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Use of Lactulose as Prebiotic and Chitosan Coating for Improvement the Viability of *Lactobacillus* sp. FM4.C1.2 Microencapsulate with Alginate

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Abstract: Lactic acid bacteria (LAB) constitute the microbial group most used as probiotics; however, many strains reduce their viability during their transit through the body. The objective of this study was to evaluate the effect of two microencapsulation techniques, as well as the incorporation of lactulose as a prebiotic and the use of chitosan coating on the microcapsules, on the viability of the *Lactobacillus* sp. strain FM4.C1.2. LAB were microencapsulated by extrusion or emulsion, using 2% sodium alginate as encapsulating matrix and lactulose (2 or 4%) as the prebiotic. The encapsulation efficiency was evaluated, and the capsules were measured for moisture and size. The encapsulation efficiency ranged between 80.64 and 99.32% for both techniques, with capsule sizes between 140.64 and 1465.65 µm and moisture contents from 88.23 to 98.04%. The microcapsules of some selected treatments (five) were later coated with chitosan and LAB survival was evaluated both in coated and uncoated microcapsules, through tolerance to pH 2.5, bile salts and storage for 15 days at 4 °C. The highest survival of the probiotic strain under the conditions of pH 2.5 (96.78–99.2%), bile salts (95.54%) and storage for 15 days (84.26%), was found in the microcapsules obtained by emulsion containing 4% lactulose and coated with chitosan. These results demonstrate the possible interaction of lactulose with alginate to form better encapsulating networks, beyond its sole probiotic effect. Additional research may shed more light on this hypothesis.

Keywords: encapsulation efficiency; emulsion; extrusion; sodium alginate; viability

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1. Introduction

Probiotics are live microorganisms that, supplied in sufficient quantities, have a beneficial effect on the health of the host [1]. The regular consumption of probiotics improves the general state of health of those who consume them. This is mainly because they colonize the intestine, produce metabolites that inhibit the growth of pathogens [2], strengthen the immune system [3], and it has even been reported that they produce substances with an anticancer effect [4,5]. Lactic acid bacteria (LAB) specifically the genera Lactobacillus and Bifidobacterium are the microorganisms mostly used as probiotics [6].

The probiotic must be live and present in sufficient quantity (>10^6 CFU g^{-1} or 10^6 CFU mL^{-1}) to confer a host benefit [7]. However, the viability of some LAB decreases during the digestive process due to gastric acidity, digestive enzyme activity, and bile acids [8]. Likewise, the storage temperature, the oxygen content and the composition of the food matrix have negative effects on the viability of the microorganism [9].

To counteract the decrease in the viability of probiotics derived from hostile environmental conditions, microencapsulation is an alternative. Microencapsulation is a process in which the probiotic microorganism is encapsulated within a polymeric matrix to protect it from its surrounding environment [10]. By being trapped by an encapsulating matrix,

probiotics can increase their viability, as well as reduce cell damage and allow controlled release at their site of action [11].

The encapsulating matrix is frequently a biopolymer since it is reported that these offer a greater advantage over the protection of probiotics, increasing viability, stability, productivity and protection against mechanical damage, as well as against contamination [9]. Sodium alginate has been reported as the most widely used encapsulating matrix due to its ease of handling, zero toxicity, high biocompatibility, and low cost [11]. However, it has been reported that sodium alginate capsules tend to be porous and sensitive to low pH conditions, making them susceptible to rapid release of their contents, as well as decreasing their physical stability in the presence of chelating agents and monovalent ions [9,10]. An alternative to improve the mechanical and chemical stability of microcapsules is the use of coatings based on other biopolymers [12]. Chitosan is a cationic, non-toxic, and biocompatible polysaccharide [7] that has been used as a coating material since it improves the stability of probiotics against simulated gastrointestinal conditions and storage conditions [13,14]. Another factor that increases the viability of microencapsulated probiotics is the use of prebiotics, which improve resistance to adverse conditions and promote cell proliferation [15]. Prebiotics are indigestible food components that stimulate the growth of beneficial bacteria present in the intestine [16]. In addition, the incorporation of prebiotic material significantly improves the efficiency of encapsulation and the controlled release of the probiotic [17]. Inulin, lactulose, resistant starch, and fructo-oligosaccharides are some of the most widely used prebiotics [18–20]. The use of three components (alginate-lactulose-chitosan) acting as an encapsulation-coating system is a novel approach to the trapping processes of probiotic cells.

On the other hand, to improve the efficiency of encapsulation, the most appropriate technique must be found based on the probiotic nature. There are a variety of techniques, however, not all are applicable to different probiotics. Extrusion is a simple and effective technique, whose reported advantages include that it decreases cell damage and increases encapsulation efficiency, avoiding extreme temperatures and pressures that compromise the viability of the microorganism; furthermore, the capsules are relatively soft to ensure high values of viable cells [11,21]. *Lactobacillus casei* has been encapsulated by extrusion, obtaining 4.7×10^8 CFU g^{-1} of microcapsules, compared to a value of 1.0×10^6 CFU g^{-1} in non-encapsulated material. Furthermore, when the capsules were subjected to simulated gastric fluid for 60 min, there was no significant decrease in viability compared to free bacteria [22]. The emulsion is another method that has been widely used because it is relatively simple, it increases the protection of probiotics using hydrocolloid matrices, and the diameter of the microcapsules obtained is acceptable compared to other techniques of encapsulation. The encapsulation of *Bifidobacterium longum* by emulsion technique showed 95% encapsulation efficiency [7]. The microcapsules obtained by these methods make them acceptable to be added to food products without affecting their texture and sensory properties [20].

Galvez-Medina et al. [23] reported that the LAB strain FM4.C1.2 inhibits the growth of *Staphylococcus aureus*, *Salmonella* spp. and *Listeria monocytogenes*. Furthermore, this strain is susceptible to several antibiotics [24], which makes it a probiotic bacterium. However, this strain is susceptible to pH 2.5 [24]. Therefore, the objective of this work was to evaluate the effect of two microencapsulation techniques, as well as the incorporation of lactulose as a prebiotic and the use of chitosan coating on the microcapsules, on the viability of the probiotic bacterium FM4.C1.2.

2. Materials and Methods

2.1. Biological Material

The lactic acid bacterium FM4.C1.2, isolated from fresh cheese [23], was provided by the strain collection of the Instituto de Biociencias of the Universidad Autónoma de Chiapas (IBC-UNACH), Mexico.

2.2. Culture and Identification of the Probiotic Strain

The strain was reactivated by continuous culture in liquid Man, Rogosa and Sharpe (MRS) medium at pH 6.5 for 48 h and growth kinetic was performed. For this, a batch of the microorganism was taken, cultivating it in 5 mL of MRS broth (Difco™, Detroit, MI, USA) at 37 °C for 48 h, later this volume of inoculum was added to a flask with 100 mL of MRS broth and incubated under the same conditions described above. Aliquots were taken from the flask at 12, 24, 36 and 48 h and the absorbances (560 nm) were measured (spectrophotometer JENWAY model 7315, Cole Parmer Ltd., Eaton Socon, UK) to determine the time to reach the highest optical density. When the highest optical density was reached (24 h), an aliquot was taken, serially diluted, and a plate count was performed to estimate the number of UFC mL^{-1}. The LAB were identified by sequence analysis of the 16S rDNA [25] using the universal primers 27f (5′-AGAGTTTAGTCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′).

2.3. Microencapsulation

To evaluate the effect of microencapsulation on the viability of the bacteria, factors were combined using a type 2^4 factorial design. Two encapsulation techniques were studied, two prebiotic concentrations, two extruder (syringe) diameters, two extruder-solution distances, two speeds and two stirring times, depending on the requirements of the techniques (Table 1).

Table 1. Factors and levels evaluated to microencapsulate the probiotic in alginate matrices.

Treatments	Factor A	Factor B	Factor C	Factor D
1	Extrusion	2% lactulose	Dia-0.3	Dis-5
2	Extrusion	2% lactulose	Dia-0.3	Dis-10
3	Extrusion	2% lactulose	Dia-0.6	Dis-5
4	Extrusion	2% lactulose	Dia-0.6	Dis-10
5	Extrusion	4% lactulose	Dia-0.3	Dis-5
6	Extrusion	4% lactulose	Dia-0.3	Dis-10
7	Extrusion	4% lactulose	Dia-0.6	Dis-5
8	Extrusion	4% lactulose	Dia-0.6	Dis-10
9	Emulsion	2% lactulose	S-300	T-10
10	Emulsion	2% lactulose	S-300	T-30
11	Emulsion	2% lactulose	S-600	T-10
12	Emulsion	2% lactulose	S-600	T-30
13	Emulsion	4% lactulose	S-300	T-10
14	Emulsion	4% lactulose	S-300	T-30
15	Emulsion	4% lactulose	S-600	T-10
16	Emulsion	4% lactulose	S-600	T-30

Factor A: Encapsulation method (extrusion and emulsion). Factor B: Concentration of prebiotic. Factor C: Dia, extruder diameter (0.3 and 0.6 mm) and S, stirring speed (300 and 600 rpm). Factor D: Dis, extruder-solution distance (5 and 10 cm) and T, stirring time (10 and 30 min).

To obtain the amount of cells needed for the probiotic, a volume of the bacterial cell culture (in MRS medium cultivated for 24 h at 37 °C) was centrifuged at 3381× *g* for 10 min at 4 °C (centrifuge Beckman Coulter model Allegra™ 64R, Fullerton, CA, USA). The cell pellet was washed with saline solution (0.9% *w*/*v* NaCl); subsequently, it was resuspended in 250 µL of saline solution for later use.

2.3.1. Extrusion

Under aseptic conditions, 20 mL of sodium alginate (molecular weight of 22,500 g mol^{-1}, Sigma-Aldrich Co., St. Louis, MI, USA) solution (2% *w*/*v*) were prepared, the required amount of factor B (Table 1) and the number of cells required based on the final volume to achieve a concentration of 2.77 × 10^8 CFU mL^{-1}. This mixture was gently shaken and placed inside a sterile syringe with a needle of variable diameter (23G or 30G, BD Ultra-Fine™, Becton Dickinson, Franklin Lakes, NJ, USA) according to factor C (Table 1) and extruded at a

constant speed against 20 mL of a 0.1 M solution of calcium chloride ($CaCl_2$, Meyer reagents, Mexico city, Mexico) at two extruder-solution distances according to treatment (Table 1). The $CaCl_2$ solution was kept stirring (300 rpm) for 30 min. Subsequently, the microcapsules were collected by filtration using Whatman #4 paper and washed with saline solution (0.9% *w*/*v*) to finally be placed in peptone water (0.1% *w*/*v*) at 4 °C until use (no more than 8 h) [26].

2.3.2. Emulsion

Under aseptic conditions, 20 mL of sodium alginate solution (2% *w*/*v*) was prepared, the required amount of factor B (Table 1) and the number of cells required based on the final volume to achieve a concentration of 2.77×10^8 CFU mL^{-1}. The mixture was added to 100 mL of soybean oil (Nutrioli®, Monterrey, Mexico) contained in a 600 mL beaker. Tween™ 80 (0.2% *v*/*v*, Thermo Scientific™, Waltham, USA) was added to the oil as an emulsifier. The mixture was stirred according to factor C (Table 1) for the time established according to factor D (Table 1) to achieve a uniform emulsion without evidence of a free aqueous phase. Subsequently, 100 mL of calcium chloride (0.1 M) was added slowly through the walls of the vessel containing the emulsion, and it was left to rest for 20 min for the separation and sedimentation of the microcapsules. The oil layer that formed on the surface was removed with the aid of a pipette. Finally, the microcapsules were collected by centrifugation (300× *g*, 10 min), filtered using Whatman #4 paper and washed two times with saline solution (0.9% *w*/*v*). The microcapsules were stored in peptone water (0.1% *w*/*v*) at 4 °C until use (no more than 8 h) [20,27].

2.4. Encapsulation Efficiency

One gram of microcapsules was added to 9 mL of a sterile sodium citrate solution (0.1 N). Subsequently, the sample was shaken for 10 min using a vortex mixer (Vortex-Genie®2, Scientific Industries, Bohemia, NY, USA), after which time the sample was subjected to serial dilutions in sterile peptone water (pH 7.2). A volume of 1 mL of the -4, -5 and -6 dilutions was deposited in a Petri dish containing MRS agar. The plates were incubated under anaerobic conditions at 37 °C for 48 h. After incubation, the colonies were enumerated, and the results were expressed in CFU g^{-1}. The EE (%) encapsulation efficiency was calculated according to the equation:

$$EE\ (\%) = N/No \times 100 \quad (1)$$

where N is the logarithmic number of viable cells (CFU) released from the microcapsules, and No is the logarithmic number of free viable cells (CFU) added to the biopolymer mixture during microcapsule production [28].

2.5. Characterization of Microcapsules

2.5.1. Microcapsule Moisture

The moisture of the microcapsules was determined by gravimetry [29]. One gram of the sample was weighed, in triplicate, in crucibles previously set to constant weight, which were placed in a Felisa® (Zapopan, Mexico) oven for 3 h at 70 °C. Once the time elapsed, the crucibles were removed from the oven, allowed to cool in a desiccator for 10 min, and then weighed. The moisture percentage was obtained by weight difference using the equation:

$$\text{moisture}\ (\%) = [\text{sample weight} - (\text{final weight} - \text{crucible weight})/\text{sample weight}] \times 100 \quad (2)$$

2.5.2. Size

The size of the microcapsules was determined using an optical microscope, equipped with an AxioCam ERc 5s digital camera (Zeiss, Göttinger, Germany), using the Zen™ program (Zeiss, Göttinger, Germany); 100 mg of microcapsules were placed on a slide and

randomly measured with an objective of 40× the diameter of 10 microcapsules. Capsule size was expressed in micrometers (µm).

2.6. Coating with Chitosan

Of the 16 evaluated treatments, five treatments (T3, T9, T14, T15, T16) were selected according to the most desirable characteristics to be coated with chitosan. Low molecular weight chitosan and 75–85% deacetylation (MW = 50–190 KDa, Sigma®, St. Louis, MI, USA) was used. An aqueous solution of chitosan (0.4% *w*/*v*) was prepared in distilled water acidified with 0.4% (*v*/*v*) of glacial acetic acid. After stirring for 2 h at 250 rpm, the pH was adjusted to 5.5–6.0 by adding NaOH (1 M). The solution was filtered through Whatman #4 paper, and the volume adjusted to 100 mL. This solution was sterilized at 121 °C for 15 min. The previously prepared microcapsules (25 g) were washed with saline solution and later submerged in 100 mL of the chitosan solution, they were shaken for 20 min at 100 rpm using an orbital shaker HZ-300 (Luzeren®, PROLAB, Tlajomulco, Mexico). Finally, the chitosan-coated microcapsules were harvested using Whatman #4 paper and washed with sterile peptone water before use [30].

2.7. Viability of Chitosan-Coated Microencapsulated LAB

2.7.1. Resistance to pH 2.5

Resistance to pH was measured for free, microencapsulated, and microencapsulated-coated bacteria. Free bacteria were obtained as described in Section 2.3. One milliliter of free bacteria was resuspended in 9 mL of MRS broth adjusted to pH 2.5 and 6.5. The cultures were incubated for 3 h at 37 °C. After incubation, serial dilutions were made and inoculated with MRS agar to estimate the number of viable cells. For microencapsulated and microencapsulated-coated bacteria, 1 g of microcapsules were resuspended in 9 mL of MRS broth adjusted to pH 2.5 and 6.5. Immediately, they were incubated for 3 h at 37 °C. For the enumeration of microencapsulated and microencapsulated-coated probiotic organisms, bacteria were released as described in Section 2.4. Survival was determined by comparing the final counts against the initial counts for each of the pH and the percentage of resistance to pH was estimated [31] using the following equation:

$$\text{Resistance to pH (\%)} = \text{CFU at pH 2.5/CFU at pH 6.5} \times 100 \quad (3)$$

2.7.2. Resistance to Bile Salts

Free bacteria were obtained as described in Section 2.3. One milliliter of the culture was inoculated into tubes with 9 mL of MRS broth with 1.5% ox bile (Meyer® reagents, Mexico City, Mexico) and the control was MRS without bile salts. Incubation was carried out for 3 h at 37 °C under static conditions. The same procedure was performed for microencapsulated and microencapsulated-coated cells. For the enumeration of microencapsulated and microencapsulated-coated probiotic organisms, bacteria were released as described in Section 2.4. The percentage of survival with respect to the control was estimated [28] with the following equation:

$$\text{Resistance to bile salts} = (\text{CFU in MRS + bile salts/CFU in MRS}) \times 100 \quad (4)$$

2.7.3. Storage at 4 °C

The viability of the cell-free, microencapsulated, and microencapsulated-coated was evaluated after storage for 15 days at 4 °C. Free bacteria were resuspended in 1 mL of saline solution (0.145 N). For microencapsulated and microencapsulated-coated bacteria, 1 g was taken and added to 1 mL of saline solution (0.145 N). Finally, the samples were stored at 4 °C for 15 days. Assays were performed in triplicate. The survival rate (%) of free, encapsulated, and encapsulated-coated FM4.C1.2 was calculated using the equation:

$$\text{Survival rate (\%)} = \text{Nt/No} \times 100 \quad (5)$$

where No (CFU g^{-1}) is the number of viable cells before storage and Nt (CFU g^{-1}) is the number of viable cells at the end of storage [31].

2.8. Data Analysis

The data obtained from all measurements were subjected to analysis of variance and subsequent comparison of means by Tukey's test ($p < 0.05$). In addition, to form groups with similar characteristics and select the treatments with the most desirable characteristics of the microcapsules, cluster analysis was performed with the measurements of the microcapsules. The analyses were carried out using the InfoStat software version 2018.

3. Results and Discussion

The analysis of the 1120 bp consensus sequence of the 16S rRNA gene of the LAB showed 100% homology with genera *Lactobacillus;* however, due to total coverage (91%) we cannot infer the species. The growth kinetics (OD) of the FM4.C1.2 strain revealed that after 24 h of fermentation in MRS broth, the highest OD was reached (1.114 absorbance units); equivalent to a cell concentration of 2.77×10^8 CFU mL^{-1}.

3.1. Characterization of Microcapsules

3.1.1. Encapsulation Efficiency

The encapsulation efficiency (EE) of each of the evaluated treatments is shown in Figure 1. The results of the treatments coded as T5–T8, all carried out with the extrusion technique, are not presented because during the process the capsules were not formed. These treatments have in common the concentration of lactulose (4%), which suggests that at this concentration, lactulose can alter the ionic interaction between alginate and the Ca^{2+} ions when extrusion is carried out, as has been suggested to occur in the interaction of alginate with some carbohydrates [9]. The EE values of the remaining 12 treatments revealed high efficiencies; however, differences were found among treatments ($p < 0.05$). The extrusion treatments T3 and T4, as well as the emulsion treatments T10 and T16, presented the highest values, above 97%.

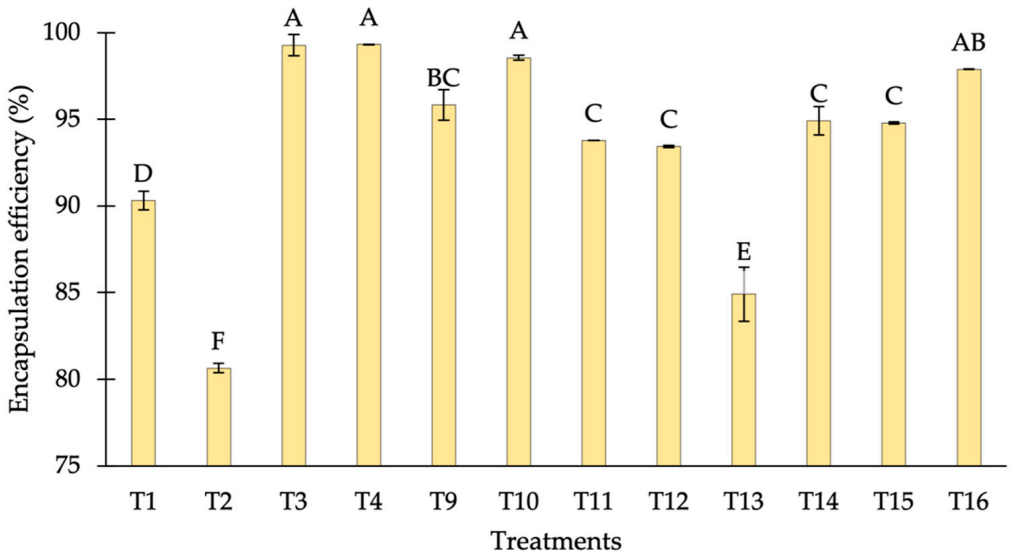

Figure 1. Encapsulation efficiency of probiotic FM4.C1.2. Bars with the same letter are not significantly different ($p > 0.05$). The lines above the bar show the standard deviation. For details of the codes in the treatments, see Table 1.

When encapsulating by extrusion, the efficiency is affected by the nature of the encapsulating matrix and the concentration of the crosslinking agent [32]. Silva et al. [33] found efficiencies like those of the present study, above 83% while Ta et al. [9] obtained a slightly lower efficiency (79%), using the same prebiotic (lactulose) but a different encapsulating matrix. Our study showed efficiency greater than 80% in all treatments, which are values higher than those reported when only calcium chloride is used as an encapsulant where values as low as 20% EE are reported [34]. The high EE values may, therefore, be a consequence of the combination of alginate with 2% lactulose since it has been reported that the combination of alginate with other substances modifies the physical properties of the encapsulating wall. This result also demonstrates that the ionic interactions between alginate and calcium were not affected by 2% lactulose when a 0.6 mm diameter extruder (T3 and T4) was used, since these extrusion treatments presented EE close to 100%. Frakolaki et al. [11] used alginate mixed with various encapsulating agents, obtaining efficiencies of 58.61% up to 100% and reported that the lowest efficiency was attributed to the use of alginate alone; however, when combined with other polymeric agents, the efficiencies increased relatively. This may explain the fact that adding 2% lactulose increased the encapsulation efficiency.

The EE using the emulsion technique is like other reports but uses different encapsulating agents. Elvan et al. [35] found EE above 90%, when using whey protein concentrate and xylan, while da Silva et al. [36] obtained lower efficiencies (81.1 and 89.61%) using porcine gelatin as the encapsulating matrix. Studies using sodium alginate as an encapsulating agent show variable results, Gul and Dervisoglu [37] report efficiencies of 86.71% and 95.25%, while in another study [36] they obtained <1.0%. The high efficiency reported in our study may be due to the use of alginate in combination with a prebiotic (lactulose), as mentioned for the extrusion technique, since it could act as a co-encapsulating agent, improving barrier properties. In addition, during the encapsulation process, agitation is a determining factor since it can decrease the viability of the probiotic bacteria, and therefore, decrease the encapsulation efficiency. This suggests that the speed used here was optimal.

3.1.2. Moisture

The microcapsules that had the lowest moisture were those of the treatments T9, T11, T12, T13, T15 and T16 with significant differences ($p < 0.05$) from the rest (Figure 2). The lower water content in the microcapsules of these treatments may be a consequence of the oil and/or emulsifier incorporated since these capsules all come from the emulsion treatments. The moisture of the microcapsules obtained by the extrusion technique is like those reported in the literature, Poletto et al. [38] found results similar to ours, where the moisture of the microcapsules was between 81.64% and 94.94%, this was attributed to the different prebiotics that were used. The microcapsules made by extrusion (T1–T4) had significant equality ($p > 0.05$) in the water content. However, for the microcapsules obtained by emulsion, the differences ($p < 0.05$) observed in the water content may be due to the other factors evaluated, thus the capsules with lower moisture are obtained by emulsion with a stirring speed of 600 ppm for 10 min, either with 2% (T11) or 4% lactulose (T15). The moisture values in these treatments are like those in other studies (50–60%) with alginate-Tween capsules [39]. The treatments that were found to be less moisture could be good candidates to keep LAB alive, since by reducing the water that interacts with the bacteria, their metabolic processes can be slowed down and their stability increased [40], without resorting to expensive processes of spray drying or freeze drying. The lower moisture content in the emulsified capsules is because at the time the emulsions were formed, fewer interactions with the surfactant were promoted; therefore, less water was trapped in the capsule.

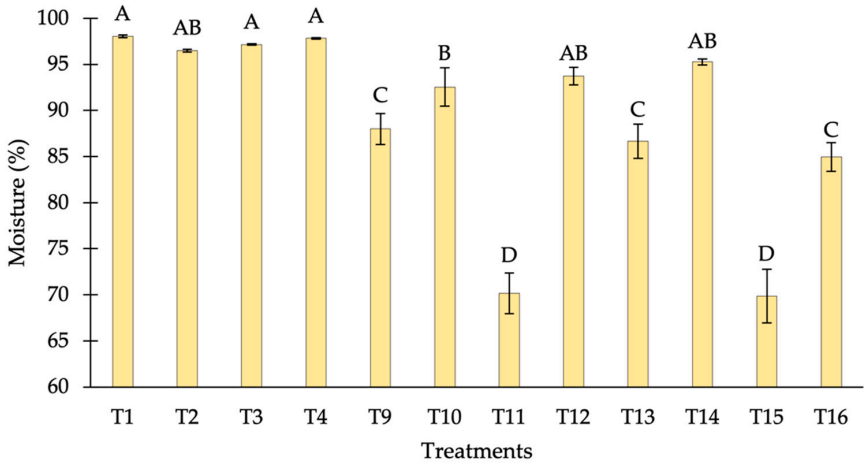

Figure 2. Moisture of microcapsules. Bars with the same letter are not significantly different ($p > 0.05$). The lines above the bar show the standard deviation. For details of the codes in the treatments, see Table 1.

3.1.3. Size

As expected, the sizes of the microcapsules varied depending on the technique used (Figures 3 and 4). The microcapsules of the extrusion technique treatments (T1–T4) had the highest values, being significantly different from the rest ($p < 0.05$). The microcapsules of treatments 11 and 12 (of the emulsion technique) presented a smaller average size but greater heterogeneity, which was reflected in high values of standard deviations (Figure 3). The size of the microcapsules is decisive in the protection of probiotics and in the texture of food [41]. In the extrusion process, in addition to the diameter of the needle, the size can be influenced by factors such as the concentration of the prebiotic, extruder-solution distance, stirring speed of the hardening solution, alginate and calcium chloride concentration [42]. The difference in the size of the microcapsules due to the diameter of the needle was significant (T1–T2 vs T3–T4, $p < 0.05$). However, when performing a contribution analysis of the factors, except for the diameter of the needle, the other variables had no significant effect ($p > 0.05$) on the size of the microcapsules. Farez et al. [43] reported similar results using a 0.6 mm diameter needle, finding sizes in the range of 1312 and 1343 µm. Lucan and Oroian [44] reported microcapsule sizes greater (between 1.86 and 2.25 mm) than our results, using a needle diameter of 0.8 mm. These reports also demonstrate the low effect of the different concentrations of inulin used as a prebiotic on the size of the microcapsule.

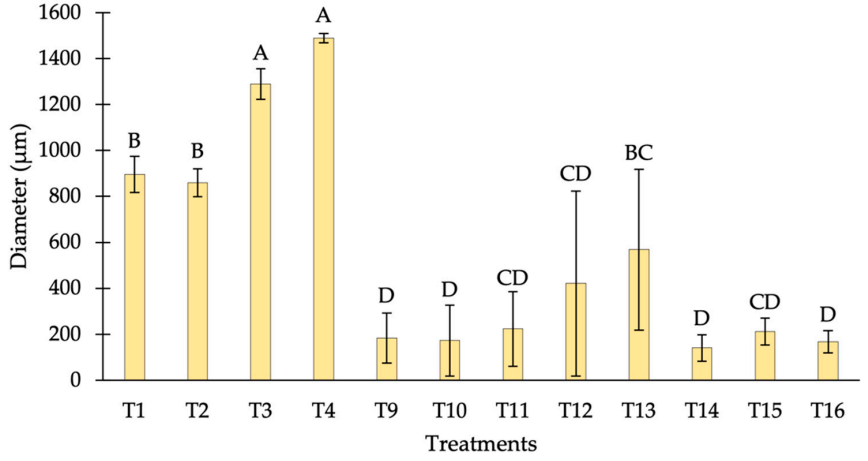

Figure 3. Size of the microcapsules. Bars with the same letter are not significantly different ($p < 0.05$). The lines above the bar show the standard deviation. For details of the codes in the treatments, see Table 1.

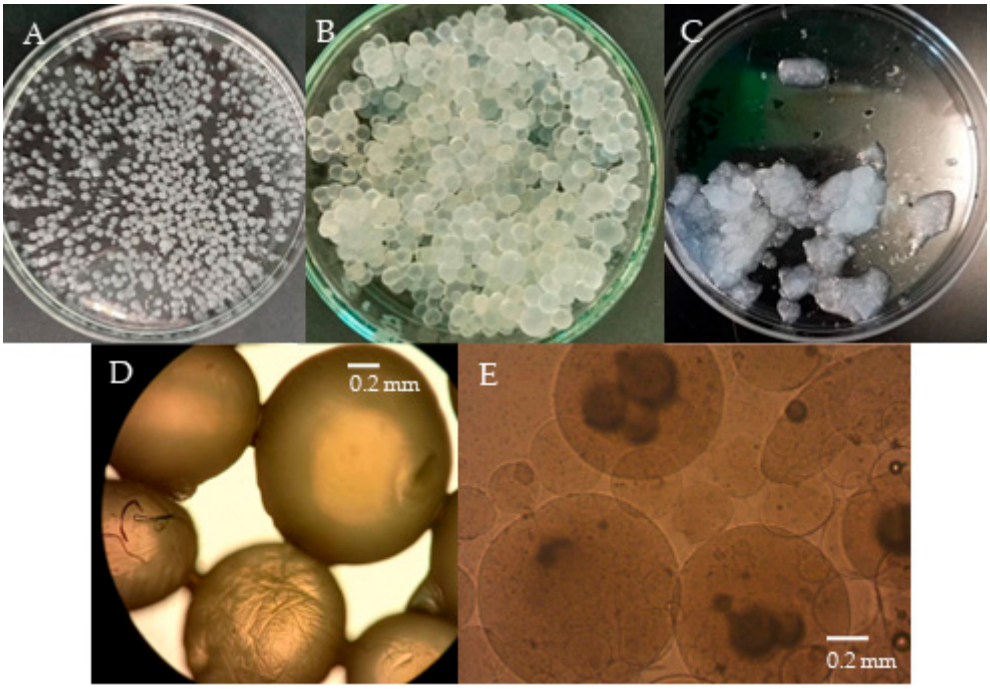

Figure 4. Aspect macroscopic (**A**–**C**) and microscopic (**D**,**E**) of the microcapsules from the extrusion (**A**,**B**,**D**) and emulsion procedure (**C**,**E**). Fresh microcapsules (**A**) and after determining moisture (**B**). Microcapsules from the treatments 3 (**D**) and 13 (**E**).

Despite the heterogeneity (Figures 3 and 4E), the size of the microcapsules obtained by the emulsion technique is within the suggested range to be used in food, since it is considered that smaller than 1000 µm offer greater protection to probiotics [45]. Song et al. [46] reported sizes like ours, with values ranging between 35 and 863 µm. The larger size was attributed to a greater dispersion of the alginate droplets when colliding with the calcium chloride, a phenomenon that may have occurred in our research. The size of the microcapsules, using this technique, is influenced by the ratio of oil: water, surfactant concentration, and time and speed of agitation [6]. Ashwar et al. [47] state that the stirring speed used (1500 rpm) influences the size of the microcapsules, finding average sizes of 45.43, 49.29 and 47.12 µm. Although the factor contribution analysis revealed that there was no significant effect ($p > 0.05$) of any of the factors individually evaluated on this variable, there seems to be a trend towards increasing the variability in the size of the microcapsules when 2% lactulose is used, since those treatments containing this concentration (T9–T12) presented the largest standard deviation to the average value (Figure 3).

3.2. Cluster Analysis

The cluster analysis of the results of the encapsulation efficiency, diameter and moisture of the microcapsules allowed the construction of a dendrogram based on the Euclidean distance (Figure 5). When four groups were requested, the dendrogram separated them with a distance >1.56. Two treatments of the extrusion process (T3 and T4, blue color) and two of the emulsions (T11 and T15, green color) were grouped together and outside of the rest of the treatments. The third group (purple color) contained five treatments and the last cluster (red) contained three treatments. The major contribution of the factors for this grouping seems to be the efficiency of encapsulation (Figure 1) and the size of the microcapsule (Figure 3). Based on this analysis and given that the results of all the treatments were acceptable, for the next stage of the study (microcapsule coating) treatments T3, T9, T14, T15 and T16 were selected, each one taken from a different group (except for red cluster) with in order to observe the behavior given the difference between them.

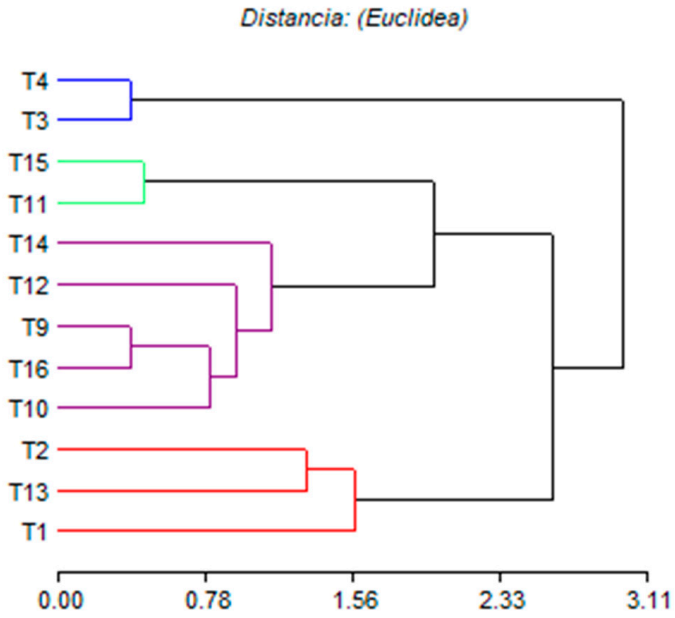

Figure 5. Dendrogram derived from the cluster analysis of the different treatments based on characteristics they share. Each color represents one cluster.

3.3. Viability of LAB Microencapsulated and Coated with Chitosan

3.3.1. Resistance to pH 2.5

The viability of free, microencapsulated, and microencapsulated-chitosan-coated bacteria when exposed to pH 2.5 is shown in Figure 6. Free cells (T0) did not show survival after 3 h of exposure to acidic pH, as previously reported for the strain FM4.C1.2 [28]. Likewise, the microencapsulated bacteria from treatments T3 and T9 did not present viability after 3 h of exposure to acidic pH. These two treatments come from different encapsulation techniques, but they have in common that both treatments contained 2% lactulose, in contrast to the other treatments that contained 4% of the prebiotic. It has been reported that alginate forms structures that can present porosity and allow the passage of acid, causing decreased viability of the bacteria [45]; however, the results obtained with microencapsulated cells for treatments T14–T16 (4% of lactulose) reveal that lactulose at this concentration could be acting as a protective agent against acidity or, where appropriate, interacting with the alginate to form a more impermeable barrier to protons. This same behavior observed in the first stage of the study could verify the action of lactulose. On the other hand, an increase in viability was observed for all treatments that were coated with chitosan at acidic pH after 3 h of incubation. The coating provided greater protection to the bacteria compared to free and microencapsulated cells (Figure 6). This is because it forms strong complexes with alginate, and this improves the stability of the microcapsules in the presence of chelating agents, it also reduces the porosity and consequently the passage of substances that decrease the viability of the cells [27]. Fareez et al. [43] proved that microencapsulation with alginate and xanthan gum and, also coating with chitosan improved the viability of *Lactobacillus plantarum* LAB12 by up to 95% when exposed to simulated gastric juice at pH 1.8 for 120 min. In contrast with our results, Castro-Rosas et al. [31] reported that the viability of free *Lactobacillus paracasei* exposed to pH 2 was 96% after 3 h of exposure, while for microencapsulated bacteria it was 97%. The null viability of the FM4.C1.2 strain, both free and microencapsulated after exposure to pH 2.5, is due to the low tolerance to this adverse condition, previously reported [24].

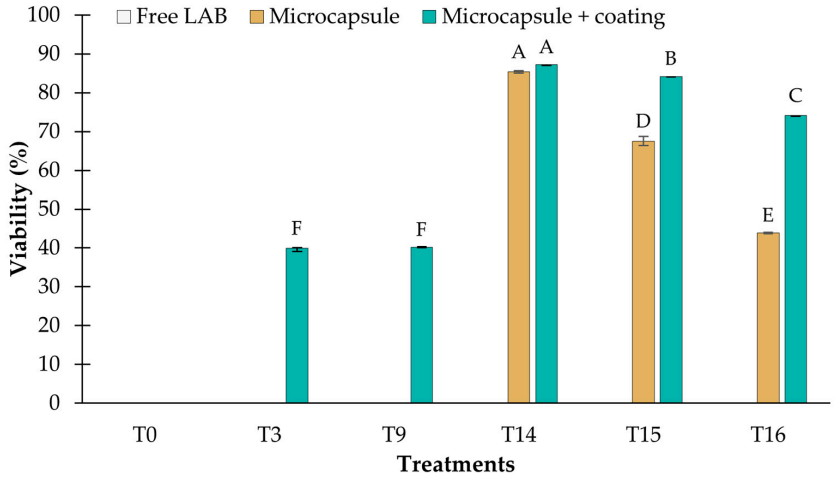

Figure 6. Viability of free (T0), microencapsulated, and microencapsulated-chitosan-coated bacteria FM4.C1.2 subjected to pH 2.5 for 3 h. Bars with the same letter are not significantly different ($p < 0.05$). The lines above the bar show the standard deviation. For details of the codes in the treatments, see Table 1.

3.3.2. Resistance to Bile Salts

Figure 7 shows the viability of free, microencapsulated, and microencapsulated-coated with chitosan bacteria exposed to bile salts (1.5%) for 3 h of incubation. The free probiotic bacteria (T0) exhibited tolerance to bile salts, maintaining viability above 80% during the 3 h of exposure. This behavior reveals that the strain FM4C1.2 evaluated here has high activity of the bile salt hydrolase (BSH) enzyme, responsible for deconjugating bile acid, thus reducing its toxic effect [48] and, also may contribute to the metabolism of fatty acids and cholesterol. The microencapsulation and coating processes improved the viability of the strain. The average value for all treatments when LAB were microencapsulated was 90%, while the average value in all microencapsulated-coated treatments increased to 95.54%. Ding et al. [49] exposed various free, microencapsulated probiotic strains, coated with palm oil and a poly-L-lysine secondary coating, to ox bile salts. They report values from 60% (free bacteria), and around 85% for microencapsulated cells and for bacteria coated with poly-L-lysine. In contrast to our study, the authors used a higher concentration of bile salts (3%) and a longer incubation time (4 h), which could be influencing the exhibiting greater viability of the FM4.C1.2 strain under this condition. These results also show that chitosan maintains viability better than poly-L-lysine whose effect was limited.

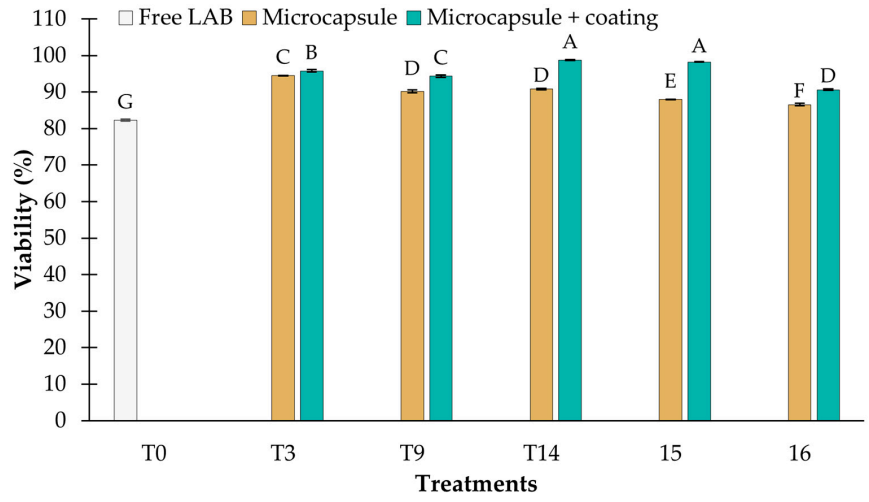

Figure 7. Viability of free, microencapsulated, and microencapsulated-chitosan-coated bacterium FM4C1.2 subjected to bile salts for 3 h. Bars with the same letter are not significantly different ($p < 0.05$). The lines above the bar show the standard deviation. For details of the codes in the treatments, see Table 1.

3.3.3. Storage at 4 °C

The survival of free probiotic bacteria decreased to 72.29% after 15 days of storage at 4 °C (Figure 8). For the microencapsulated treatments T3 and T9, higher values than free cells (88.33 and 78.43%, respectively) were found in viability. The treatments T14–T16 presented viability values higher than 89%, being the highest among all the microencapsulated treatments. This behavior is similar to that reported for tolerance to pH 2.5. This may be due to the higher content of lactulose present (4%), since in addition to the structural effect it may be having, this prebiotic could have established symbiosis with the strain FM4.C1.2, increasing viability and stability [50]. For the microencapsulated-chitosan-coated treatments, a slight increase in viability was generally observed with respect to the microencapsulated treatments. The exception to this behavior occurred in treatment 16, whose viability was lower in the microencapsulation-coating process. These results demonstrate that both the chitosan coating and the addition of 4% prebiotic influenced the maintenance of LAB viability after 15 days of storage. This is in agreement with other studies [51,52] reporting that adding prebiotics to an alginate matrix and coating them improves the survival rate during storage at 4 °C. Valero-Cases and Frutos [42] found high survival values of *L. plantarum* microencapsulated with 2% inulin during 30 days of storage at 4 °C, indicating that the percentage of prebiotic used influenced maintaining the viability of the probiotic bacteria since it acted as a source of energy.

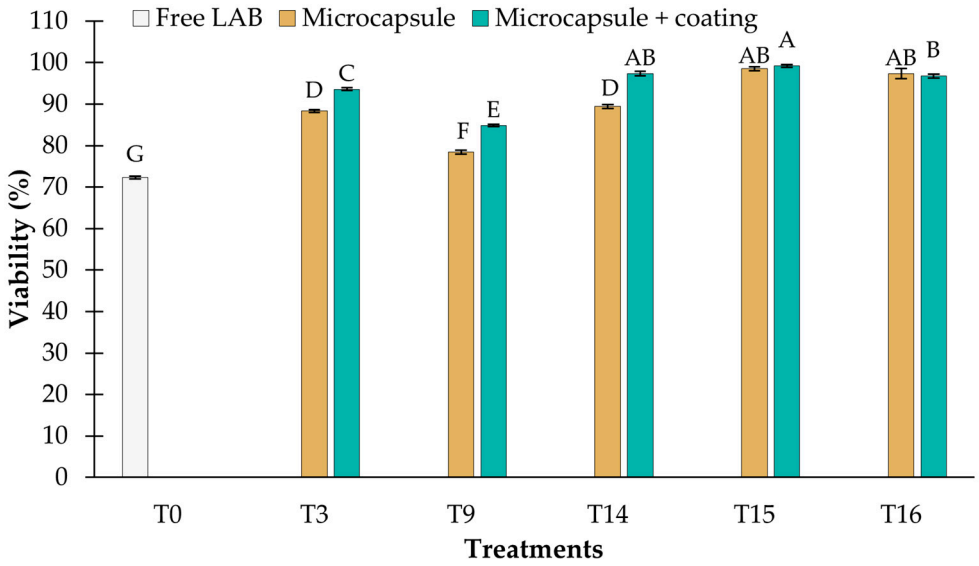

Figure 8. Viability of free, microencapsulated, and microencapsulated-chitosan-coated probiotic bacteria during 15 days of storage at 4 °C. Bars with the same letter are not significantly different ($p < 0.05$). The lines above the bar show the standard deviation. For details of the codes in the treatments, see Table 1.

4. Conclusions

Of the two techniques evaluated, microencapsulation by emulsion combined with the addition of 4% of prebiotic maintained the viability of *Lactobacillus* sp. strain FM4.C1.2 at higher levels. This procedure allowed obtaining capsules with a size between 140.64 and 1465.65 µm. In addition, when coating the microcapsules with chitosan, greater tolerance was observed when exposed to pH 2.5, bile salts and storage at 4 °C. The results of this study showed that lactulose when added at a concentration of 4% (*w*/*v*), acted as a prebiotic for *Lactobacillus* sp. strain FM4.C1.2, and possibly as an encapsulating agent interacting with sodium alginate.

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