4%). It has already been proposed that thermostability of caseins due to their high flexible structure could be related to thermal protection, which could be advantageous for applications in the food industry.[53] Thus, we hypothesized that caseins from the employed encapsulating matrix could confer stability to our lactobacilli strains when exposed to the tested temperatures. Moreover, different recent studies have demonstrated that application of milk proteins as encapsulating materials (reconstituted skim milk, casein protein, sweet whey) conferred improved thermal stability to probiotic bacteria and higher survival rates than other materials.[17,24,25]

Application of mid–high temperatures, such as those tested in this study, is common during the manufacturing of cereal bars, chocolate bars, candies, sausages or cheeses that may contain selected beneficial bacteria. For this reason, the high resistance of microencapsulated lactobacilli to the mid-heat treatments tested in our study is relevant due to their potential application for production of certain foods that require mild heating processes, and even pasteurization (62.5 °C for 30 min). Thus, the thermal protection conferred to lactobacilli by microencapsulation in a matrix of milk

proteins would increase the range of food products in which microencapsulated lactobacilli could be applied.

Effect of simulated gastrointestinal condition on microencapsulated lactobacilli

Before reaching the intestinal tract, probiotic bacteria must survive the passage through the stomach, where pH values range between 2.0 and 3.0, and the time that food (and microorganisms) remain is of around 2–3 h.[13] These conditions were simulated *in vitro* to evaluate the effectiveness of the microcapsules for protecting the beneficial *Lactobacillus* spp. Both free lactobacilli strains showed a decrease in viability when they were subjected to low pH values. L23 counts decreased from an initial value of 9.03 log CFU mL^{-1} to 6.75 and 6.96 log CFU mL^{-1} after 120 min of exposure to pH 2 and 2.5, respectively (Fig. 4 (A,B)). With free L60 strain, the bacterial population decreased to values of 6.11 and 6.72 log CFU mL^{-1} after treatment at pH 2 and 2.5 for 120 min, respectively, from an initial count of 8.67 log CFU mL^{-1} (Fig. 4 (C,D)). When both lactobacilli were microencapsulated, the mean log bacterial counts were higher than 8.5 log CFU mL^{-1} under both tested acid conditions (Fig. 4 (A–D)), without showing significant differences compared with the initial mean log counts ($P > 0.05$). Viability values of microencapsulated lactobacilli were significantly higher compared with free bacteria ($P < 0.05$). The survival rates achieved by microencapsulated lactobacilli ranged between 96.61% and 97.74%. There were no significant differences between survival rates of both microencapsulated strains ($P > 0.05$). These high survival values are similar to those reported by Bosnea *et al.*,[22] although those authors tested a complex coacervation method using whey proteins and gum arabic as encapsulating materials. In contrast, our results differ from those reported by Afzaal *et al.*[4] and Fazilah *et al.*,[54] who worked with other microencapsulated lactic acid bacteria (LAB) in different materials and observed a reduction in cell viability up to 4.28 log after 2 h in simulated gastric juice. In another study, Afzaal *et al.*[55] reported 1.68 and 2.77 log reduction of *Bifidobacterium* spp. microencapsulated with sodium alginate and k-carrageenan after exposure to simulated gastric conditions. It is important to highlight that the results of our study showed survival values significantly higher with microencapsulated lactobacilli in comparison with recent studies, which used other microencapsulation methods but obtained survival rates ranging from 75% to 82.86% with different bacterial strains.[19,46,56] The high survival rates found in this study (>96.5%) could be due to the buffering capacity of milk proteins used as encapsulating material, as has already been reported by Iqbal *et al.*[18] and Ramos *et al.*[20] Thus, milk proteins could increase the local pH value within the protein matrix of microcapsules, improving the survival of lactobacilli under the extreme conditions of low pH tested.

The presence of bile in the upper parts of the small intestine is one of the main factors affecting the viability of probiotic

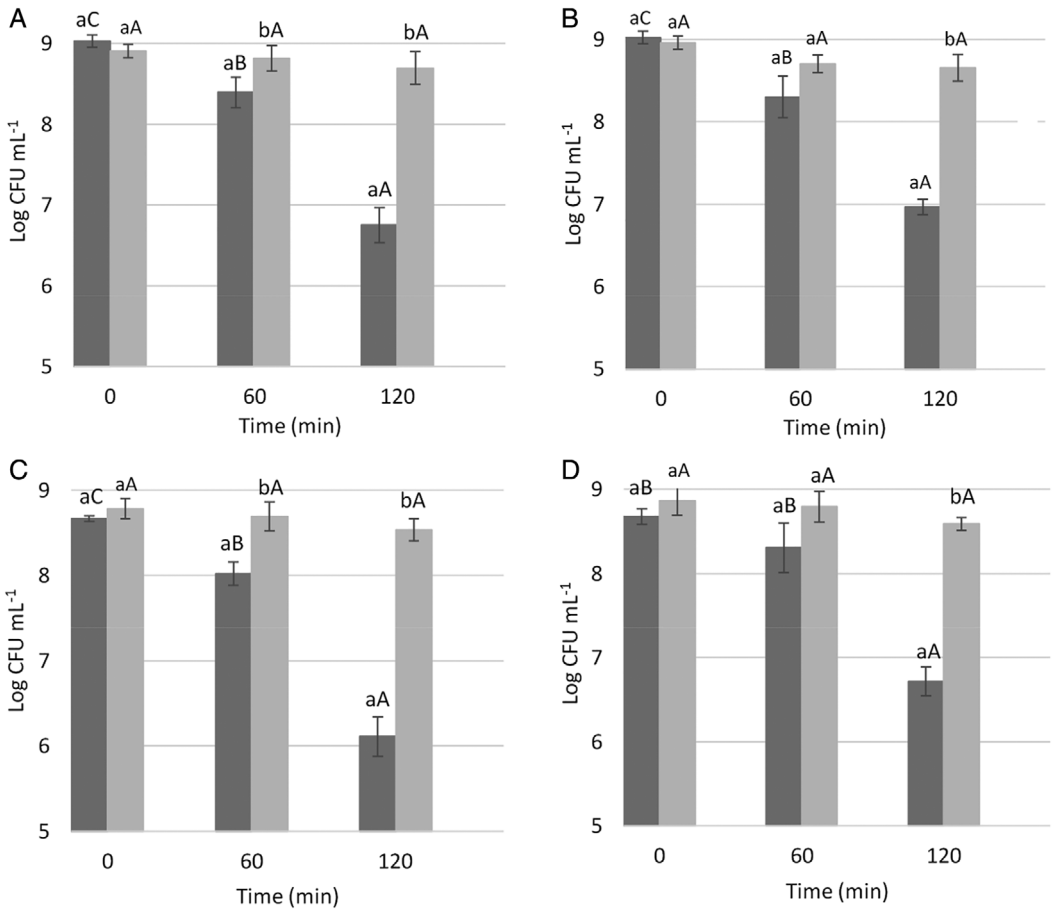

Figure 4. Survival of free and microencapsulated lactobacilli under simulated gastric conditions: (A) *L. fermentum* L23, pH 2 ; (B) *L. fermentum* L23, pH 2.5 ; (C) *L. rhamnosus* L60, pH 2 ; (D) *L. rhamnosus* L60, pH 2.5. ■, free lactobacilli counts; ■microencapsulated lactobacilli counts. Results are the mean of three replicates and error bars represent the standard deviation. Different lower-case letters indicate significant differences between different color bars; different upper-case letters indicate significant differences between the same color bars. All statistical studies were performed using Tukey's test ($P < 0.05$).

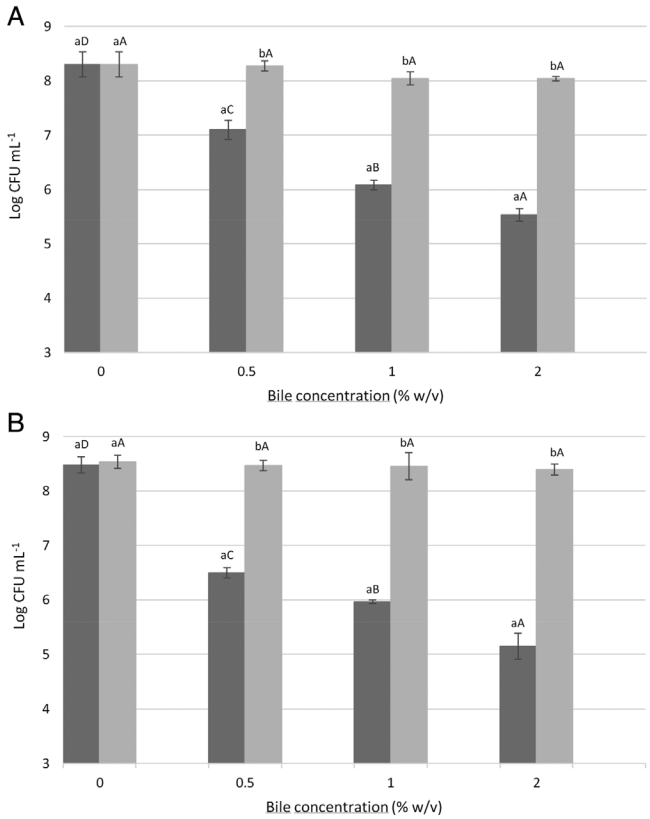

Figure 5. Survival of free and microencapsulated *Lactobacillus* after exposure to different bile concentrations during 120 min: (A) *L. fermentum* L23; (B) *L. rhamnosus* L60. ■, free lactobacilli counts; ■, microencapsulated lactobacilli counts. Results are the mean of three replicates and error bars represent the standard deviation. Different lower-case letters indicate significant differences between different color bars; different upper-case letters indicate significant differences between the same color bars. All statistical studies were performed using Tukey's test ($P < 0.05$).

microorganisms. Under normal physiological conditions, the intestine has bile salt concentrations ranging from 0.05% to 2%.[12] Thus, the effect of different bile concentrations on the viability of microencapsulated lactobacilli compared with controls (free lactobacilli) was evaluated (Fig. 5). In this experience, the initial concentrations of *L. fermentum* and *L. rhamnosus* L60 were 8.3 and 8.5 log CFU mL^{-1}, respectively. After treatments with 0.5%, 1% and 2% bile, free *L. fermentum* L23 showed a decrease in bacterial counts of 1.2, 2.2 and 2.8 log CFU mL^{-1}, respectively. For free *L. rhamnosus* L60, the bacterial counts were reduced in 1.98, 2.51 and 3.33 log CFU mL^{-1} after treatment with increasing concentrations of bile. In contrast, the bacterial population of both microencapsulated lactobacilli had a decrease lower than 0.3 log CFU mL^{-1}, maintaining counts higher than 8 log CFU mL^{-1} at all bile concentrations tested. Both microencapsulated lactobacilli showed mean bacterial counts significantly higher than controls ($P < 0.05$) for all tested bile concentrations. The survival rates of microencapsulated L23 and L60 strains were higher than 96% in all treatments, without statistically significant differences in relation to the initial log counts ($P > 0.05$). Both microencapsulated strains showed similar survival rates after the different bile treatments. The findings of this study did not coincide with those observed by Fazilah *et al.*,[54] who reported that another microencapsulated LAB strain had a marked viability reduction (~3 log CFU mL^{-1}) at 0.6% (w/v) bile salts. In this study, we demonstrated that the lactobacilli microencapsulation in the tested encapsulating material effectively protected *L. fermentum* L23 and *L. rhamnosus* L60 strains when exposed to simulated gastrointestinal conditions. Indeed, the microencapsulation process carried out would represent a valuable strategy potentially applicable in certain foods for the effective delivery of these probiotic bacteria.

CONCLUSIONS

Microencapsulated *L. fermentum* L23 and *L. rhamnosus* L60 exposed to long-term refrigerated storage, mid–high processing temperatures and simulated gastrointestinal conditions showed higher survival rates in comparison with free bacteria. Therefore, the microencapsulation process together with the matrix of milk proteins used in this study protected lactobacilli strains against these first simulated technological and physiological conditions. These findings suggest that this microencapsulation method could contribute to securing optimal amounts of living lactobacilli cells able to reach the intestine. Nevertheless, further *in situ* studies are being carried out to evaluate the viability and functionality of these microencapsulated bacteria, applied in different food matrixes.

ACKNOWLEDGEMENTS

This work was supported by the Secretaría de Ciencia y Técnica, Universidad Nacional de Río Cuarto, Córdoba, Argentina. MJ García has a postdoctoral fellowship from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Argentina.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1 Sarao LK and Arora M, Probiotics, prebiotics, and microencapsulation: a review. *Crit Rev Food Sci Nutr* **57**:344–371 (2017).
2 Tiani KA, Yeung TW, McClements DJ and Sela DA, Extending viability of *Lactobacillus plantarum* and *Lactobacillus johnsonii* by microencapsulation in alginate microgels. *Int J Food Sci Nutr* **69**:155–164 (2018).
3 Douillard FP and de Vos WM, Biotechnology of health-promoting bacteria. *Biotechnol Adv* **37**:e107369 (2019). https://doi.org/10.1016/j.biotechadv.2019.03.008.
4 Afzaal M, Saeed F, Arshad MU, Nadeem MT, Saeed M and Tufail T, The effect of encapsulation on the stability of probiotic bacteria in ice cream and simulated gastrointestinal conditions. *Probiotics Antimicrob Proteins* **11**:1348–1354 (2019).
5 Dey G, Non-dairy probiotic foods: innovations and market trends, in *Innovations in Technologies for Fermented Food and Beverage Industries. Food Microbiology and Food Safety*, ed. by Panda S and Shetty P. Springer, Cham, pp. 159–173 (2018). https://doi.org/10.1007/978-3-319-74820-7_9.
6 Terpou A, Papadaki A, Lappa IK, Kachrimanidou V, Bosnea LA and Kopsahelis N, Probiotics in food systems: significance and emerging strategies towards improved viability and delivery of enhanced beneficial value. *Nutrients* **11**:e1591 (2019). https://doi.org/10.3390/nu11071591.
7 Vecchione A, Celandroni F, Mazzantini D, Senesi S, Lupetti A and Ghelardi E, Compositional quality and potential gastrointestinal behavior of probiotic products commercialized in Italy. *Front Med* **5**:e59 (2018). https://doi.org/10.3389/fmed.2018.00059.
8 Phoem AN, Chanthachum S and Voravuthikunchai SP, Applications of microencapsulated *Bifidobacterium longum* with *Eleutherine americana* in fresh milk tofu and pineapple juice. *Nutrients* **7**:2469–2484 (2015).
9 Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B *et al.*, International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* **11**:506–514 (2014).

10 Iravani S, Korbekandi H and Mirmohammadi SV, Technology and potential applications of probiotic encapsulation in fermented milk products. *J Food Sci Technol* **52**:4679–4696 (2015).
11 Vijaya Kumar B, Vijayendra SV and Reddy OVS, Trends in dairy and non-dairy probiotic products: a review. *J Food Sci Technol* **52**:6112–6124 (2015). https://doi.org/10.1007/s13197-015-1795-2
12 Ruiz L, Margolles A and Sánchez B, Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Front Microbiol* **4**:e396 (2013). https://doi.org/10.3389/fmicb.2013.00396.
13 Ilha EC, da Silva T, Goulart Lorenz J, de Oliveira Rocha G and Sant' Anna ES, *Lactobacillus paracasei* isolated from grape sourdough: acid, bile, salt, and heat tolerance after spray drying with skim milk and cheese whey. *Eur Food Res Technol* **240**:977–984 (2015). https://doi.org/10.1007/s00217-014-2402-x
14 Ashwar BA, Gani A, Gani A, Shah A and Masoodi FA, Production of RS4 from rice starch and its utilization as an encapsulating agent for targeted delivery of probiotics. *Food Chem* **239**:287–294 (2018).
15 El-Salam MHA and El-Shibiny S, Preparation and properties of milk proteins-based encapsulated probiotics: a review. *Dairy Sci Technol* **95**:393–412 (2015). https://doi.org/10.1007/s13594-015-0223-8
16 Shori AB, Microencapsulation improved probiotics survival during gastric transit. *HAYATI J Biosci* **24**:1–5 (2017). https://doi.org/10.1016/j.hjb.2016.12.008
17 Gul O and Atalar I, Different stress tolerance of spray and freeze dried *Lactobacillus casei* Shirota microcapsules with different encapsulating agents. *Food Sci Biotechnol* **28**:807–816 (2018). https://doi.org/10.1007/s10068-018-0507-x
18 Iqbal UH, Westfall S and Prakash S, Novel microencapsulated probiotic blend for use in metabolic syndrome: design and *in-vivo* analysis. *Artif Cells Nanomed Biotechnol* **46**:S116–S124 (2018).
19 Yasmin I, Saeed M, Pasha I and Zia MA, Development of whey protein concentrate–pectin–alginate based delivery system to improve survival of *B. longum* BL-05 in simulated gastrointestinal conditions. *Probiotics Antimicrob. Proteins* **11**:413–426 (2018). https://doi.org/10.1007/s12602-018-9407-x
20 Ramos PE, Cerqueira MA, Teixeira JA and Vicente AA, Physiological protection of probiotic microcapsules by coatings. *Crit Rev Food Sci Nutr* **58**:1864–1877 (2018).
21 Moghaddas Kia E, Alizadeh M and Esmaiili M, Development and characterization of probiotic UF Feta cheese containing *Lactobacillus paracasei* microencapsulated by enzyme based gelation method. *J Food Sci Technol* **55**:3657–3664 (2018).
22 Bosnea LA, Moschakis T and Biliaderis CG, Complex coacervation as a novel microencapsulation technique to improve viability of probiotics under different stresses. *Food Bioproc Technol* **7**:2767–2781 (2014). https://doi.org/10.1007/s11947-014-1317-7
23 Soukoulis C, Behboudi-Jobbehdar S, Yonekura L, Parmenter C and Fisk I, Impact of milk protein type on the viability and storage stability of microencapsulated *Lactobacillus acidophilus* NCIMB 701748 using spray drying. *Food Bioproc Technol* **7**:1255–1268 (2014). https://doi.org/10.1007/s11947-013-1120-x
24 Pinto SS, Verruck S, Vieira CRW, Prudencio ES, Amante ER and Amboni RDMC, Influence of microencapsulation with sweet whey and prebiotics on the survival of *Bifidobacterium*-BB-12 under simulated gastrointestinal conditions and heat treatments. *LWT – Food Sci Technol* **64**:1004–1009 (2015). https://doi.org/10.1016/j.lwt.2015.07.020
25 Dianawati D, Lim SF, Ooi YBH and Shah NP, Effect of type of protein-based microcapsules and storage at various ambient temperatures on the survival and heat tolerance of spray dried *Lactobacillus acidophilus*. *J Food Sci* **82**:2134–2141 (2017).
26 Burgain J, Corgneau M, Scher J and Gaiani C, Encapsulation of probiotics in milk protein microcapsules, in *Microencapsulation and Microspheres for Food Applications*, ed. by LMC S. Academic Press, London, pp. 391–406 (2015).
27 Pascual LM, Daniele MB, Giordano W, Pájaro MC and Barberis IL, Purification and partial characterization of novel bacteriocin L23 produced by *Lactobacillus fermentum* L23. *Curr Microbiol* **56**:397–402 (2008).
28 Pascual LM, Daniele MB, Ruiz F, Giordano W, Pájaro C and Barberis L, *Lactobacillus rhamnosus* L60, a potential probiotic isolated from human vagina. *J Gen Appl Microbiol* **54**:141–148 (2008).
29 Pascual L, Ruiz F, Giordano W and Barberis IL, Vaginal colonization and activity of the probiotic bacterium *Lactobacillus fermentum* L23 in a murine model of vaginal tract infection. *J Med Microbiol* **59**:360–364 (2010).
30 Ruiz F, Efecto terapéutico de las bacteriocinas producidas por las cepas de *Lactobacillus fermentum* y *Lactobacillus rhamnosus* sobre microorganismos causales de infecciones genitales. Doctoral thesis, Universidad Nacional de Río Cuarto. Río Cuarto, Córdoba, Argentina (2013).
31 Gerbaldo G, Barberis C, Pascual L, Dalcero A and Barberis L, Antifungal activity of two *Lactobacillus* strains with potential probiotic properties. *FEMS Microbiol Lett* **332**:27–33 (2012).
32 Ruiz F, Gerbaldo G, García MJ, Giordano W, Pascual L and Barberis IL, Synergistic effect between two bacteriocin-like inhibitory substances produced by lactobacilli strains with inhibitory activity for *Streptococcus agalactiae*. *Curr Microbiol* **64**:349–356 (2012).
33 Ruiz F, Pascual L, Giordano W and Barberis L, Bacteriocins and other bioactive substances of probiotic lactobacilli as biological weapons against *Neisseria gonorrhoeae*. *FEMS Pathog Dis* **73**:1–10 (2015).
34 García MJ, Ruíz F, Asurmendi P, Pascual L and Barberis L, Searching potential candidates for development of protective cultures: evaluation of two *Lactobacillus* strains to reduce *Listeria monocytogenes* in artificially contaminated milk. *J Food Saf* **40**:e12723 (2020). https://doi.org/10.1111/jfs.12723.
35 Champagne CP and Kailasapathy K, Some current food products with microencapsulated probiotics. *Agro Food Ind Hi Tech* **22**:54–56 (2011). http://www.agro-food-industry.com
36 De Prisco A and Mauriello G, Probiotication of foods: a focus on microencapsulation tool. *Trends Food Sci Technol* **48**:27–39 (2016). https://doi.org/10.1016/j.tifs.2015.11.009
37 FAO/WHO (Food and Agriculture Organization of the United Nations and World Health Organization). Food Standards Programme. Codex Committee on Nutrition and Foods for Special Dietary Uses (Fortieth session). Berlin, Germany (2018).
38 Heidebach T, Först P and Kulozik U, Microencapsulation of probiotic cells by means of rennet-gelation of milk proteins. *Food Hydrocolloids* **23**:1670–1677 (2009).
39 Ansari F, Pourjafar H, Jodat V, Sahebi J and Ataei A, Effect of Eudragit S100 nanoparticles and alginate chitosan encapsulation on the viability of Lactobacillus acidophilus and Lactobacillus rhamnosus. *AMB Express* **7**:1–8 (2017). https://doi.org/10.1186/s13568-017-0442-x.
40 Martin MJ, Villoslada FL, Ruiz MA and Morales ME, Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716. *LWT – Food Sci Technol* **53**:480–486 (2013). https://doi.org/10.1016/j.lwt.2013.03.019
41 Favarin L, Laureano-Melo R and Luchese RH, Survival of free and microencapsulated *Bifidobacterium*: effect of honey addition. *J Microencapsul* **32**:329–335 (2015).
42 Chen MY, Zheng W, Dong QY, Li ZH, Shi LE and Tang ZX, Activity of encapsulated *Lactobacillus bulgaricus* in alginate–whey protein microspheres. *Braz Arch Biol Technol* **57**:736–741 (2014). https://doi.org/10.1590/S1516-8913201402377
43 Pan LX, Fang XJ, Yu Z, Xin Y, Liu XY, Shi LE *et al.*, Encapsulation in alginate–skim milk microspheres improves viability of *Lactobacillus bulgaricus* in stimulated gastrointestinal conditions. *Int J Food Sci Nutr* **64**:380–384 (2013). https://doi.org/10.3109/09637486.2012.749841
44 Wang M, Wang C, Gao F and Guo M, Effects of polymerized whey protein-based microencapsulation on survivability of *Lactobacillus acidophilus* LA-5 and physiochemical properties of yoghurt. *J Microencapsul* **35**:504–512 (2018).
45 Afzaal M, Saeed F, Hussain S, Mohamed AA, Alamri MS, Ahmad A *et al.*, Survival and storage stability of encapsulated probiotic under simulated digestion conditions and on dried apple snacks. *Food Sci Nutr* **8**:5392–5401 (2020).
46 Olivares A, Silva P and Altamirano C, Microencapsulation of probiotics by efficient vibration technology. *J Microencapsul* **34**:667–674 (2017).
47 Acordi Menezes LA, de Almeida MCA, Mattarugo NMS, Ferri EAV, Bittencourt PRS, Colla E *et al.*, Soy extract and maltodextrin as microencapsulating agents for *Lactobacillus acidophilus*: a model approach. *J Microencapsul* **35**:705–719 (2018).
48 Riaz T, Iqbal MW, Saeed M, Yasmin I, Hassanin HAM, Mahmood S *et al.*, *In vitro* survival of *Bifidobacterium bifidum* microencapsulated in zein-coated alginate hydrogel microbeads. *J Microencapsul* **36**:192–203 (2019).

49 Jouki M, Khazaei N, Rashidi-Alavijeh S and Ahmadi S, Encapsulation of *lactobacillus casei* in quince seed gum–alginate beads to produce a functional synbiotic drink powder by agro-industrial by-products and freeze-drying. *Food Hydrocoll* **120**:1–10. https://doi.org/10.1016/j.foodhyd.2021.106895
50 Maciel GM, Chaves KS, Grosso CRF and Gigante ML, Microencapsulation of *Lactobacillus acidophilus* La-5 by spray-drying using sweet whey and skim milk as encapsulating materials. *J Dairy Sci* **97**:1991–1998 (2014).
51 Tarifa MC, Piqueras CM, Genovese DB and Brugnoni LI, Microencapsulation of *Lactobacillus casei* and *Lactobacillus rhamnosus* in pectin and pectin–inulin microgel particles: effect on bacterial survival under storage conditions. *Int J Biol Macromol* **179**:457–465 (2021).
52 Corbo MR, Bevilacqua A, Speranza B, di Maggio B, Gallo M and Sinigaglia M, Use of alginate beads as carriers for lactic acid bacteria in a structured system and preliminary validation in a meat product. *Meat Sci* **111**:198–203 (2016).
53 Fathi M, Donsi F and McClements DJ, Protein-based delivery systems for the nanoencapsulation of food ingredients. *Compr Rev Food Sci Food Saf* **17**:920–936 (2018).
54 Fazilah NF, Hamidon NH, Ariff AB, Khayat ME, Wasoh H and Halim M, Microencapsulation of Lactococcus lactis Gh1 with gum arabic and Synsepalum dulcificum via spray drying for potential inclusion in functional yogurt. *Molecules* **24**:1–21 (2019). https://doi.org/10.3390/molecules24071422.
55 Afzaal M, Saeed F, Saeed M, Ahmed A, Ateeq H, Nadeem MT *et al.*, Survival and stability of free and encapsulated probiotic bacteria under simulated gastrointestinal conditions and in pasteurized grape juice. *J Food Process Preserv* **44**:e14346 (2020). https://doi.org/10.1111/jfpp.14346.
56 Qi W, Liang X, Yun T and Guo W, Growth and survival of microencapsulated probiotics prepared by emulsion and internal gelation. *J Food Sci Technol* **56**:1398–1404 (2019).

