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THE EFFECT OF MICROENCAPSULATION AND POTATO STARCH ON THE SURVIVAL OF *LACTOBACILLUS* STRAINS

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Abstract: *The effectiveness of encapsulating ofactive bacteria in releasing probiotics depends on cell survival. In this study, a prebiotic - potato starch was used to evaluate its influence on the viability of probiotics microcapsulated. Lactobacilluscasei, Lactobacillus rhamnosus and Lactobacillus plantarumwas cultured for 16 hours and after that, the cells were added to an emulsion with 1.5% sodium alginate, supplemented with 1% prebiotics and encapsulated. Initial tests revealed that the initial culture concentration is not relevant for prebiotic supplementation. The testing of fresh microcapsules recorded a load of 3.31 × 10 ¹³ cfu / g. After 7 days of storage, the probiotics that survived were 2.53 × 10¹¹ colony-forming unit/g (cfu / g) and, after 30 days1.7 × 10^7cfu / g. The probiotics stored for 7 days at 4 °C were tested for achieving the survival rate of the probiotics in the simulated intestinal juice for 1 hour and it was determined a survival rate of 10^8cfu / g. The simulation for 4 hours in the intestinal juice reported a survival of 10^6cfu / g. In conclusion, starch is an effective material for encapsulating Lactobacillus cultures.*

Keywords: *probiotics, prebiotics, Lactobacillus rhamnosus, L.plantarum, L.casei*

1. Introduction

From Metchnikof onwards, studies of lactic acid bacteria (LAB) began to be increasingly numerous. This group of bacteria are actually natural inhabitants of the human gastrointestinal tract and play an important role in maintaining the microbial ecosystem of the colon. LAB are considered extremely advantageous non-pathogenic species for the human population that play important roles in nutrition, metabolism, immunity and defense against pathogens. According to the definition of probiotics given by (Fao / Who, 2002): living microorganisms that when administered in adequate amounts confer beneficial effects to the host, many species and genera of LAB are considered to be probiotics[1].Probiotic explorations have led to the development of prebiotics, which are a source of carbon and at the same time bioprotectors for probiotics. The prebiotic sources are represented by vegetables, fruits, grains from which we can mention: starch, inulin, oligofructose, xylose, galactose, etc. Although prebiotics are not easily digested in the human intestine, they have a selective role in stimulating the growth or the activity of beneficial bacterial species in the intestine, being nutrients that modify the intestinal microbial flora [2].Besides supporting growth, survival of probiotics and suppressing the development of pathogenic micro-organisms, a symbiotic interaction indicates a great potential for improving the efficiency of the functional food class. Taking into account the FAO/WHO, 2001recommenddations, that at the time of food consumption the concentrations of live microorganisms must be above 10^6-10^7CFU g^{-1} or mL^{-1} then it is necessary to ensure the viability of probiotic strains. This viability may be affected during

processing and storage: temperature, chemical additives, dissolved oxygen, pH, hydrogen peroxide and many others; then after this they will have to survive the storage temperatures, the storage time; and last but not least, it should survive the transit through the digestive tract, low pH values, gastric juice, bile juice, pancreatic juice and intestinal juice, etc.[3].Over the years many methods have been conducted in order to protect the probiotics and to improve their survival against the above mentioned harmful factors. One of these methods is the creation by a mechanical process of a physical-chemical barrier around probiotic cells. Microencapsulation is a process that tries to improve and maintain the survival of the cells, so that their release to the site of action is in the optimal number for human and animal health. Several encapsulation techniques have been developed, of which the most economically accessible and which do not impose harmful solutions and can be performed in both aerobic and anaerobic conditions, is the extrusion encapsulation technique [4-7].At the same time, besides the encapsulation method, an important criterion for the production of resistant probiotic microcapsules is the selection of the appropriate encapsulation material at appropriate concentrations, so that it can resist all harmful factors [8]. Therefore, the aim of this study was to evaluate the effect of the addition of an prebiotic (potato starch) in microcapsules produced by the ionic extraction / gelling technique, which contain a mixture of *Lactobacillus strains*(*L. casei 431, L. plantarum and L. rhamnosus*). Evaluation was performed on microcapsules stored at 4 °C for 30 days.

2. MATERIALS AND METHODS

Media and substrates

For encapsulation the reagents used were: potato starch soluble (STH), Tween 80, De Man – Rogosa – Sharpe (MRS) broth, Manganese (II) sulfate hydrate, potassium chloride, sodium chloride sodium citrate, peptone from casein, meat extract, yeast extract, magnesium sulfate heptahydrate and glucose which werepurchased from Sigma-Aldrich, Saint Louis, USA; calcium chloride hex hydrate was purchased from Lach-Ner Company - Czech Republic; Sunflower oil, alginic acid Sodium salt was purchased from AppliChem GmbH, MRS agar waspurchased from VWR International bvba/sprl; acetic acid-ammonium salt, sodium salt trihydrate, sodium phosphate, acetic acid, and sodium phosphate dibasic hydratewere purchased from Across organics-Spain.

Bacterial strain and culture conditions

The bacterial strains used in this study were *Lactobacillus plantarum and Lactobacillus rhamnosus* (Bioprox RP 80, France, *Lactobacillus* cells count = 7±2x10e10) and *L. casei* 431 (Christian Hansen). From *L. casei* 431 strain, pure cultures were obtained after 3 reactivations in MRS broth and stored at -20 °C in glycerol.The lyophilized frozen bacterial strains were reactivated 24 hours at 37°Cin MRS broth, then for another 24 hours at 37°C in modified MRS broth - carbon source, glucose, was replaced with a prebiotic likepotato starchto obtain a densitometry of 5 McFarland units for *L. casei* and 10 McFarland units for the other 2 strains (*Lactobacillus plantarum and Lactobacillus rhamnosus*) grown together.These values were determined on a McFarland densitometer with McFarland measuring range of 0.3 - 15.0 at wavelength $\lambda = 565 \pm 15$ nm. The three strains were inoculated for 24 hours at 37°C and raised together in the modified MRS broth media environment. From the latter culture a 16-hour culture was obtained. This one was centrifuged at 2500

× g for 10 min at 4 ℃ ± 1 and the pellet was used for micro-encapsulation.

Encapsulation by extrusion

The extrusion technique was used for the preparation of alginate beads. The alginate was added together with 1% prebiotic, 10% vegetable oil in 85% distilled water and mixed using a magnetic stirrer (1400 rpm), with heating until the mixture was completely dissolved. The alginate solution (1.75%) was then autoclaved for 15 minutes at 121°C and allowed to cool to 25 °C. This mixture was stirred at 10000 U/min for 30min. 15 ml of the bacterial pellet was added to the emulsion. This mixture was stirred again for 15 min at 600 rpm / min. Then, the emulsion was injected using a peristaltic pump with a power of 3.2 rpm / ml through a needle with a diameter of 0.4 mm. The suspension was dropped into aliquots of sterile calcium chloride solution (2 %) from different heights: 1.5-2.5cm, and 10 - 15 cm. The droplets formed gel spheres immediately. The beads were allowed to stand for 30 min to harden, then there were harvested using a vacuum pump. The whole procedure was performed using autoclaved (121°C, 15 min) materials and under sterile conditions in a horizontal laminar air-flow cabinet. All solutions and media were prepared with distilled water (Aquatron A4000D, Cole-Parmer Ltd) and all glassware and reagents used in the experiment were sterilized before use.

Viable cell count entrapped within micro-capsules

The viability of the lactobacillus strains was determined by homogenizing 0.1 g of fresh microcapsules in 9.9 ml of 1% sterile sodium citrate solution at pH 6.0, by gentle shaking at room temperature for 10-12 minutes. After the release of the cells from the microcapsules,serial dilutions were performed in sterile distilled water and then inoculated in MRS agar. The plates were incubated in anaerobiotic condition at 37 °C for 72 hours. The determination was made in triplicate, and the results were expressed in colony forming units (cfu) / g^{-1} of microcapsules.

Bacterial enumeration and survival during storage

The microcapsules resulting from the encapsulation process were recovered from calcium chloride, washed and then stored in sealed, sterile Petri plates at 4 °C for 30 days. Evaluation of survival during 30 days at 4 °C of the *Lactobacillus (L. casei, L. plantarum, L. rhamnosus)*strains in various concentrations of alginate microcapsules and various carbon sources was made at 0, 7, 14, 30 days in triplicate. For the evaluation of the number of viable cells, the protocol described above was followed. The results were expressed as cfu / g of microcapsules.

Survival of microencapsulated cells to simulated intestinal condition

The survival of microencapsulated bacteria was analyzed by counting the viable cells after treatment with simulated intestinal condition.Simulated intestinal juice was prepared by dissolving bile salts in intestinal solution (0.65% NaCl, 0.0835% KCl, 0.022% $CaCl_2$ and 0.1386% $NaHCO_3$) pH 7.5 to final concentrations of 0.3% [9]. For thisanalysis it wasused beads which were stored for 7 days at 4 °C.Allthedeterminationwere made in triplicate andincubated at 37 °C for 60, 120, 180and 240 min. Aftereachincubation, survival of microencapsulatedcells was immediately counted by pour plating on MRS agar following the protocol described above.

Microencapsulation efficiency (ME %) andencapsulation yield (EY%)

Microencapsulation efficiency is the survival rate of microorganisms during the microencapsulation process and calculated according to:

$$ME\ \% = N_1 / N_0 \times 100 \quad (1)$$

as proposed by Martinet et al. [10], where N^1_1 is the number of viable cells (log CFU g^{-1}) released from the microcapsules and N^0_0 is the number of viable cells (log CFU g^{-1}) in the cell concentrate used for microencapsulation.

The encapsulation yield (EY) was calculated according to the formula used by [9, 11, 12]:

$$EY = = \log_{10}N_1 / \log_{10}N_0 \times 100$$

Where $\log_{10}N_0$ represents the number of viable cells trapped in the capsule and $\log_{10}N_1$represents the amount of free viable cells added to the emulsion during the encapsulation process and the result of the equation is expressed as number of CFU / ml. This formula, EY, represents a criterion for measuring how the encapsulation process influences the number of viable cells [12].

Characterization of the microcapsules

The dimensions and the morphological characterization of a number of 50 randomly chosen micro-capsules were performed using optical microscopy and electron microscopy. The surface morphology of the capsules was determined by scanning electron microscope (SEM).

Statistical analysis

The results were computed, the means submitted to a normality test and reported as the means and standard deviations. All the variables were submitted to a Two-way ANOVA analysis followed by Tukey's multiple comparisons test with a significance level of 5% ($p < 0.05$).

3. RESULTS AND DISCUSSIONS

Morphological characterization of micro particles

The characterization of the particle morphology helped us to a better understanding of the possible morphological or structural changes of the microcapsules during the micro-encapsulation process. The size of the microcapsules can be influenced by factors such as: alginate concentration, calcium chloride concentration, needle diameter, pump pressure, distance between the needle and calcium chloride solution.Following the study of the morphology of the microcapsules with the help of the optical microscope, it was observed different forms of microcapsules resulted from the microencapsulation process but the most abundant were the round ones. It was also observed that the vast majority are not adherent to each other and the dimensions are much smaller than 1 mm than in other studies such as those conducted by Mokarram et al., [13]; Hansen et al. [14] who obtained microcapsules with dimensions > 1mm using the same encapsulation technique and the same material for the capsule matrix.At the same time, the study of the morphology of the micocapsules was also performed with the help of the scanning electron microscope where it was also observed that there are different forms (figure 1 – A,B, C;) but very few adhesions between them. With the help of electron microscopy it was observed that on the surface of the microcapsules, from the study, there are no free lactobacillus cells, which leads to the idea that the methodology approached for micro-encapsulation is improved.

Evaluation of the mean diameter and distribution of microparticles size

Figure 2 shows the dimensional distributions of the microcapsules that have STH in their matrix.On the other hand, the histogram shows the presence of

two well-separated groups of sizes, one corresponding to the dimensions between 450-500µm and the other between 550-600µm. According to table 1 it can be seen that the most common are microcapsules with size 483µm.Factors such as stirring speed, the concentration of surfactant or the ratio between water and oil (v / v) can influence the distribution and the size of the microcapsules. The capsule compression can also be explained by the inner water partial expulsion due to addition of the cellular pellet in the emulsion and the expulsion of the emulsion in calcium chloride with gel layer forming[10, 15].

Table1. Beads size expressed as mean of 50 microcapsules ± standard deviation.

	size in micrometers (µm)
Mean	504.1 ± 119
Median	497.5
Mode	483
Minimum	259
Maximum	920
Count	50

Comparing with other studies, it was found that the method approached in this study is improved because smaller microcapsules were obtained. For example Lenton et al., [16] obtained an average size for microcapsules of 2.9 mm using also microencapsulation by extrusion; then in another study in which a needle similar to the one in the present study was used, Muthukumarasamy et al., [17] obtained an average size of 2.37 mm; in another study it has obtained an average of the microcapsules 1.86mm [18] obtained by microencapsulation by extrusion.

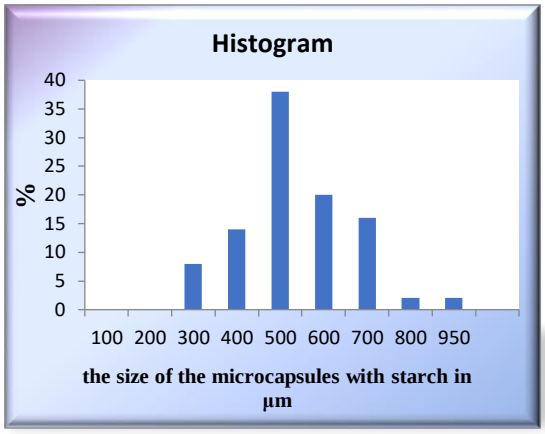
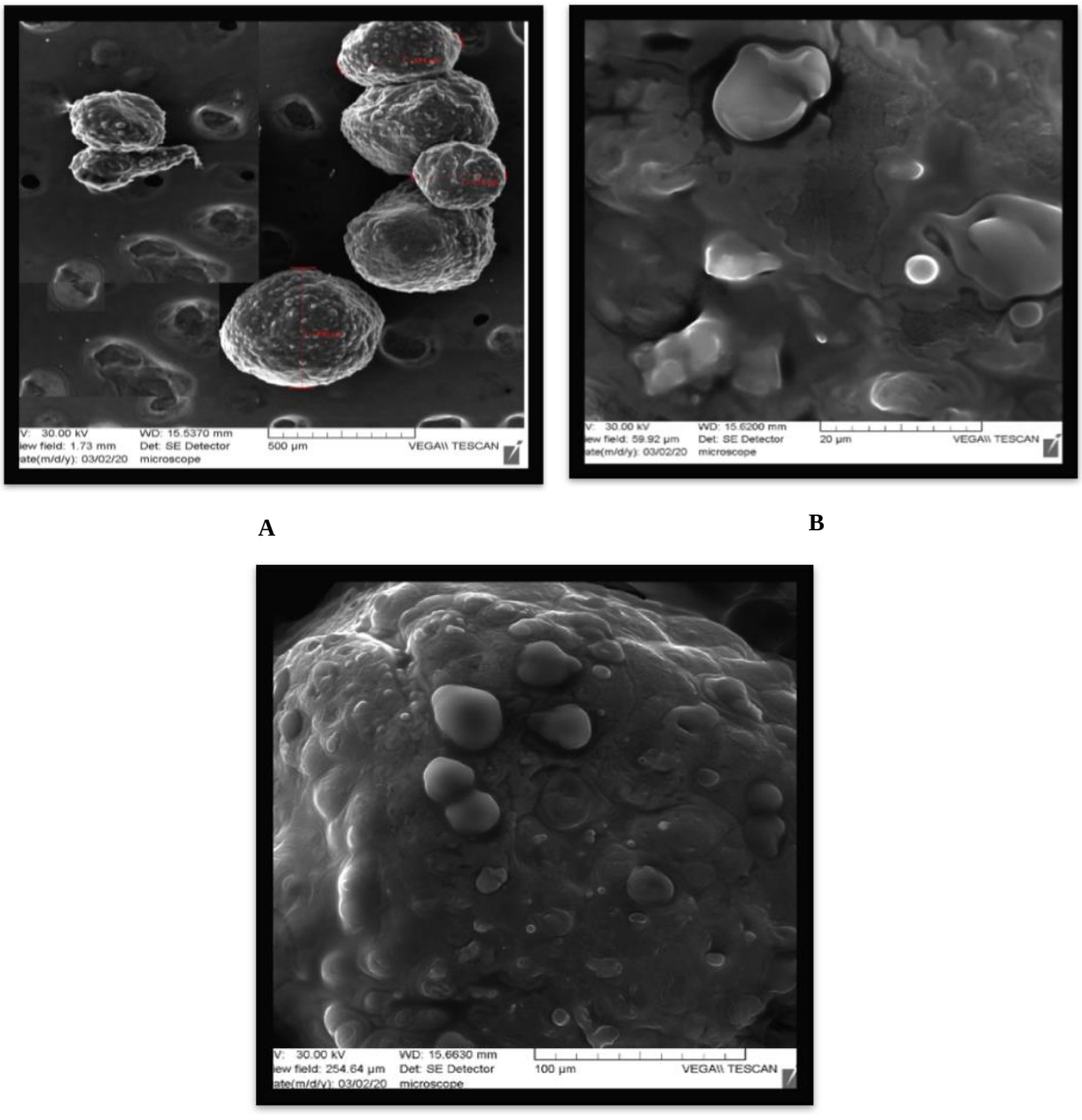


Fig. 2. Particle size distribution expressed as percentage of total particles. (n =50)

Viable cell count and micro-encapsulation efficiency and yield

To determine the efficiency of the matrix with the chosen prebiotic, the way the probiotic cells were protected during encapsulation, i.e. the efficiency of encapsulation, was analyzed. Table 2 shows the results obtained for the starch microcapsules and it can be observed that they have an optimal encapsulation efficiency of 100%.The results showed an encapsulation efficiency of over 100%, this occurs due to the fact that the capsules are a suitable medium for the growth of encapsulated bacteria. This percentage, over 100% is due to the fact that the matrix of the capsules contains prebiotics and that the enumeration of the encapsulated bacteria was performed approximately 2 hours after encapsulation. In other studies it was observed that after 4-6 hours from encapsulation the growth rates for probiotic strains of *Lactobacillus plantarum and L. rhamnosus or L. casei* can be about 1 log [19, 20]. Analyzing other authors such as Raddatz et al. [8] who used as an encapsulation technique internal emulsification / gelling, they

obtained an EY% between 82.65% and 91.24%; and the authors Jantarathin et al. [21] using the extrusion technique with *L. acidophilus* and using as material for the encapsulation matrix sodium alginate and inulin had an EY% = 88.19%.Other authors obtained an even lower EY between 43% and 50 % using the internal gelling technique for the encapsulation of *Bifidobacterium bifidumF-35*[22]. It is very important to maintain cell viability following the encapsulation process regardless of the type of encapsulation.



A **B**

C

Fig.1. Scanning electron microscope photographs of fresh beads. A) bead with STH; B, C) surface of a bead with STH

Table 2.
Number of cells entrapped compared to initial number of cells and encapsulation yield

Probiotic population before encapsulation (cfu/ml)	Encapsulated probiotic population (cfu/g)	EY %= $\log_{10}N_1/\log_{10}N_0$
2.40E+13±4.6	3.31E+13±2.7	101,04

Viability of encapsulated bacteria during storage

The cell viability of starch microcapsules stored in the refrigerator for 30 days at 4° C is shown in Figure 3. The viability of the cells between the values obtained at the same time was statistically analyzed and it was found that there are significant differences ($p < 0.05$) and these differences persisted over the 30 days.

If in the first 7 days after encapsulation a reduction of 2.0 log cfu / g was observed, in the next 7 days the decrease of the cellular viability of the microcapsules was

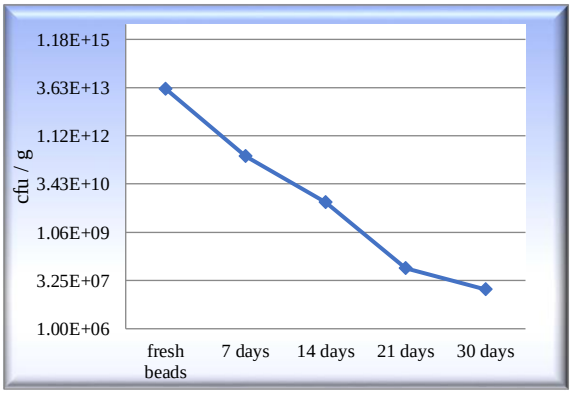


Fig.3. Stability of microparticles of *Lacto-bacillus* during 30 days at 4ºC

smaller, by 1.45 log cfu / g, so the cellular viability decreased by approximately 3.53 log cfu / g from time 0.Therefore, the viability decreased in the first 7 days by 0.77% and between 7 and 14 days of storage the percentage was much higher, 3.68%.After 21 days of storage, a decrease in cell viability of 2.04 log cfu / g in microcapsules was observed compared to day 14 and a decrease of 5.57 log cfu / g compared to the time of encapsulation. This decrease will not be the same after 30 days when the reduction was only 0.64 log cfu / g compared to the previous period.After 30 days, the survival rate of *Lactobacillus* cells in the microcapsules indicates a decrease of 6.21 log cfu / g. Data obtained attest to the fact that the presence of a carbon source in the microcapsule matrix offers the possibility of the existence of a metabolic activity of probiotic cells at 4 °C.The decrease in cell viability is not due to the encapsulation process, but we believe that some of the main causes would be the time, the consumption of the nutrient substrate, the presence of compounds resulting from the metabolic process, such as metabolic acids and bacteriocins.On the other hand, at each sampling the Petri dishes were opened, which could have led to the increase of humidity and implicitly to the increase of water activity and here the presence of residual water from microcapsules must be taken into account [14].Humidity is also known to have a negative effect on cell viability [23]. Analyzing other studies that used the same encapsulation method but different strains (*L. gasseri and Bifidobacterium bifidum*),it was found that a decrease in viability was reported in the first 11 days of 4.11 cfu log / ml; and after 14 days they observed no survival [11].In order to survive and reach the intestine at the site of action in sufficient quantities to facilitate colonization, a large number of the initial cells must be trapped in the microcapsules. Tabel 3 shows the cell viability following the microcapsules of the simulated intestinal conditions. It was found that after 4 hours cell viability falls within the limits recommended by the

FAO. However, in order to survive the passage through all the dull conditions of the digestive tract and to benefit from a beneficial action, the cellular concentration can be increased during encapsulation.This would lead to an increased number of bacterial survivors at the end of incubation in simulated gastric conditions.

Table 3.

The effect of exposure to simulated intestinal juice on the survival of microcapsulated cells

Number of cells entrapped (log cfu/g)		time (min)			
		simulated intestinal juice			
0 days	7 days	60	120	180	240
13.52 ± 1.27	11.40 ± 0.426	7.93 ± 0.561	7.74 ± 0.343	7.72 ± 0.621	6.27 ± 0.987

4. Conclusions

The present study showed that the microencapsulation of Lactobacillus strains with a combination of ALG (1.5%) and starch (1%) ensures a good protectionof probiotic bacteria during the 30 days of storage as well as in simulated intestinal conditions and at the same time it improves their viability. The results of this study also indicate that starch microcapsules can be used as a micrometric delivery vehicle due to an improved encapsulation method. Investigating the stability of these microcapsules in food models during storage is an area that requires further research.

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