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MICROENCAPSULATION OF *Lactobacillus acidophilus* LA-5 INCREASES RELATIVE SURVIVAL UNDER SIMULATED GASTROINTESTINAL TRACT STRESS

LA MICROENCAPSULACIÓN DE *Lactobacillus acidophilus* LA-5 INCREMENTA LA SOBREVIVENCIA BAJO LAS CONDICIONES SIMULADAS DE ESTRÉS GASTROINTESTINAL

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Abstract

This paper focused on increasing the relative survival of *Lactobacillus acidophilus* LA-5 when exposed to a sequential simulated gastrointestinal tract stress by the immobilization in microparticles of alginate formed by an emulsification and external gelation method. The aqueous phase consisted of 4% alginate and the oil phase consisted of mineral oil and Span 80 at 1%. The microparticles were manufactured at 250 rpm, at 20°C for 30 min. The free microorganism did not survive on the all simulated gastrointestinal track stress tested. In contrast, after stress the relative survival of *L. acidophilus* LA-5 microencapsulated was 46.9%. The microparticles performed had the ability to protect and liberate the probiotics cells sensitive to gastrointestinal stress, making then suitable for use as protective agents, which may have applications in the food or pharmaceutical industries.

Keywords: microencapsulation, viability, controlled release, gastrointestinal transit, microencapsulation, probiotics.

Resumen

Este trabajo tuvo el objetivo de incrementar la sobrevivencia relativa de *L. acidophilus* LA-5 cuando fue expuesta a estrés del tracto gastrointestinal simulado de manera secuencial por medio de la inmovilización en micropartículas de alginato formadas por el método de emulsificación y gelación externa. El sistema de inmovilización consistió en una fase acuosa de alginato al 4% y una fase de aceite mineral y 1% de Span 80. Las micropartículas fueron elaboradas con una velocidad de agitación de 250 rpm a 20 °C durante 30 min. Las bacterias libres no sobrevivieron a todas las condiciones de estrés gastrointestinal simulado. En contraste, después del estrés *L. acidophilus* LA-5 microencapsuladas, mostró una sobrevivencia relativa del 46.9%. Las partículas producidas presentaron la habilidad de proteger y liberar las células probióticas sensible al estrés gastrointestinal, por lo que estas partículas actúan como un agente protector, lo que presenta interesantes perspectivas para la industria de alimentos y farmacéutica.

Palabras clave: microencapsulación, viabilidad, liberación controlada, tránsito gastrointestinal, probioticos.

1 Introduction

Probiotics are living organisms that, when ingested in adequate doses, produce beneficial health effects in the host (Trabelsi *et al*., 2013; Smilkov *et al*., 2014). Consequently, probiotics, particularly *Lactobacillus* and *Bifidobacterium* spp. (Trabelsi *et al*., 2013), are available in various foods, dietary supplements and medicinal foods (Bhadoria and Mahapatra, 2011) due to the many benefits they can provide. In order for a probiotic exert a beneficial effect in the host, at least 10^8 CFU of the food product should be consumed to yield the minimum daily intake (Chopde *et al*., 2014). However, some probiotics are further encounter sequential several obstacles during gastro-intestinal transit (pH, enzymes, bile salts), that limit the survival and functionality of probiotic for the conveyance of health benefits (González-Vázquez *et al*., 2014).

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Lactobacillus acidophilus LA-5 is a probiotic bacterium, used due to its influence on recolonization and modulation of the intestinal microbiota (Matijasic *et al*., 2016) and improved health (Tonucci *et al*., 2017). However, it shows limited ability to survive under gastrointestinal tract conditions, Gebara *et al*. (2013) observed a 6 log decrease in *L. acidophilus* LA-5 viability after exposure to simulated gastric juice (pH 1.2) for 1 h and a 5 log reduction after incubation in simulated intestinal juice (pH 7) for 5 h.

Microencapsulation is used to protect probiotics from the gastrointestinal stress, and provides a barrier to diffusion of environmental components of the simulated gastrointestinal tract thus achieving a greater number of viable cells at the site of action (Gbassi and Vandamme, 2012; Mendoza-Madrigal *et al*., 2017). There are several methods available to protect probiotic cells, such as extrusion, emulsification, spray drying, fluid bed, freeze drying and complex coacervation (Rokka and Rantamäki, 2010; Gerez *et al*., 2012). Extrusion and emulsification are the most widely used methods for the protection of *Lactobacillus* (Trabelsi *et al*., 2013). Both methods are based on the immobilization of the microorganism within a biocompatible material (Gbassi and Vandamme, 2012) such as calcium alginate, which is frequently used because of its easy handling, low cost and non-toxicity, characteristics that make it appropriate for use as a food additive (Li *et al*., 2009; Trabelsi *et al*., 2013). One advantage of immobilization by emulsification is the production of particle sizes less than 1000 μm, allowing these to be incorporated into food without affecting its texture and organoleptic properties (Hansen *et al*., 2002). Microparticles formed during microencapsulation refers to a particle of size ranged between 1 - 1000 μm, made of solids or small droplets of liquids surrounded by a wall formed of a variety of materials of varying thickness and degree of permeability, used as reservoir for biological molecules with a controlled release rate (Sharma *et al*., 2016; Fuentes-Ortega *et al*., 2017). Viscosity, mixing speed, mixing time and mixing temperature are some of important variables during microparticle production, that have an impact on the size of the particle (Silva *et al*., 2006; Wang *et al*., 2008) and load capacity, which are important parameters to ensure that the viable probiotic cells reach the site of action (Chandramouli *et al*., 2004; Mandal *et al*., 2006; Smilkov *et al*., 2014).

In this context, to contribute to technological solutions for the development of a system for microparticles formation and the adequate release and survival of probiotic cells, this study focused on increasing the relative survival of *L. acidophilus* LA-5 when exposed to sequential simulated gastrointestinal tract stress fluids. The microparticles loaded with *L. acidophilus* LA-5 were prepared by an emulsification and external gelation method, at a controlled speed, mixing time and mixing temperature.

2 Materials and methods

2.1 Materials

Sodium alginate (Sigma-Aldrich, USA), lysozyme and calcium chloride (CaCl2) (Merck, Germany); sodium hydroxide and hydrochloric acid (JT Baker, Mexico), potassium phosphate monobasic (Fermont, Mexico), NF55 mineral oil food grade (Droguería Cosmopolita, Mexico), Span 80® (Fluka, Germany), Man Rogosa Sharpe (MRS) culture broth and agar (Difco, USA), pepsin and oxgall (Meyer, Mexico) were used. *L. acidophilus* LA-5 was kindly donated by Christian Hansen Laboratories, Mexico City.

2.2 L. acidophilus LA-5 growth conditions

The lyophilized *L. acidophilus* LA-5 cells were added to MRS culture broth. After aerobic incubation at 37 °C for 48 h, the culture was centrifuged at 13130 × g at 4 °C for 15 min using a Sorvall® RC-5B centrifuge (DuPont Instrument, USA). The pellet was suspended in fresh medium supplemented with 5% dextrose and incubated under aerobic conditions at 37 °C for 48 h (Gebara *et al*., 2013). Afterwards, the cells were concentrated at 13130 × g for 15 min using the Sorvall® RC-5B and stored in MRS broth containing 20% glycerol at -20 °C until use. A modified method of Reyes-Nava *et al*. (2016) was used to determine the viable cell count. Briefly, six decimal dilutions were performed using sterile water and 5 μL of each dilution were plated onto MRS agar medium, in triplicate. The plates were incubated at 37 °C for 24 h and the cell viability then assessed. The results are expressed as CFU/mL.

2.3 Optimization of mixing conditions for microparticle formation

A 2^3 full factorial design, with three replicates, was used to optimize the conditions for microparticle formation.

Table 1 Coding and values of each independent experimental variable evaluated.

Treatments	Aleatory order	Level of mixing			Values of mixing			Particle size
		speed (rpm)	time (min)	Temperature (°C)	speed (rpm)	time (min)	Temperature (°C)	Mean (μm)
bc	7	-1	1	1	250	45	20	89.3
ab	4	1	1	-1	350	45	15	56.8
abc	8	1	1	1	350	45	20	67.8
a	2	1	-1	-1	350	30	15	70.2
1	1	-1	-1	-1	250	30	15	77
c	5	-1	-1	1	250	30	20	93.4
b	3	-1	1	-1	250	45	15	84.5
ac	6	1	-1	1	350	30	20	56.1

The independent experimental variables were mixing time, mixing speed and mixing temperature. In all experiments, $CaCl_2$ and alginate were 170 mM and 4%, respectively (Chandramouli *et al*., 2004; Mandal *et al*., 2006).

The response variable was the mean particle size. Table 1 shows both the factors and levels of the experimental design. The results were used to determine the manufacturing conditions that could produce microparticles in range of 100 μm because it is known that particle sizes less than 1000 μm are amenable to the immobilization of probiotics intended for use in foods without causing adverse effects on their organoleptic properties (Hansen *et al*., 2002). In this work, microparticles were defined as the material in the order of micrometers, constituted by alginate with the aim of immobilizing and protect probiotics.

2.4 *Microparticle formation by emulsification*

The microparticles were formed without and with *L. acidophilus* LA-5. Microparticles without the probiotic were prepared by an emulsion (W/O, 32:68) formed by mixing 16 mL sodium alginate (4%) with 35 mL mineral oil and Span 80 at 1%, using a propeller mixer (IKA® RW20, USA) with a 50 mm diameter propeller (IKA® R1342, USA), under the conditions described in Table 1. The temperature was maintained by using a thermostatic bath circulator (LAUDA Alpha RA 12, USA). Once the emulsion was formed, 44 mL $CaCl_2$ (170 mM) was added at a rate of approximately 10 mL/min, with stirring that continued for 15 min to produce a suspension of the microparticle system (MS). Gelation was completed by refrigeration (4 °C) of the MS for 18 h.

After analyzing the results obtained from the treatments in Table 1, it was decided to use the following conditions for the microparticles with the probiotic: mixing speed 250 rpm, mixing temperature 20 ºC, and mixing time 30 min. To form the MS, the same methodology to form microparticles without the probiotic was followed, and 600 μL of a concentrated solution of *L. acidophilus* LA-5 (9.6×10^7 CFU) was added to the sodium alginate solution (4%).

2.5 *Quantification of immobilized cells*

The plate count method was used to determine the number of microorganisms in the MS. In brief, 100 μL of the MS was suspended in 900 μL of sterilized water and serial dilutions were conducted. Then, 5 μL of each dilution was plated onto MRS agar medium and incubated at 37 ºC for 24 h (Reyes-Nava *et al*., 2016).

The number of immobilized microorganisms was determined by the difference between the probiotics in the MS (1.01×10^6 CFU/mL of MS) and the values determined by the plate count, considering them as non-immobilized since no treatment was performed to release them. This difference was determined in this way because to disintegrate the microparticles is necessary an acid process, which in turns would affect the viability of microorganisms.

2.6 *Determination of size and morphology of the microparticles*

The sizes of the microparticles were evaluated using a laser scattering particle size distribution analyzer (HORIBA LA-950V2, Japan), with distilled

water as the dispersing medium and following the manufacturer's instructions. The mean diameter and size distribution of microparticles were measured at 25.0 °C ± 0.5 °C by a laser diffraction particle sizer (LA-950V2, Horiba Instruments Inc., Irvine, CA, USA) with distilled water as the dispersion media. To ensure even distribution of each composition, 4 mL of sample was added to 16 mL deionized water. This was added drop-wise to the dispersion media in the cell particle sizer to achieve the desired red transmission between 92% and 89% (approximately 4 ml). Volume mean diameter was calculated from three measurements and the coefficient of variation was determined. Measurements were carried out under continuous magnetic agitation.

Microparticle morphology was observed using an optical microscope (Olympus BH2-UMA, Japan) at 10x, and by scanning electron microscopy (SEM) (JEOL6010-LV, USA). A sample of the microparticles was oven-dried at 70 °C for 1 min before it was attached to adhesive tape and placed on a sample holder for analysis.

2.7 *Effect of SSF, SGF and SIF on immobilized and free L. acidophilus LA-5*

The ability of *L. acidophilus* LA-5 to survive the SSF, SGF and SIF conditions was determined by the plate count method in the free cells of *L. acidophilus* LA-5 and in immobilized cells before and after exposure to each fluid.

Simulated saliva fluid (SSF) was prepared by using a sterilized solution of 60 mM phosphate buffer (pH 6.2) and 0.01% (w/v) lysozyme (≥ 250 KU/mL) (Merck, Germany). Simulated gastric fluid (SGF) consisted of sterilized 0.05 g/L NaCl (pH 2.0) solution and 3 g/L pepsin (2000-3500 U/g) (Meyer, México). Simulated intestinal fluid (SIF) consisted of a sterilized phosphate buffer solution (pH 6.8) supplemented with 0.5% (w/v) oxgall (Meyer, México). All the simulated fluids were prepared according to Reyes-Nava *et al*. (2016).

To test the ability of free cells of *L. acidophilus* LA-5 to survive the simulated gastrointestinal fluids, 3×10^5 CFU were suspended in 1 mL SSF and incubated at 37 °C for 5 min. The temperature was maintained by using a thermostatic bath circulator (LAUDA Alpha RA 12, USA) for 5 min with stirring speed around 400 rpm using a magnetic stirrer (IKA RO 5, Germany). The cell suspension was centrifuged at 9000 × g for 5 min at room temperature using a mini spin plus (Eppendorf, USA) then the pellet was suspended in 1 mL of SGF and incubated at 37 °C for 90 min with stirring, using the same equipment as in the SSF test. Afterwards, the cell suspension was centrifuged under the above mentioned conditions; the pellet was then suspended in 1 mL of SIF and incubated at 37 °C for 150 min. All the experiments were conducted in triplicate (González-Vázquez *et al*., 2015; Reyes-Nava *et al*., 2016).

To test the ability of immobilized *L. acidophilus* LA-5 to survive the simulated gastrointestinal fluids, 1 mL of sterile water and 9 mL of MS were placed in a Falcon tube and the mixture was centrifuged at 44.7 × g for 5 min at room temperature using a Sol-Bat centrifuge (model 5094, Mexico) to separate the microparticles. The pellet was washed twice with 4 mL of sterile water. Then, the microparticles were suspended in 1 mL of SSF. Finally, the concentration in this SSF was 1×10^6 CFU of *L. acidophilus* LA-5). After incubation time, the suspension was centrifuged at 44.7 × g for 5 min at room temperature using a Sol-Bat centrifuge (model 5094, Mexico) and the pellet then suspended in 1 mL of SGF and incubated at 37 °C for 90 min with shaking. Afterwards, the solution was centrifuged under the abovementioned conditions to separate the pellet, which was then suspended in 1 mL SIF and incubated at 37°C for 150 min (González-Vázquez *et al*., 2015; Reyes-Nava *et al*., 2016). All the experiments were conducted in triplicate and the temperature was maintained at 37 °C by using a thermostatic bath circulator (LAUDA Alpha RA 12, USA) for 5 min with stirring speed around 400 rpm using a magnetic stirrer (IKA RO 5, Germany).

2.8 *Statistical analysis*

The experimental design and data were analyzed using Statgraphics Centurion XV software (Virginia, USA). Statistical analysis of the results was performed using analysis of variance (ANOVA) with $P \leq 0.05$. The response was adjusted to a linear function of the independent variables using equation 1 follows:

$$Y = \mu_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y is the predicted response; μ_0 is the intercept; $\beta_1 - \beta_3$ are the linear coefficients; β_{12}, β_{13} and β_{23}, are the interactions or cross-product coefficients and X_i is the coded independent variable. The data obtained using this equation was used to construct the response surface and contour plots.

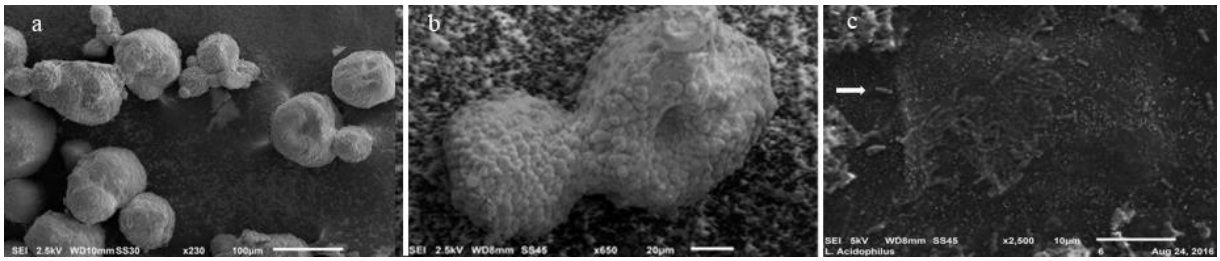

Fig. 1 Scanning electron microscopy: (a) microparticles with *L. acidophilus* LA-5, x230; (b) microparticles with *L. acidophilus* LA-5, x650; (c) *L. acidophilus* LA-5, x2500.

3 Results and discussion

3.1 Optimization of mixing conditions for microparticle formation

The statistical analysis of the data obtained from the experimental design showed that the only variable that significantly impacted the particle size was the mixing speed (P = 0.0001). As the mixing speed increased, the particle size decreased. The response was adjusted to a linear function of the independent variables. The approximate function of the first-order model is expressed as equation 2.

$$response = 39.5 + 0.26 * M_s - 1.04 * M_t + 4.83 * M_T - 0.002 * M_s * M_t - 0.024 * M_s * M_T + 0.09 * M_t * M_T \quad (2)$$

where M_s = Mixing speed; M_t = Mixing time and M_T = Mixing temperature.

The analysis of variance indicates that the only factor that influences the response variable is the mixing speed (P = 0.0001). Therefore, we do not consider necessary to make an adjustment to the linear model by addition of central points, since the lack of fit test in the improved ANOVA is greater than 0.05 (P = 0.3763). Therefore, this means that first-order model seems to be adequate for the data observed at the 95.0% confidence level. The average of the results, in triplicate, under these conditions was 75 μm. Thus, it was decided to use 250 rpm, 20 °C and 30 min to immobilize the probiotic.

Optical microscopy and SEM revealed that the particles loaded with *L. acidophilus* LA-5 were spherical (Fig. 1a), with average size of 80 μm. The surface of the microparticles with probiotic showed protrusions (Fig. 1b), whose dimensions were between 1 and 4 μm. The average size of *L. acidophilus* LA-5 determined by SEM was 2 x 0.5 μm (Fig. 1c). Therefore, the protrusions could be due to the presence of *L. acidophilus* LA-5. Holkem *et al*., (2016) reported that microcapsules charged with *Bifidobacterium animalis* subsp *Lactis* BB12 produced by emulsification/internal ionic gelation showed an elliptical and swelling shape with multi cavities distributed throughout the surface, similar to our results. This irregular shape is probably due to the production process and system components

3.2 Microparticle formation by emulsification

The final concentration in the MS was 1.01×10^6 CFU/mL, due to the dilution performed during manufacturing of both the microparticles with and non-immobilized cells. In contrast, the viable count of the non-immobilized probiotics cells in the MS was 1.93×10^5 CFU/mL (19.1%). Therefore, the microparticles contained approximately 8.17×10^5 CFU/mL, corresponding to 80.9%, which represent the efficiency of the encapsulation process. In comparison, Gebara *et al*. (2013) used pectin as the polymeric material and whey protein as the coat to encapsulate *L. acidophilus* LA-5 using the ionotropic gelation method and obtained an encapsulation yield of 84.35%. We observed that, although different methods were used, the yield was similar. This may be because the probiotic is tolerant to the working conditions or that the process conditions are not damaging to the microorganisms.

3.3 Effect of SSF, SGF and SIF on L. acidophilus LA-5

During the sequential passage of the free cells of *L. acidophilus* LA-5 through the three stages of the simulated intestinal tract conditions, the relative survival of *L. acidophilus* LA-5 after SSF was close to 100% (Fig. 2).

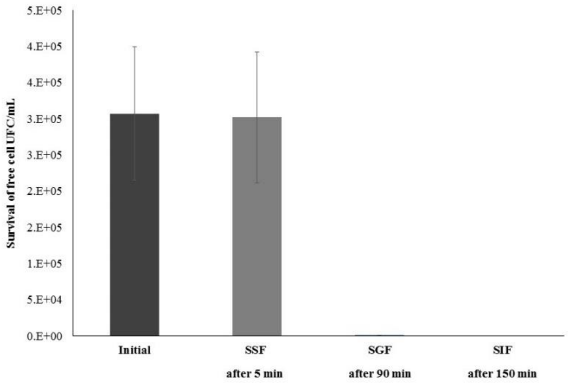

Fig. 2. Viability of free cells of *L. acidophilus* LA-5 following exposure to simulated gastrointestinal stress conditions. SSF simulated saliva fluid; SGF simulated gastric fluid; SIF simulated intestinal fluid.

After SGF, the mortality was 99.9%. In the context of the definition of a probiotic, the cells must be able to resist gastrointestinal stress, arriving in a suitable concentration at the site of action to exert their beneficial effect, which in the case of *Lactobacilli* is the small intestine (Fooks and Gibson, 2002). In the present work, *L. acidophilus* LA-5 did not show resistance to simulated gastrointestinal stress. Therefore, it would benefit from protection via immobilization by microparticles. Similarly, Gebara *et al*. (2013) reported a comparable trend in their findings.

3.4 Effect of SSF, SGF and SIF on immobilized L. acidophilus LA-5

Microparticles containing approximately 1.01×10^6 CFU/mL *L. acidophilus* LA-5 cells were placed under SSF conditions. After incubation in SSF, the viable count was 2.04×10^5 CFU/mL (20.2% of the initial cells) (Fig. 3), which represented the probiotics that were not immobilized but that were in the MS. The remainder (79.8%) are the immobilized probiotic cells, which could be considered as de efficiency of immobilization.

The progress of the loaded microparticles through the simulated gastric stress conditions was determined by centrifuging the SSF treated microparticles and then exposing them to SGF. After incubation in SGF, 1.99×10^5 CFU/mL (19.6% of the initial count of viable cells) was detected, which represents the probiotics that were released from the microparticles and that resisted the SGF. In contrast, no free cells of *L. acidophilus* LA-5 survived under this stress condition (Fig. 2). In the presence of acid, the alginate-Ca^{2+} complex is damaged to form alginic acid, which is soluble in an aqueous medium; and probably the structure of the microparticles was partially maintained and the cell liberation is start (Chandramouli *et al*., 2004; Rafi and Mahkam, 2015). Therefore, it was supposed that the liberation could be due to the acidity of the SGF and the stirring applied during incubation and. The released probiotics did not die could be due to the alginate that surrounding the cell which protect them or because the liberation of the cell have occurred near to the end of the incubation time. In this work, the hardened time was 18 h which could aloud a hardy microparticle. However, this needs further confirmation.

The microparticles obtained at the end of the SGF stress were separated and then placed in SIF. After 2.5 h under these conditions, 4.74×10^5 CFU/mL were recorded as a final count, which represented 46.9% of the microorganisms contained in the MS and liberated during the SIF condition (Fig. 3). Therefore, a relatively higher percentage of release occurred under conditions representing the intestine, compared to oral and gastric conditions. Also, this result shows that cells were immobilized and targeted to an environment that simulated the conditions of the small intestine, where the genus *Lactobacillii* exerts its benefits; in this instance, they must be released from the carrier matrix (microparticles) to exert their benefits. Thus, the release of microorganisms in SIF should be considered as an advantage because, if the microparticles did not release the microorganisms under the physiological conditions of the intestine, then the probiotic could not exert its beneficial effect. Also, despite disintegration of the microparticles exposed to sequential SSF, SGF and SIF, the presence of the alginate surrounding the probiotic or in the medium may protect the microorganisms from the acidity and the presence of bile salts that would otherwise destroy the cells. Chandramouli *et al*. (2004) reported that the viability of immobilized bacteria in simulated gastric conditions increased with increased microparticle size (200 μm) and that microparticles less than 100 μm did not significantly protect the probiotic cells; nevertheless, in this work using the selected conditions showed in Table 1, it was possible to produce microparticles of 80 m size, which were able to immobilize, protect and release viable cells of *L. acidophilus* LA-5 in an stressing environment that simulated the conditions of intestinal tract, where these bacteria exert their benefits.

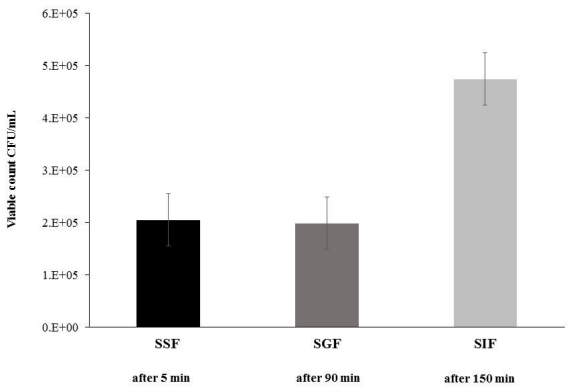

Fig. 3. Viable count of *L. acidophilus* LA-5 in SSF considered as not-immobilized probiotics and, in SGF and SIF, considered as probiotics released from microparticles.

In this work, the hardened time was 18 h, while Chandramouli *et al*. (2004) used a hardened time of 30 min, which could be implicated in the capacity of the microparticle to protect the probiotic bacteria. In addition, in this work it was used a 4% of alginate and emulsification method that have been reported as good for probiotic protection in SGF Smilkov *et al*. (2014) showed 22.4% protection with microparticles of 8.84 μm with 4% sodium alginate further coated with 3% whey protein. The difference in the protective effect between the microparticles obtained in this work (46.9 %), Mandal *et al*. (2006) (4.57%) and Smilkov *et al*. (2014) (22.4%), could be attributed to several factors. One of them could be pH; in this work, SGF at pH 2 was used, whereas in Mandal *et al*. (2006) and Smilkov *et al*. (2014) the solution was prepared at pH 1.5. It is worth noting that the pH of the stomach is highly variable; during fasting, it can go down to a pH of 1 and after food intake, the pH can increase up to 5. Therefore, bioactive substances such as penicillin G or probiotic bacteria that are sensitive to acidity, need to be protected (Cook *et al*., 2013). Another factor that can influence cell survival is the pH sensitivity of the probiotic. Mandal *et al*. (2006) used *L. casei* NCDC 298 and Smilkov *et al*. (2014) used L. casei 01 and in this work was used *L. acidophilus* LA-5. There are several studies in which the encapsulation in alginate for bacterial protection was used. For instance, Chandramouli *et al*. (2004) tested the survival of *L. acidophilus* CSCC 2400 using 1.8% (w/v) alginate and observed 0.1% viability following incubation in SGF at pH 2.0 for 3 h. Lee *et al*. (2004) investigated the effect of alginate microparticles coated with chitosan of various molecular weights on the survival of *Lactobacillus bulgaricus* KFRI 673 in SGF (pH 2, 60 min). They found that microencapsulation in alginate microparticles improved the survival of acid-sensitive *L. bulgaricus* KFRI 673 in SGF, with the high molecular weight chitosan coating affording the highest protection. Using the extrusion method with a mixture of 2.0% sodium alginate, 0.15% xanthan gum, 3% maltose, 11% MRS agar medium and 5.5% glycerol, Trabelsi *et al*. (2013) observed 28.8% survival of L. plantarum TN8 after exposure to SGF (10 mL pancreatin at pH 2) for 4 h. In another study, Zhao *et al*. (2012) prepared microparticles by extrusion using 3% sodium alginate, 8% skim milk and the probiotic Lactobacillus reuteri DPC16. The particle size ranged from 3-4 mm and the cell viability decreased by less than one log cycle after incubation in SGF (pH 1.2, pepsin 3.2 g/L) for 2 h. Furthermore, there was only a slight decrease in the viable count in SIF (glucose 10 g/L glucose, 5 g/L extract yeast,10 g/L pancreatin and 8 g/L bile salts in 0.1 M Tris buffer, pH 7.4) despite the fragmentation of microparticles. Moreover, Rodklongtan *et al*. (2014) used an alginate-chitosan mixture to form a non-covalent network reinforced with a second network formed by the interaction of alginate with Ca^{2+}, where the viability of microencapsulated *L. reuteri* KUB-AC5 showed a decrease of one log cycle after incubation in SGF (pH 1.8) for 180 min.

In this work, we observed that alginate microcapsules performed using the selected conditions, protect and release viable cells of *L. acidophilus LA-5* in simulated conditions where these bacteria exert their benefits.

Conclusions

The present work represents the immobilization of *L. acidophilus* LA-5 by emulsification and external gelation using alginate (4%) and emulsion (W/O, 32:68) as a sustainable option for the protection of probiotics that display limited survival under sequential simulated gastrointestinal tract conditions, reflecting those experienced in the human digestive system.

Additionally, the generated microparticles are an alternative for the survival of probiotics during their passage through the gastrointestinal tract, since the generated microparticles delivered the probiotic alive to an environment that simulated the conditions of the small intestine, where *Lactobacillii* exert their

benefits. It is worth mentioning that the cells were successfully immobilized, protected and delivered to an environment that simulated the conditions of the small intestine. Moreover, the microparticles were simple to prepare and amenable to production on an industrial scale because the materials were economical and readily obtained, without requiring additional coatings, thus avoiding a prolonged processing time.

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Abbreviations

CFU	Colony forming units
MS	Microparticle suspension
SEM	Scanning electron microscope
SSF	Simulated saliva fluid
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
W/O	water in oil emulsion

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