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Survival of alginate-microencapsulated *Lacticaseibacillus paracasei* under gastrointestinal conditions in models *in vitro* and *in vivo*

Supervivencia de *Lacticaseibacillus paracasei* microencapsulado en alginato en condiciones gastrointestinales en modelos *in vitro* e *in vivo*

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Abstract

The aim of this work was to evaluate the probiotic potential of the strain *Lacticaseibacillus paracasei*, both free and microencapsulated by ionic gelation, using *in vitro* and *in vivo* models. The strain was microencapsulated with different alginate concentrations (1, 2, 3, and 4 %). After encapsulation, the following were evaluated: survival (%), morphology, particle size (μm), zeta potential, resistance to pH and bile salts, and adhesion of free and encapsulated bacteria in CD-1 mice. The highest resistance to simulated pH 2 and bile salt conditions was obtained with 2 % alginate. The microsphere size ranged between 47.43 and 72.56 μm, with zeta potential from -11.7 to -22.00 (Mv) and oval morphology. The quantification results in mice intestines showed that free and encapsulated bacteria adhered at concentrations of 3 and 6 Log CFU/g ($p \leq 0.05$), respectively. *L. paracasei* is a potential probiotic in the food and pharmaceutical industries.

Keywords: alginate, probiotics, CD-1 mice, microencapsulation, zeta potential.

Resumen

El objetivo de este trabajo fue evaluar el potencial probiótico de la cepa *Lactobacillus paracasei*, libre y microencapsulada mediante gelificación iónica, en modelos *in vitro* e *in vivo*. La cepa se microencapsuló mediante gelificación iónica con diferentes concentraciones de alginato (1, 2, 3 y 4 %). Después de la encapsulación, se evaluaron: sobrevivencia (%), morfología, tamaño de partícula (μm), potencial zeta, resistencia a pH y sales biliares, y adhesión de bacterias libres y encapsuladas a intestinos de ratones CD-1. El tamaño de las microesferas fue de 47.43 a 72.56 μm y el potencial zeta, de -11.7 a -22.0 (mV) con morfología ovalada. Los resultados de la cuantificación en intestinos de ratones mostraron que la bacteria libres y encapsuladas se adhirieron en concentraciones de 3 y 6 Log UFC/g ($p \leq 0.05$), respectivamente. *L. paracasei* es un probiótico potencial en las industrias de alimentos y farmacéutica.

Palabras clave: alginato, probióticos, ratones CD-1, microencapsulación, potencial zeta.

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1 Introduction

Probiotics are a group of bacteria defined by FAO/WHO (2002) as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". They can be used in several conditions, such as diarrhea, candida vaginitis, urinary tract infections, immune disorders, lactose intolerance, hypercholesterolemia, and food allergies (Mombelli and Gismondo, 2000). The recommended concentration of viable cells for probiotic microorganisms to have a beneficial effect on health is commonly > 10^6 – 10^8 CFU/g or 10^8 – 10^{10} CFU/day of the product during the time of consumption (Champagne *et al*., 2011). However, the viability during processing, storage, and passage through the gastrointestinal tract (GIT) is questionable since the bacteria are susceptible to adverse factors; for example, oxygen concentration, acidic pH, and high concentrations of bile salts (Castro-Rosas *et al*., 2020). The microencapsulation of probiotic bacteria consists in protecting the bacteria using a physical barrier against adverse environmental conditions (Martín *et al*., 2015). The production of microcapsules by ionic gelation (IG) is of great interest since it does not demand the use of high temperatures nor organic solvents (Martín *et al*., 2015; Kurozawa and Hubinger, 2017; Quiroz *et al*., 2020). In IG, probiotic organisms (bacteria) are added to a polymeric solution that is dripped through a nozzle or syringe on a cationic hardening solution as calcium chloride (Solanki *et al*., 2013). Alginate is the main polymer used in IG; it is obtained from brown seaweeds (Wang *et al*., 2022) and consists of two molecules: β-d-mannuronic acid (M) and α-L-guluronic acid (G). The molecules constituting M-, G-, and MG- sequential block structures for alginate polymers can form gels that are uniform, water-insoluble, thermo-irreversible, and transparent at room temperature. The gels are produced by chemically cross-linking G with di- or tri-valent ions, and calcium chloride solutions are commonly used in the process (Funami *et al*., 2009; Comaposada *et al*., 2015), which results in microencapsulated probiotics. To produce microspheres with alginate, probiotic cells are mixed in a solution with alginate polymer and homogenized with a sodium alginate solution. The mixture is then dripped into a solution containing multivalent cations, and the droplets form gel spheres quickly and spontaneously, entrapping cells and forming a three-dimensional network (Martín *et al*., 2015).

Extrusion has been recently used to produce capsules (micrometric scale) using IG due to its simplicity, low cost, and flexibility in terms of formulation conditions, which ensure high cell viability (Chun *et al*., 2014; Martín *et al*., 2015). Several studies have focused on the encapsulation of probiotic bacteria using IG (Zou *et al*., 2011; Gebara *et al*., 2013; Shi *et al*., 2013; Holkem *et al*., 2017; Falfán-Cortés *et al*., 2022; Escamilla-Montes *et al*., 2023), evaluating resistance to simulated *in vitro* conditions in the GIT. They have obtained favorable results for the survival of several genera with probiotic potential (Yao *et al*., 2020). Still, the *in vitro* model describes a different portion of the GIT in the reported works, and it is difficult to extrapolate the results to an *in vivo* case. *in vitro* models are more practical to rapidly examine bacterial probiotic potential, yet they cannot precisely simulate the human intestine. Therefore, promising candidates for probiotic delivery systems must be tested in more precise *in vivo* models (Yao *et al*., 2020). Studies on probiotics commonly involve three main components: identification of survival in GIT, safety for human or animal consumption, and establishment of probiotic activity/benefit for the consumer (Ramos *et al*., 2013).

The *in vivo* adhesion to intestine in animal models represents a key parameter for the probiotic action of a microorganism as a prerequisite of GIT colonization (Saxami *et al*., 2012). However, few studies report adhesion using *in vivo* models of encapsulated probiotics (Lee *et al*., 2004; Cook *et al*., 2012; Saxami *et al*., 2012; Ding *et al*., 2023). Then, the aim of this research was to encapsulate *Lacticaseibacillus paracasei* and evaluate the survival of free and encapsulated bacteria against the effect of adverse conditions in models *in vitro* and *in vivo*.

2 Materials and methods

The rifampicin-resistant *Lacticaseibacillus paracasei* bacteria was provided by the Food Microbiology Laboratory at the Autonomous University of the State of Hidalgo. The sodium alginate utilized was acquired from the food additives company FMC Ingredientes Alimenticios (Protanal RF 6650, Mexico).

2.1 Microencapsulation process

The activation of *L. paracasei* cells was carried out according to Hernández-López *et al*. (2018), with a double transfer in MRS broth (BD Difco) to obtain the cell package (1×10^9 CFU/mL). The microencapsulation was performed according to Castro-Rosas *et al*. (2021) with modifications. The solutions were prepared from alginate (1, 2, 3, 4 % w/v) and sunflower oil (1.25 w/v) solutions and homogenized in a 25-SI Ultra Turrax homogenizer (IKA Works, Wilmington, NC, USA) at 14,000 rpm for 5 min. The *L. paracasei* cell package (1×10^9 CFU/mL) was added to the solution and homogenized again at 3000 rpm for 5 s. The final mixture of

alginate-bacteria was atomized into a NaCl solution (1, 2, 3, 4 % w/v) at 9.2 mL/min. The air pressure was maintained at 7.5 psi using a double-fluid atomizer (1 mm diameter, stainless steel). For the hardening stage, the microspheres were maintained in NaCl solution. They were then separated, washed with deionized water (pH 4, 200 mL), and sieved through steel mesh (Ø53 μm). The microspheres were placed in a container and kept under refrigeration at 7 °C until further analysis.

2.2 *Encapsulation efficiency (%)*

The encapsulation efficiency (%) was determined according to Rajam and Anandharamakrishnan (2015). Prior to the IG encapsulation process, an aliquot of the alginate-bacteria mixture was used in serial dilutions (10^{-9}) as follows: The aliquot was mixed with MRS agar (BD Difco) added with rifampin and incubated for 48 h to perform the CFU count (No). After the encapsulation (IG) process, 1 g microspheres was destroyed using a sodium citrate solution (2% w/v). Serial dilutions (10^{-9}) were done and the samples were mixed in MRS agar (BD Difco) added with rifampin using the pour-plate technique. The plates were incubated at 37 °C for 48 h, and the CFU count (No) was performed. The encapsulation efficiency (EE %) was calculated according to Rajam and Anandharamakrishnan (2015):

$$(EE\%) = \frac{N}{No} \times 100 \tag{1}$$

2.3 *Morphology of microspheres with Lacticaseibacillus paracasei*

The morphology of the microspheres was analyzed under an optical microscope (Motic, Xiamen, China). Wet microcapsules (1 g) were suspended in 1 mL sterile water at pH 4, and a sample (20 μL) was taken using a micro pipette and placed on a slide. Each sample was observed at 40 X magnification (Castro-Rosas *et al*., 2021).

2.4 *Average size and zeta potential of microspheres*

The average size and zeta potential of the microspheres were identified according to Castro-Rosas *et al*. (2021). Microspheres with different concentrations (1, 2, 3, 4 %) were dispersed (0.5 g) in 9 mL sterile deionized water at pH 4. The average size of the alginate microspheres was measured in a Mastersizer 2000 equipment (Malvern, Worcestershire, WR, UK) with an LS 13320 Laser Diffraction Particle Size Analyzer and deionized water (pH 4) as dispersing solution. The zeta potential was also determined in a nano ZS90 zetasizer (Malvern, Worcestershire, WR, UK). All measurements were done by triplicate.

2.5 *Lacticaseibacillus paracasei resistance to pH and bile salts in vitro*

Resistance (%) to pH and bile salts *in vitro* was evaluated using free (unencapsulated) and encapsulated bacteria, according to Hernández-López *et al*. (2018). The free bacteria were activated by double transfer in MRS broth (BD Difco) incubated at 37 °C for 24 h. One mL of the culture (10^9 CFU/mL) was used for viable count in MRS agar (BD Difco) added with rifampicin. Free cells (1 mL) were resuspended in 9 mL MRS broth adjusted to pH 2, 3, and 6.5 (control). The cultures were incubated at 37 °C for 3 h, and viable cell count was performed using MRS agar with rifampin. The same process was carried out with microencapsulated bacteria, using microspheres (1 g) resuspended in 9 mL MRS broth adjusted to pH 2, 3, and 6.5 (control). The same methods were followed for free and encapsulated cells to evaluate their resistance to bile salts. The culture (10^9 CFU/mL) was mixed with MRS broth added with bile salts at a concentration of 0.5 and 1.5 %, while the control contained no bile salts (0 %). The resistance to pH and bile salts was calculated according to Kociubinski *et al*. (1999) and using Equations 2 and 3:

$$\% \text{ Resistance to pH} = \frac{\text{CFU pH 2 or 3}}{\text{CFU pH 6.5 control}} \times 100 \tag{2}$$

$$\% \text{ Resistance to bile salts} = \frac{\text{CFU + bile salts}}{\text{CFU pH 6.5 control}} \times 100 \tag{3}$$

2.6 *Quantification of Lacticaseibacillus paracasei in CD-1 mice feces*

The vivarium of the Autonomous University of the State of Hidalgo, Mexico, donated CD-1 male mice aged 8 weeks (n = 8). The animals were assigned to three groups and were kept under controlled temperature (22 ºC ± 2) and 12-h light/dark cycle. They were fed a standard diet and had free access to water (Le *et al*., 2019). After 1 week of acclimation, the mice were placed in individual cages and orally administered daily doses (1×10^9 CFU/mL) of free and encapsulated *Lacticaseibacillus paracasei* (100 μL phosphate buffer as vehicle) via an intragastric tube for 7 days. The control group did not receive the bacteria. Fecal samples were collected daily (every 24 h) for microbiological analyses of *L. paracasei*. The samples (0.05 g each) were diluted in 4.5 mL peptone diluent; 10^{-8} dilutions were done and plated on the selected MRS media added with rifampicin.

The samples were finally incubated at 37 °C for 48 h before enumeration by plate pouring.

2.7 Lacticaseibacillus paracasei adhesion in CD-1 mice intestines

The animal groups in section 2.6 were euthanized by cervical dislocation, and small and large intestines were removed to determine the presence and adherence of *L. paracasei*. Intestines of all groups were washed with phosphate buffer (100 mL) to remove excess feces. Once cleaned, they were homogenized in a peptone diluent solution (1:9 ratio) and serial dilutions were done. Subsequently, *L. paracasei* was quantified (CFU/g) following microbiological plate pouring in MRS agar with rifampicin.

2.8 Statistical analysis

For studies *in vitro* (n = 3) and *in vivo* (n = 8) the differences between the means of the treatments were determined through one-way analysis of variance (one-way ANOVA) and post-hoc Tukey's HSD, with Statistica software v 7.0.

3 Results and discussion

3.1 Encapsulation efficiency (%)

Figure 1 shows the EE % of the microspheres obtained by IG with alginate at different concentrations. The EE % increased by 100 % when the alginate concentration was 4%. However, percentages above 90 were obtained using all the concentrations, making this technique adequate for the encapsulation of *Lacticaseibacillus*. These results were better than those reported by Gebara *et al*. (2013) and Sandoval-Castilla *et al*. (2010). The first authors used the same technique to obtain EE % of 84.35 ± 0.60 % when using pectin and whey as wall materials to encapsulate *Lactobacillus acidophilus* La5. The second group of authors used the same extrusion technique to encapsulate *Lactobacillus casei* with alginate as biopolymer (54.3 ± 3.7 %). In this work, the alginate matrix resulted in higher efficiencies, although *Lacticaseibacillus paracasei* showed more resistance to the encapsulation process. On the other hand, Voo *et al*. (2011) state that extrusion is the principal method used to encapsulate probiotics, and the size and encapsulation yield (%) of microspheres obtained by extrusion can be affected by factors such as nozzle diameter, polymer concentration, and composition chemistry.

Shi *et al*. (2013) microencapsulated *L. bulgaricus* by extrusion, using alginate and milk as wall materials,

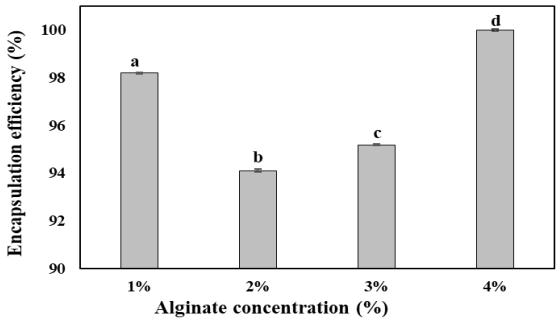


Figure 1. Encapsulation efficiency percentages of microspheres obtained by ionic gelation with alginate at different concentrations (1, 2, 3, and 4 %). Different letters indicate highly significant differences ($p \leq 0.05$).

to obtain EE % around 100. The high efficiencies involved factors as strain and wall material.

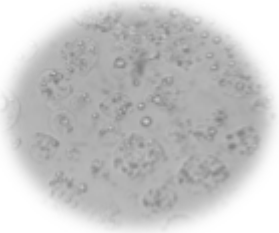
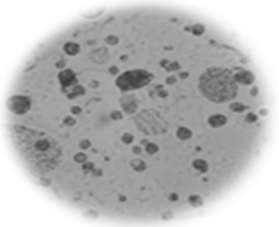
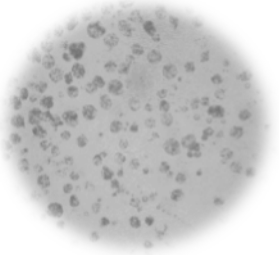
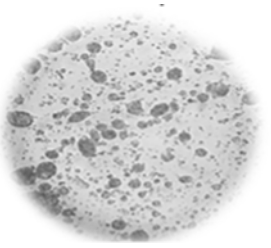
3.2 Morphology, size, and zeta potential of Lacticaseibacillus paracasei microspheres

The microspheres were oval (2 and 4 %) and spherical (1 and 3%), as shown in Table 1. Those with a higher alginate concentration (4 %) measured 47.43 μm, and those with 1% alginate measured 72.56 μm. Therefore, the capsule size is likely related to the alginate concentration in the mix, while higher concentrations result in a smaller contact surface for capsule formation. Rodrigues *et al*. (2014) reported that microcapsules obtained by IG-extrusion using pectin and protein showed particle sizes of 240-260 μm, evidencing that the wall material is key to determine the microparticle size. Tello *et al*. (2015) and Falfán-Cortés *et al*. (2020) reported similar results in encapsulates with alginate.

Recently, Mahmoud *et al*. (2020) microencapsulated using different wall materials (skim milk, dextrin, denatured whey protein, or coated with chitosan) mixed with alginate, and reported particle sizes between 501.541 and 800.739 μm. According to the results obtained in the present work, the size could be adequate to introduce the microspheres in a food matrix without affecting the sensory and physical characteristics of the product. However, it is necessary to carry out tests that show the sensorial effect of particle size in different food matrices (Falfán-Cortés *et al*., 2020).

The zeta potential of the microspheres ranged from -11.7 ± 0.05 to -22.0 ± 0.03, and similar mV values from -0.68 to -16 have been reported for alginate microspheres (You *et al*., 2001; Tello *et al*., 2015). This potential is the product of the reaction between sodium alginate and calcium chloride at different concentrations. In it, (Ca^{2+}) cations interact with alginate COO^- groups.

Table 1. Morphology, size, and zeta potential of *Lactobacillus paracasei* microspheres.

Alginate concentrations (%)	Morphology	Average size (μm)	Zeta potential (mV)
1		72.56 ± 5.2^{a}	-11.7 ± 0.05^{a}
2		52.71 ± 6.2^{b}	-17.35 ± 0.03^{b}
3		58.57 ± 5.0^{b}	-22.0 ± 0.05^{c}
4		47.43 ± 3.0^{c}	-18.05 ± 0.05^{d}

Different superscripts within the same column indicate that the means differ significantly ($p \leq 0.05$). All the experiments were carried out in triplicate.

Then, the value obtained for the 4 % concentration might indicate a saturation of the substituent groups.

3.3 Lacticaseibacillus paracasei resistance to pH and bile salts

Figure 2 shows the resistance (%) of *Lacticaseibacillus paracasei* to pH 2 and 3 (Figure 2a) and different bile salt concentrations (2b). Survival (CFU) was increased by over 50 % at the different alginate concentrations vs free bacteria. The best treatments ($p \leq 0.05$) for resistance against pH 2 were 2, 3, and 4 % alginate ($p \leq 0.05$). These results are similar to those reported by Castro-Rosas *et al*. (2021), who encapsulated *Lactobacillus paracasei* using 2 % alginate by IG-extrusion with percentages of 97-100 % using the same technique for extrusion. Resistance to cells free of bile salts was low (under 40 %) at both concentrations ($p \leq 0.05$). Figure 2b shows that 2 % alginate was the best treatment (> 90 %) for resistance against bile salts with high concentration (1.5%) ($p \leq 0.05$). Then, this treatments was selected for *in vivo* studies. Regardless of *in vivo* tests, *in vitro* studies are important to identify the bacterial potential, as established in the guidelines for the evaluation of strains as probiotic candidates (Byakika *et al*., 2019).

3.4 Lacticaseibacillus paracasei quantification in CD-1 mice feces and intestines

Figure 3 shows the results of *L. paracasei* quantification in CD-1 mice feces. The sampling was carried out across 7 days, and the concentrations of free and encapsulated bacteria varied from 2.5 to 7.5 Log CFU/g feces.

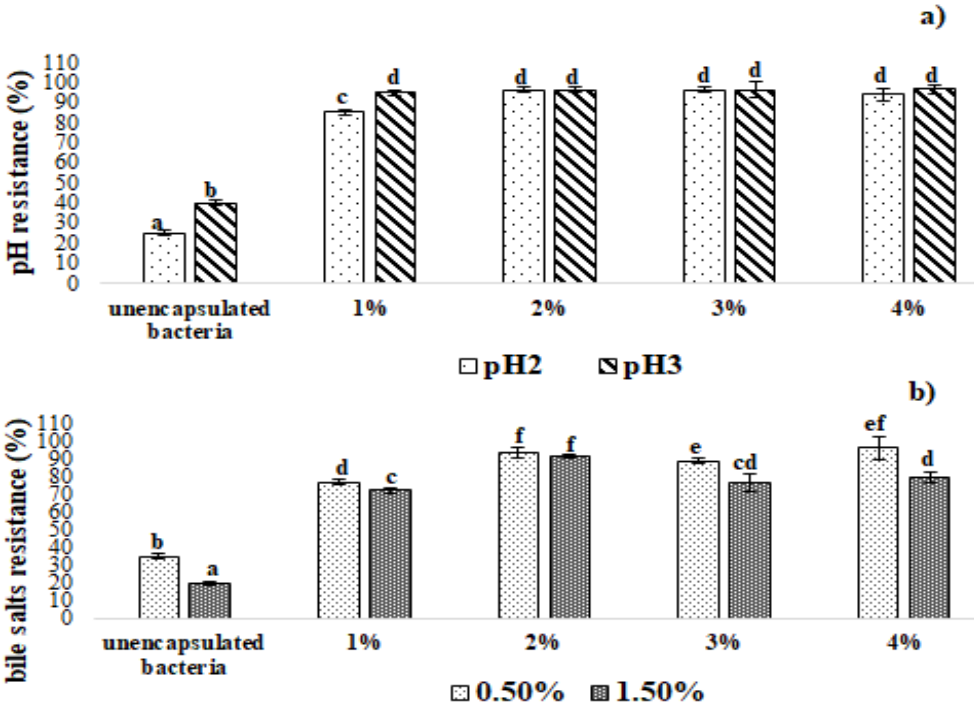


Figure 2. Resistance (%) of free and encapsulated *Lacticaseibacillus paracasei* at pH 2 and 3 (Figure 2a), and high concentrations (0.50 and 1.5 %) of free and encapsulated bile salts (2b). Different letters indicate significant differences ($p \leq 0.05$).

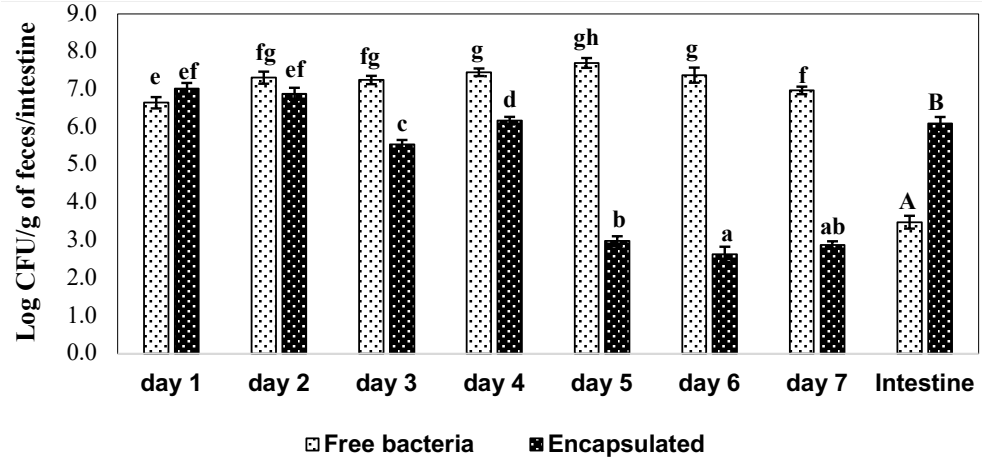


Figure 3. Quantification of free and encapsulated *L. paracasei* in CD-1 mice feces and intestines. Different lowercase letters indicate a significant difference ($p \leq 0.05$) in bacterial concentration in feces and capital letters, in mice intestines ($p \leq 0.05$).

At the end of the experiment, the cell concentration in feces was lower in encapsulated bacteria. Nevertheless, after the animals were euthanized, bacteria in the small and large intestines were quantified. *L. paracasei* was not detected in animals that were not administered with the bacteria, while the largest *L. paracasei* concentration was observed in samples from mice administered the encapsulated bacteria as compared to those given free bacteria ($p \leq 0.05$). Encapsulation likely allowed the bacteria to arrive and adhere at a higher rate than that of free cells, so feces presented the lowest eliminated concentrations. Adhesion is the first major criterion for probiotic microorganisms beneficial to health. Kučan *et al*. (2012) reported that probiotic quantification in feces is a viable method to determine whether the strains survive the conditions of passage through the GIT *in vivo*. According to the results they obtained,

the bacteria showed resistance to passage through mouse GIT. Similar results were reported by Saxami *et al*. (2012) in *Lactobacillus casei* ATCC 393. They found that the concentration of bacteria adhered to Wistar rat intestines was 2-6 Log CFU/g after administration for 7 days. The authors explained that adhesion to the GIT was transitory; therefore, daily consumption was suggested to maintain levels with effective concentrations.

Ouwehand and Salminen *et al*. (2003) stated probiotic bacteria adhesion depends on several factors *in vitro* and *in vivo*. In the first case, the variables include bacterial concentration (CFU), growth buffer, incubation time, and culture; while the second considers normal intestinal microbiota and food matrix (probiotic vehicle). Servin and Coconnier (2003) explain that "although it is believed that the maximum probiotic effect is achieved if the organisms adhere to intestinal mucosal cells, there is little evidence that exogenously administered probiotics in fact do this". They add that, in order to achieve a probiotic effect, the intake of the microorganism must be continuous. Adhesion to epithelial cells is complex because two membranes cells are involved: host and microbial. Then, it has been proposed that adhesion depends on the chemical and physicochemical composition of the cell surface of the probiotic strain, as a result of the equilibrium of electrostatic charge and Van der Waals interactions on the host's surface (Duary *et al*., 2011; Melo-Pereira *et al*., 2018). Furthermore, the mechanisms of bacterial adhesion are multiple. Bacteria can synthesize a large number of extracellular components, as exopolysaccharides and proteins, to form an S layer. The layer works as a protector against the hostile environment and helps in the adaptation of cells to different stress factors. Ouwehand and Salminen (2003) describe that most of the models used to assess probiotic adhesion *in vitro* represent simplifications of *in vivo* conditions.

It is important to follow the guidelines across phases of criteria to characterize strains with probiotic potential. Few reports evaluate the adhesion of microencapsulated probiotic bacteria in models *in vivo*. Nambiar *et al*. (2018) evaluated the survival of *Lactobacillus plantarum* HM47 microencapsulated by spray drying when the capsules were added to milk chocolate. The authors used a Swiss albino mice model to evaluate acute oral toxicity in mice when administering the encapsulated bacteria. They reported that the administration of the probiotic powder in milk chocolate showed no adverse effects in hematological parameters and vital organs of mice. In addition, the bacterial count of intestinal lactic acid was improved while enteric pathogenic bacteria were reduced in the subjects, suggesting the intestinal colonization by HM47. Recent studies using models *in vivo* to report the effects of symbiotic encapsulation have proved the benefits of bacteria microencapsulated with prebiotics (Jiménez-Villeda *et al*., 2023).

Conclusions

The microencapsulation of *Lacticaseibacillus paracasei* bacteria using ionic gelation by extrusion increased the resistance to simulated gastrointestinal tract conditions. In addition, the encapsulated bacteria adhered to CD-1 mice intestines across a 7-day period. Therefore, it is evident that encapsulation allows for the increase in *L. paracasei* viability in *in vitro* and *in vivo* models. Finally, there is an area of opportunity to identify the effects of adhesion and colonization, along with benefits of probiotic bacteria in models *in vivo*.

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