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Effect of resistant starch and chitosan on survival of *Lactobacillus acidophilus* microencapsulated with sodium alginate

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ABSTRACT

Resistant starch (Hi maize) and chitosan at concentrations of 1% and 0.4% were added to the microencapsulation of *Lactobacillus acidophilus* in alginate beads by extrusion technique. Moist and freeze-dried microparticles were analyzed. The addition of prebiotics and chitosan increased the size of the moist particles, whose diameter was 70.37 μm, while the diameter of the microparticles containing alginate alone was 55.13 μm. In contrast, the freeze-dried microparticles of alginate and alginate + Hi −Maize + chitosan had diameters of 114.51 μm and 112.50 μm, respectively. Both Hi−maize and chitosan provided better protection of probiotics after exposure of the moist microparticles to simulated gastric and intestinal juice, with counts of 6.35 log CFU g^{-1}, while lower counts were observed for the freeze-dried microcapsules. Regarding the viability of the probiotic culture during the storage periods and temperatures, all treatments were viable, with suitable values to confer the probiotic effects (<6 log CFU g^{-1}), with counts up to 6 logs for at least 30 days for the microparticles stored in the freeze-dried form, and 135 days in the moist form, both under storage at room temperature (25 °C).

1. Introduction

According to World Health Organization, probiotics are defined as live microorganisms which when administered in adequate amounts (10^7 CFU g^{-1}) confer health benefits to the host (FAO/WHO, 2001).

In recent years, there is a growing demand for use of probiotics in foods aimed to increase the nutritional and therapeutic value of food products, thus various probiotic strains have been studied and commercially exploited (Franz, 2014).

However, the maintenance of microorganisms viability throughout the product shelf life is a major challenge to the food industry (Douglas & Sanders, 2008), since certain cultures are extremely sensitive to environmental factors such as acidic and oxygen (Kailasapathy & Chin, 2000). The low pH of the stomach together with the presence of bile salts in the small intestine are the main reasons for the dramatic decline in the viability of the probiotic cells after their uptake (Mortazavian & Sohrabvandi, 2007). Therefore, microencapsulation has been widely studied to protect microorganisms from acid environment, bile salts, and oxygen (Oliveira et al., 2007).

Sodium alginate is one of the polymers most used as encapsulating material, since it forms a highly versatile, biocompatible and non-toxic matrix for the protection of active ingredients, especially probiotic microorganisms and cells sensitive to heat, pH, dissolved oxygen, among other factors in which food is exposed during processing and storage (Pasin, Azón, & Garriga, 2012). This polymer is presented as a food additive in the form of white or yellowish brown powder, tasteless and odorless. It is Consisted mainly by the sodium salt of alginic acid, or that is, a mixture of polyuronic acids composed of residues of D-mannuronic and L-guluronic acid (Rowe, 2009).

The microparticles of calcium alginate can be prepared by the extrusion method by dripping a solution of sodium alginate into a solution of a calcium salt, leading to the phenomenon of external ionic gelation (Gombotz and Wu, 1998). In this technique, the

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microorganisms are added to an alginate solution and are immediately incorporated in the form of droplets in a solution of calcium chloride to hardening (Yeo, Baek, & Park, 2001). The interaction of the ions, such as Ca^{2+}, with the carboxyl groups of the polymer chains of the alginate results in the formation of an insoluble gel (Smrdel, Bogataj, Zega, Planinsek, & Mrhar, 2008). In the research conducted by Kim et al. (2008) positive results were obtained for *Lactobacillus acidophilus* ATCC 43121 encapsulated with calcium alginate, by the drip method, during exposure to the *in vitro* gastrointestinal tract and resistance to the thermal treatment.

Although sodium alginate is suitable for encapsulation, its gel is porous and sensible to extreme pH values, thus affecting both the release and protection of the compounds (Mortazavian & Sohrabvandi, 2007). There are several ways to overcome this obstacle and improve stability of microorganisms as, for example, coating the particles with ionic gelling with biopolymers through electrostatic interactions (Patil, Kamalapur, Marapur, & Kadam, 2010) and the addition of prebiotics in the capsule formulation (Chen, Chen, Liu, Lin, & Chiu, 2005).

Lee, Cha, and Park (2004) analyzed the effects of chitosan and alginate microparticles on the survival of *Lactobacillus bulgaricus* KFRI763 in simulated gastric and simulated intestinal juices and on their stability during storage at 4 and 22 °C. Studies conducted by Homayouni (2008), demonstrated that a combination of alginate with starch improves the efficiency of different bacterial cells, particularly lactic acid-producing bacteria, due to the production of granules of good prebiotic structure and effect in the microcapsules.

Therefore, this study aimed to evaluate the effect of resistant starch (Hi–maize) and chitosan on the viability of *L. acidophilus* microencapsulated with sodium alginate against the simulated digestive system and under different storage temperatures.

2. Material and methods

2.1. Inoculum

The probiotic culture *L. acidophilus* La-14 (Danisco) was activated in MRS broth (Himedia) and incubated for 15 h at 37°°C. Then, it was centrifuged at 4670 × g for 15 min and washed with NaCl solution (0.85%). The cells were suspended in saline to obtain a solution containing about 10 log CFU g^{-1}. The concentration of microorganism was adjusted by bacterial growth curve.

2.2. Production of microparticles

Microparticles were produced according to the extrusion technology developed by Liserre, Ré, and Franco (2007), with adaptations. For that, an aerograph (Size of nozzle: 0.3 mm) model EW 110 was coupled to an air compressor Model MB24/BV, on air pressure of 2.72 kgf/cm^2, using the height of 30 cm between the atomizing nozzle and the $CaCl_2$ solution.

The cultures were mixed in two solutions containing 1.0% sodium alginate (Vetec). The first solution contained only sodium alginate (ALG) was sprayed in 0.1 M $CaCl_2$, and the second was composed by sodium alginate + 1% Hi–maize (National Starch), sprayed in 0.1 M $CaCl_2$ containing 0.4% chitosan, as reported by Gaserod, Smidsrod, and Skjakbraek (1998), with adaptations, where 0.4 g of chitosan were dissolved in 90 mL of distilled water acidified with 0.4 mL of glacial acetic acid to achieve a final concentration of 0.4% (w/v). The pH was then adjusted to 5.8 ± 0.2 with 1M NaOH. The mixture was filtered through filter paper and the volume was adjusted to 100 mL. Then, it was autoclaved at 121°°C for 15 min and mixed with calcium chloride solution. The particles were kept under stirring for 30 min in $CaCl_2$ solution, and then removed from the solution using a sieve (50 μm), sterilized, and washed with sterile distilled water.

An amount of moist microparticles was stored in sterile collectors, and the remaining was freeze-dried in Liotop Lyophilizer Model L101 for 24 h.

2.3. Morphological characterization of the microparticles by optical and scanning electron microscopy

Optical microscopy of the moist microparticles was performed using a microscope MDL-150-TPI model, and a digital camera Samsung 14.2 model for image capture. The morphology of the freeze-dried microparticles was evaluated using a scanning electron microscope JEOL brand, model JM6360. The microcapsules were fixed with a double sided tape on aluminum stubs and coated with a thin layer of gold.

2.4. Evaluation of the mean diameter and size distribution of the microparticles

The average size of the moist and freeze-dried microparticles was measured in Mastersizer equipment 2000 (Malvern, Alemanha).

2.5. Viable cells count

Appropriate dilutions were transferred in triplicate to sterile Petri plates, followed by addition of MRS agar (Himedia). Plates were incubated at 37 °C for 72 h in anaerobic jars containing anaerobic generator (Oxoid). The dilution of the microparticles consisted in weighing 1 g of moist microparticles and 0.1g of freeze-dried microparticles, followed by the addition of 9 mL of sterile phosphate buffer solution (pH 7.5) according to the methodology described by Sheu, Marshall, and Heymann (1993).

2.6. Survival of microencapsulated L. acidophilus La-14 under simulated gastrointestinal conditions

This analysis was performed according to the method described by Liserre et al. (2007) with modifications. Aliquots of 1 g of moist microparticles and 0.1 g of freeze-dried microparticles were mixed with 1M HCl pH 1.8, pepsin (pepsin from porcine gastric mucosa P7000, Sigma–Aldrich), and lipase at a concentrations of 3 g L^{-1} and 0.9 mg L^{-1} (lipase from porcine pancreas 62300, Sigma–Aldrich), respectively, prior to incubation at 37°°C under continuous stirring in a refrigerated incubator shaker (model TE-421), for 2 h.

Subsequently, the pH of the samples was adjusted to 5.0. Bile (bovine bile B3883-25G, Sigma–Aldrich) and pancreatin (pancreatin from porcine pancreas P3292, Sigma–Aldrich) was added at a concentration of 1 g L^{-1} and 0.1 g L^{-1}, respectively, and incubated again at 37 °C for 2 h.

Finally, pH was adjusted to 7.5, and the bile and pancreatin concentrations were maintained. The samples were incubated at 37 °C for 2 h under continuous stirring to a total of 6 h of analysis.

Counts were performed after 5, 30, 120, 125, 150, 240, 245, 270, and 360 min of incubation. Serial dilutions were made as described in Section 2.5.

2.7. Viability of the microparticles during storage at different temperatures

Both moist (U) and freeze-dried (L) microparticles were stored at room temperature (25 °C), refrigerated (7 °C), and frozen (−18 °C), of 120 and 60 days respectively.

2.8. Statistical analysis

A completely randomized design was used. In case of significance in ANOVA ($p < 0.05$), the test for least significant difference (LSD) was performed using the Duncan Test Statistical Analysis System (SAS).

3. Results and discussion

3.1. Morphological characterization of the microparticles by optical and scanning electron microscopy

As can be seen in Fig. 1, it was possible to verify the presence of alginate (encapsulating agent) and microorganisms (active material) in the entire interior of the microparticle, characterizing it as a matrix type, once the active material is not only located in the center, but inside the particle (Azeredo, 2005; Jafari, Assadpoor, He, & Bhandari, 2008) or even on the surface.

In addition, Fig. 1b shows the internal appearance of AHQ microparticles containing apparent resistant starch granules (Hi–maize). These results corroborate those found by Iyer and Kailasapathy (2005) and Mirzaei, Pourjafar, and Homayouni (2012).

The morphology of the freeze-dried microparticles by scanning electron microscopy (Figs. 2 and 3) indicated high agglomeration, leading to a loss of spherical shape and producing a variety of sizes regardless of the treatments.

However, at a higher magnification (×1000), some fragments were detected, with slightly more spherical shape, besides the presence of microorganisms.

Veelken and Pape (1984) have reported that the sharp dehydration of freeze-dried polysaccharide gels may contribute to the formation of a porous matrix, similar to a sponge. In freeze drying process, the microcapsules are subjected to low temperatures, leading to the formation of ice crystals and ice crystal sublimation under reduced pressure, resulting in a porous dry product (Dolly, Anishaparvin, Joseph, & Anandharamakrishnan, 2011).

3.2. Mean diameter and size distribution of the microparticles

Moist microparticles of the treatments ALGU and AHQU had mean diameters of 55.13 μm and 70.37 μm respectively. These results showed that the addition of chitosan increased the microparticles diameter. Iyer and Kailasapathy (2005) also found similar results, in which the microparticles diameter increased with the chitosan coating. The sphere size to less than 100 μm would be advantageous for texture considerations and allow direct addition of encapsulated probiotics to a multitude of foods (Hansen, Allan-Wojtas, Jin, & Paulson, 2002).

In the present study, the freeze-dried microparticles ALGL and AHQL had mean diameters of 114.51 μm and 112.50 μm, respectively. The structural change caused by the freeze-drying process is often referred to cause an increase in pore size (Nakagawa, Iwamoto, Nakajima, Shonob, & Satohb, 2004), allowing a fast and complete rehydration (Fellows, 2006).

For the treatment ALGL, the size difference may be related to hydration capacity of polysaccharides. Chemical side groups such as COO- and SO_3 in polysaccharides can interact with water molecules via hydrogen bridges (Boudou et al., 2010).

With respect to the treatment AHQL, the results can be explained by the high hydrophilicity of chitosan due to the large number of hydroxyl and amino groups present on the polymer chain (Tonhi & Peplis, 2002).

3.3. Survival of microencapsulated L. acidophilus La-14 under simulated gastrointestinal conditions

When comparing the moist microparticles of the treatments ALGU and AHQU (Table 1), after increasing the pH 1.8 to 5.0, and then 5.0 to 7.5, the number of viable cells was 6 log CFU g^{-1} in both treatments, being within the requirements for probiotics benefits (FAO/WHO, 2001).

After 360 min, log reductions of 3.67 and 3.52 were observed for the microparticles ALGU and AHQU respectively, when compared to time zero, with significant differences between the treatments.

The survival of the viable cells in the simulated gastric environment was higher in chitosan-coated alginate microparticles as compared to uncoated microparticles. The protection provided by the chitosan is due to strong bonding between chitosan and alginate by electrostatic interactions, leading to formation of a membrane on the surface of the granules, which reduces the probability of migration of coating materials (Gaserod et al., 1998).

Chavarri et al. (2010) used chitosan as coating material in alginate microparticles and quercetin as prebiotics to encapsulate *Lactobacillus gasseri* and *Bifidobacterium bifidum*, and found improved survival during exposure to adverse conditions of the gastrointestinal tract.

Yu, Yim, Lee, and Heo (2001), Ding and Shah (2007) and Murata, Toniwa, and Miyamoto (1999) reported that the probiotics

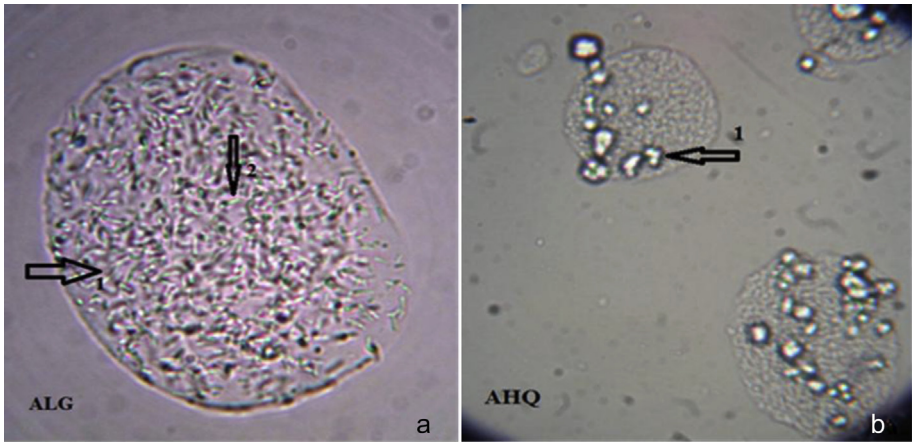

Fig. 1. Optical microscopy of both alginate and alginate + HM + chitosan microparticle (a) alginate microparticle, in which number 1 shows the sodium alginate in the interior of the particle, and number 2 indicates the microorganism within the particle (100×) (b) AHQ microparticles, in which number 1 shows the prebiotic Hi–maize (40×).

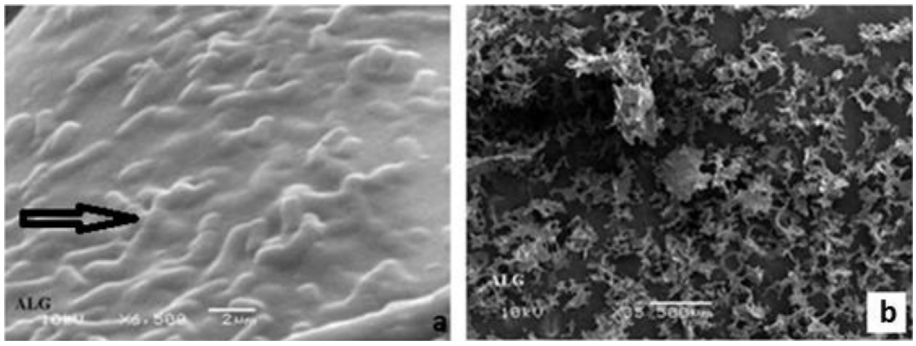

Fig. 2. Morphology and microstructure of the freeze-dried microparticles with alginate matrix (ALG), obtained by scanning electron microscopy. a. Microparticle surface showing microorganisms (6,500×); b. Particles distribution (35 ×).

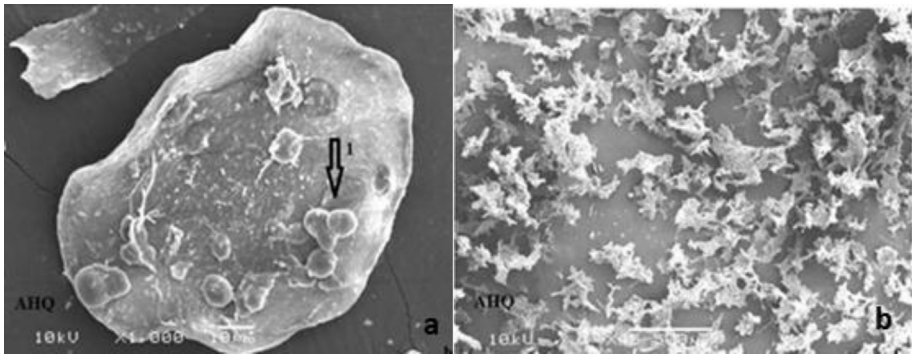

Fig. 3. Morphology and microstructure of the freeze-dried microparticles with alginate and Hi–maize + chitosan (AHQ), obtained by scanning electron microscopy. a. Microparticle aspect in which the number 1 shows the prebiotic Hi–maize (1000×); b. Particles aggregation (45×).

Table 1
Viability of the moist microparticles ALGU and AHQU under simulated gastrointestinal conditions, at different pH values for a period of 360 min.

Treatment/Time (minutes)	pH	ALGU	AHQU
0	–	9.78 ± 0.05^{aA}	9.87 ± 0.08^{aA}
5	1.8	4.87 ± 0.04^{aF}	4.58 ± 0.04^{bF}
30	1.8	4.42 ± 0.03^{aG}	4.17 ± 0.03^{bH}
120	1.8	4.14 ± 0.04^{bH}	4.25 ± 0.03^{aG}
125	5.0	4.18 ± 0.05^{bH}	4.93 ± 0.06^{aE}
150	5.0	4.61 ± 0.08^{bG}	5.18 ± 0.06^{aD}
240	5.0	5.51 ± 0.07^{aE}	5.21 ± 0.02^{bD}
245	7.5	6.10 ± 0.09^{aD}	6.17 ± 0.03^{aC}
270	7.5	6.17 ± 0.33^{bB}	6.29 ± 0.02^{aB}
360	7.5	6.11 ± 0.03^{bC}	6.35 ± 0.03^{aB}

Means followed by different uppercase letters differ statistically in column (Duncan test, $p < 0.05$). Means followed by different lowercase letters differ statistically in line (Duncan test $p < 0.05$).
ALGU = moist microparticles of sodium alginate; AHQU = moist microparticles of sodium alginate + Hi–maize + chitosan.

encapsulated in alginate particles containing chitosan showed higher viability when compared with the alginate particles without chitosan. Chitosan forms a semipermeable membrane around the negatively charged polymer that does not dissolve in the presence of Ca^{2+} or chelating agents, and thus increasing gel stability (Smidsrod & Skjak-breaek, 1990).

Hansen et al. (2002) encapsulated *Bifidobacteria* in calcium alginate capsules without chitosan coating, and failed to protect the probiotic cells against simulated gastrointestinal conditions. Mokarram, Mortazavi, Najafi, and Shahidi (2009) reported that the encapsulation of *L. acidophilus* and *Lactobacillus rhamnosus* in calcium alginate uncoated capsules did not significantly improve the survival of probiotic cells in simulated gastrointestinal conditions.

Table 2 presents the results of the viability of the freeze-dried microcapsules. After 2 h of exposure to simulated gastric conditions at pH 1.8, lower populations of *L. acidophilus* La-14 were observed with values of 4.23 log CFU g^{-1} for ALGL microparticles and 4.13 log CFU g^{-1} for AHQL microparticles, demonstrating that the lower pH may have caused a slight release of the capsule. Higher microbial counts were observed with increasing pH, with values of 5.41 log CFU g^{-1} for ALGL microparticles, and 5.10 log CFU g^{-1} for AHQL microparticles.

Murata et al. (1999) reported that alginate capsules with

Table 2
Viability of the freeze-dried microparticles ALGL and AHQL under simulated gastrointestinal conditions, at different pH for a period of 360 min.

Treatment/Time (minutes)	pH	ALGL	AHQL
0	–	6.65 ± 0.07^{aA}	6.80 ± 0.18^{aA}
5	1.8	4.23 ± 0.05^{aG}	4.13 ± 0.03^{bF}
30	1.8	4.08 ± 0.04^{bH}	4.27 ± 0.04^{aE}
120	1.8	3.92 ± 0.06^{bI}	4.51 ± 0.12^{aD}
125	5.0	4.23 ± 0.03^{aG}	3.40 ± 0.08^{bI}
150	5.0	4.47 ± 0.04^{aF}	3.58 ± 0.04^{bH}
240	5.0	4.66 ± 0.04^{aE}	3.92 ± 0.05^{bG}
245	7.5	4.87 ± 0.04^{aD}	4.23 ± 0.05^{bEF}
270	7.5	5.11 ± 0.04^{aC}	4.84 ± 0.03^{bC}
360	7.5	5.41 ± 0.07^{aB}	5.10 ± 0.02^{bB}

Means followed by different uppercase letters differ statistically in column (Duncan test, $p < 0.05$).
Means followed by different lowercase letters differ statistically in line (Duncan test $p < 0.05$).
ALGU = freeze-dried microparticles of sodium alginate; AHQU = freeze-dried microparticles of sodium alginate + Hi–maize + chitosan.

chitosan coating presented a complexation which reduces the porosity of alginate capsules and decreases the release of the encapsulated material. Gbassi, Vandamme, Ennahar, and Marchioni (2009) found that *Lactobacillus plantarum* encapsulated in calcium alginate showed a substantial loss of viability after 90 min of incubation. However, when the same author used alginate matrix combined with whey protein as coating material, an increase in bacteria survival was observed, demonstrating that the technique has been relatively effective for the protection of probiotic bacteria.

3.4. Viability of the microparticles during storage at different temperatures

Table 3 shows the effect of ambient temperature (25 °C), freezing (−18 °C) and refrigeration (7 °C), and storage time on the viability of *L. acidophilus* La-14 in the moist microcapsules.

With respect to the storage at room temperature, the number of viable *L. acidophilus* cells remained above 6 log CFU g^{-1} for all treatments, being within the requirements for probiotics benefits (FAO/WHO, 2001). Other studies have shown that the encapsulation of different probiotic bacteria using resistant starch as prebiotics and chitosan as coating material significantly increased the survival of microorganisms in up to 6 months at room temperature (Iyer & Kailasapathy, 2005).

Regarding the effect of freezing temperatures on the viability of *L. acidophilus*, it was observed that only the alginate microcapsules (ALGU) showed counts of 5.93 ± 0.04 log CFU g^{-1} after 60 days of storage. However, the treatment AHQU remained stable at the end of 135 days of storage with counts of 6.35 ± 0.22 log CFU g^{-1}, whose values are recommended for the shelf life of probiotic product, thus demonstrating that the addition of prebiotic and chitosan conferred greater protection for microorganisms up to 135 days.

Concerning the refrigeration temperature, a significant reduction (3.08 log) was observed on day 15 for the alginate microcapsules (ALGU). This reduction was also significant in the treatment containing prebiotic and chitosan (AHQ), but to a lesser extent (1.63 log). From day 75, the treatment ALG had reduced stability, with counts of 5.89 ± 0.05 log CFU g^{-1}. The treatment AHQU presented counts of 6.13 ± 0.13 log CFU g^{-1}, which remained viable up to 105 days of storage as compared to the microcapsules of alginate alone. Nualkaekul, Lenton, Cook, Khutoryanskiy, and Charalampopoulos (2012) studied the viability of both alginate and alginate + chitosan microcapsules on survival of *L. plantarum* during storage at 4 °C for 42 days, and found that the viable cells concentration remained greater than 5.5 log CFU g^{-1} in alginate + chitosan microcapsules in pomegranate juice. Brinques and Ayub (2011) reported that chitosan-alginate capsules significantly improved the viability of *L. plantarum* 011 BL under refrigerated storage at 4 °C during 38 days.

The addition of prebiotics and chitosan significantly improved the microorganism survival regardless of temperature. Studies conducted by Iyer and Kailasapathy (2005) and Sultana et al. (2000) have shown that lactic acid bacteria encapsulated with modified starch can survive for more than 6 months at room temperature under normal conditions of atmosphere and humidity, and at least 18 months when in frozen storage. The starch and alginate tend to have a synergy during gelation, thereby providing additional protection to microencapsulated cells at certain concentrations; in addition, the increase in the number of viable bacteria can be due to the prebiotic action of the modified starch (Sultana et al., 2000).

Table 4 shows the results of the viability of freeze-dried microcapsules over 60 days. It was observed that at room temperature, the microcapsules remained stable for only 30 days in the treatment ALGL. Lee et al. (2004) evaluated the stability of *L. bulgaricus* KFRI 673 in alginate microparticles coated with high molecular weight chitosan stored at 22 °C, and obtained values of 6 log CFU g^{-1} within 30 days of storage.

In the refrigeration temperature, no significant differences were observed for the ALGL microcapsules during 30 days of storage,

Table 3
Effect of ambient temperature (25 °C), freezing (−18 °C) and refrigeration (7 °C), on the viability of microencapsulated *Lactobacillus acidophilus* La-14, for different treatments, in the moist form, stored for 135 days.

Treatment/Time (days)	ALGU log 10 CFU/g	AHQU log 10 CFU/g
Temperature	Ambient (25 °C)	
0	9.78 ± 0.05^{aA}	9.87 ± 0.08^{aA}
15	9.24 ± 0.17^{aBC}	9.07 ± 0.01^{aC}
30	9.60 ± 0.13^{aA}	9.53 ± 0.06^{aB}
45	9.07 ± 0.06^{aC}	8.28 ± 0.03^{bE}
60	9.37 ± 0.04^{aB}	8.12 ± 0.02^{bF}
75	7.63 ± 0.27^{bD}	8.33 ± 0.03^{aDE}
90	7.28 ± 0.08^{bE}	8.42 ± 0.16^{aD}
105	6.95 ± 0.05^{bF}	8.29 ± 0.05^{aDE}
120	6.72 ± 0.07^{bG}	8.15 ± 0.09^{aF}
135	6.53 ± 0.12^{bG}	8.00 ± 0.02^{aG}
Temperature	Freezing (−18°)	
0	9.78 ± 0.05^{aA}	9.87 ± 0.08^{aA}
15	7.63 ± 0.05^{aB}	7.72 ± 0.06^{aB}
30	6.33 ± 0.44^{aD}	7.75 ± 0.07^{aD}
45	6.84 ± 0.10^{aC}	6.74 ± 0.16^{aD}
60	5.93 ± 0.04^{bE}	6.30 ± 0.06^{aE}
75	5.75 ± 0.14^{bEF}	7.06 ± 0.10^{aC}
90	5.90 ± 0.05^{bE}	6.35 ± 0.08^{aE}
105	5.77 ± 0.03^{bE}	6.33 ± 0.02^{aE}
120	5.48 ± 0.05^{bFG}	6.74 ± 0.05^{aD}
135	5.35 ± 0.07^{bG}	6.35 ± 0.22^{aE}
Temperature	Refrigeration (7 °C)	
0	9.78 ± 0.05^{aA}	9.87 ± 0.08^{aA}
15	6.70 ± 0.14^{bB}	8.24 ± 0.02^{aB}
30	6.48 ± 0.08^{bC}	7.45 ± 0.02^{aC}
45	6.10 ± 0.05^{bD}	6.25 ± 0.09^{aEF}
60	6.33 ± 0.16^{bC}	6.79 ± 0.04^{aD}
75	5.89 ± 0.05^{bE}	6.38 ± 0.12^{aE}
90	5.87 ± 0.02^{aE}	6.03 ± 0.03^{aG}
105	5.37 ± 0.17^{bF}	6.13 ± 0.13^{aFG}
120	5.27 ± 0.18^{bFG}	5.77 ± 0.16^{aH}
135	5.15 ± 0.11^{bG}	5.75 ± 0.20^{aH}

Means followed by different uppercase letters differ statistically in column (Duncan test, $p < 0.05$). Means followed by different lowercase letters differ statistically in line (Duncan test $p < 0.05$).
ALGU = moist microparticles of sodium alginate; AHQU = moist microparticles of sodium alginate + Hi–maize + chitosan.

Table 4
Effect of ambient temperature (25 °C), refrigeration (7 °C), an freezing (−18 °C) and on the viability of microencapsulated *Lactobacillus acidophilus* La-14, for different treatments, in the lyophilized form, stored for 60 days.

Treatment/Time (days)	ALGL log 10 CFU/g	AHQL log 10 CFU/g
Temperature	Ambient (25 °C)	
0	6.65 ± 0.07^{Aa}	6.80 ± 0.18^{Aa}
30	6.05 ± 0.12^{Ba}	5.78 ± 0.04^{Bb}
60	5.92 ± 0.03^{Ba}	5.63 ± 0.06^{Bb}
Temperature	Refrigeration (7 °C)	
0	6.65 ± 0.07^{Aa}	6.80 ± 0.18^{Aa}
30	6.56 ± 0.02^{Aa}	6.39 ± 0.03^{Bb}
60	5.80 ± 0.06^{Bb}	6.34 ± 0.07^{Ba}
Temperature	Freezing (−18 °C)	
0	6.65 ± 0.07^{Ab}	6.80 ± 0.18^{Ab}
30	6.61 ± 0.01^{Aa}	6.10 ± 0.03^{Bb}
60	5.98 ± 0.04^{Bb}	6.08 ± 0.02^{Ba}

Means followed by different uppercase letters differ statistically in column (Duncan test, $p < 0.05$). Means followed by different lowercase letters differ statistically in line (Duncan test $p < 0.05$).
ALGU = freeze-dried microparticles of sodium alginate; AHQU = freeze-dried microparticles of sodium alginate + Hi–maize + chitosan.

with a reduction of 0.63 log after this period, resulting in 5.80 ± 0.06 log CFU g^{-1}. Although the treatment AHQL (chitosan + hi–maize) exhibited significant reductions within 60 days, the results remained stable, with counts of 6.34 ± 0.07 log CFU g^{-1}. This indicated that addition of prebiotic and chitosan increased the survival ability of *Lactobacillus acidophillus* La-14.

Simpson (2005) studied the viability of 12 *Bifidobacterium* species microencapsulated by spray drying with reconstituted skim milk, with and without addition of gum arabic, and found viable cell counts higher than 6 log CFU g^{-1} after 90 days at 4 °C, and the inclusion of gum acacia had no significant affect on survival or viability. These results were better than those obtained by Pedroso, Thomazini, Heinemann, and Favaro-trindade (2012) who studied the viability of *L. acidophilus* microencapsulated by spray chilling lipid matrices, and found counts higher than 6 log CFU g^{-1} until 30 days at 7 °C.

The freezing temperature maintained the viability of the ALG microcapsules for 30 days (6.61 ± 0.01), and ensured stability in the AHQL treatment over 60 days of storage, with counts of 6.08 ± 0.02 log CFU g^{-1}.

The lyophilized microparticles of the AHQL treatment showed values above 6 log CFU g^{-1}, both in the refrigeration temperature and in freezing, being within the stipulated values for probiotic products (FAO/WHO, 2001) for 60 days of storage. The microparticles of alginate and chitosan combined prebiotic analyzed in wet form stored at room temperature, developed in this study may be an alternative and feasible means for obtaining a probiotic product to be incorporated into foods, to allow a greater survival of bacteria.

4. Conclusion

The use of the prebiotic Hi–maize (1%) and chitosan (0.4%) positively affected the survival of the microencapsulated microorganisms in both gastrointestinal resistance tests as during storage of the moist and freeze-dried microparticles.

Moist microparticles were more effective than the freeze-dried microparticles, however, the addition of cryoprotective agents is absolutely needed to optimize the drying process aimed at a better microorganism survival.

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