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Effect of different types of encapsulation on the survival of *Lactobacillus plantarum* during storage with inulin and *in vitro* digestion

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ABSTRACT

The aim of this work was to investigate the effect of different microencapsulation methods (extrusion and internal emulsion microencapsulation) and inulin on the viability of *Lactobacillus plantarum* during storage at 4 °C. The inulin was added during microencapsulation at 0, 1 and 2%. The effect of the different phases of the gastrointestinal digestion on the survival of the microorganism during storage (0, 15 and 30 days) was also investigated. In both types of microcapsules, the best protection with higher survival of *Lactobacillus plantarum* during the 30 days of storage, was observed in the presence of 2% inulin with only 0.71 and 0.47 logs reduction for extrusion and emulsion microencapsulation respectively. From 15 days of storage the internal emulsion microcapsules did not maintain their structure during the *in vitro* digestion processes. At the end of the simulated gastrointestinal conditions (30 days), the number of cells were 7.40 and 6.53 log CFU g^{-1} for extrusion and emulsion microencapsulation respectively. In both microencapsulation methods, *Lactobacillus plantarum* showed a high survival ($\geq 10^6$ CFU g^{-1}). However for long storage periods, the best method for increasing the survival of *Lactobacillus plantarum* to the gastrointestinal digestion was the extrusion microencapsulation.

1. Introduction

Probiotics are described by The Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) as "Live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient number confer one or more specified demonstrated health benefits for the host" (FAO/WHO, 2001). To exert beneficial effects, the concentration of live microorganisms in the product should be above 10^6–10^7 CFU g^{-1} or mL $^{-1}$ at the moment of consumption (FAO/WHO, 2001) and be able of survive to the digestive processes. The lactic acid bacteria (LAB) are the probiotic microorganisms more important linked to the human gastrointestinal tract. However their viability could be influenced by changes in temperature, pH, acidity, dissolved oxygen and hydrogen peroxide (Anal & Singh, 2007).

To provide the protection and survival of the LAB during storage and digestion, different microencapsulation methods have been developed, where probiotics are retained in a matrix that must be generally recognized as safe (GRAS) and insoluble to ensure its integrity in either the food and the upper part of the gastrointestinal tract (Krasaekoopt, Bhandari, & Deeth, 2003; Nazzaro, Orlando, Fratianni, & Coppola, 2012).

The material most widely used for the extrusion and emulsion microencapsulation techniques is the alginate, that is a natural polymer extracted from seaweed, consisting of 1 → 4 linked β-D-mannuronic and α-L-guluronic acids (Rinaudo, 2008). The extrusion microencapsulation technique is performed using a syringe with a needle which contains a hydrocolloid solution (alginate) with the microorganism, and by extrusion the cell suspension is dropped over a hardening $CaCl_2$ solution (Krasaekoopt et al., 2003). The emulsion microencapsulation originates microcapsules formed by the water in oil emulsion. The solution contains alginate, vegetable oil, calcium carbonate and a surfactant, and an organic acid that reacts with the calcium carbonate to form the microcapsules (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012; Krasaekoopt et al., 2003)

Prebiotics are described by Gibson, Probert, Loo, Rastall, and Roberfroid (2004) as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well

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being and health". Inulin that is classified as soluble fiber, is one of the most known prebiotics. Is a reserve carbohydrate that is found in many fruits and vegetables as onion, garlic and banana among the most common ones (Flamm, Glinsmann, Kritchevsky, Prosky, & Roberfroid, 2001). This prebiotic reach the large intestine without modification and is available to be metabolized by microorganisms as *lactobacillus* and *bifidobacteria.* The combination of probiotics and prebiotics is known as symbiotic and is used in food products to take advantage of their synergic effects (Al-Sheraji et al., 2013).

The comparative studies of *in vitro* digestion among the different encapsulation methods during storage are scarce. The main aim of this work has been the study of the effect of two microencapsulation methods with different inulin concentrations on the survival of *Lactobacillus plantarum* during storage, and to test the influence of the *in vitro* digestion during that period on the release and survival of *L. plantarum*.

2. Material and methods

2.1. Bacterial strain and culture conditions

L. plantarum CECT 220 (ATCC 8014) was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain).The bacterial strain was prepared by growing the lyophilized culture in de Man Rogosa Sharpe (MRS) broth (Oxoid; Madrid, Spain) for 24 h at 37 °C under aerobic conditions to obtain an initial cell density about 10^8 Colony Forming Units per mL (CFU mL^{-1}). The cells were centrifuged at 2000 × g for 10 min at 4 °C, the pellet was washed sterile phosphate buffer saline (PBS) and was re-suspended in appropriate volume of PBS, resulting in a cell concentrate of about 9 log and were kept refrigerated 4 °C.

2.2. Microencapsulation of L. plantarum

2.2.1. Extrusion microencapsulation (EM)

The microencapsulation was made according to the method described by Nazzaro, Fratianni, Coppola, Sada, and Orlando (2009). Solutions were made with 2% Na-alginate, 5.5% MRS broth, 5% glycerol, 0.15% xanthan gum and with different amounts of the short chain artichoke inulin with a degree of polymerization 10 (Farma-química; Málaga, Spain), was added 0% (EM), 1% (EMI1%) and 2% (EMI2%), and were sterilized at 121 °C for 15 min. The solutions were cooled at 25 °C and *L. plantarum* was added at a concentration of 1:10 (microbial culture: alginate solution). Suspension was dropped through a 21G needle into sterile 0.05 M $CaCl_2$ (Scharlau; Barcelona, Spain) and was allowed to harden for 30 min. The microcapsules were washed with sterile NaCl solution at 0.9%, immersed in aseptic ultrapure water and stored at 4 °C.

2.2.2. Internal emulsion microencapsulation (IM)

The internal emulsion microencapsulation was made according to the method of Rodriquez-LLimos, Chiappetta, Szelig, Fernández, and Bregni (2003) with modifications: The Na-alginate solution was prepared following the same procedure as for extrusion microencapsulation. Inulin was also added at different proportions: 0% (IM), 1% (IMI1%) and 2% (IMI2%).

L. plantarum was added at a concentration of 1:10 (microbial culture:Na-alginate solution) under continuous magnetic stirring at 500 rpm together with 0.4% of $CaCO_3$ (Panreac; Barcelona, Spain). The emulsion was heated at 45 °C and 200 mL of olive oil and 4 mL Tween 80 (Panreac; Barcelona, Spain) were added at 45 °C under magnetic stirring at 500 rpm for 15 min to obtain the water/oil emulsion. For the internal ionic gelation, 1.7 mL of glacial acetic acid (Panreac; Barcelona, Spain) was added and stirred for 15 min at 500 rpm. The microcapsules formed were washed with a sterile NaCl solution at 0.9% and stored in aseptic ultrapure water at 4 °C.

2.3. Size and structure of microcapsules

The diameters of 100 microcapsules for each treatment were measured using an electric digital micrometer (Insize IP65, Spain). The results were expressed in mm.

The shape and surface of the microcapsules before and after digestion, was observed with a stereomicroscope Leica MZ95 (Leica, Spain)

2.4. Bacterial enumeration and survival during storage

The survival of *L. plantarum* microencapsulated with different amounts of inulin was evaluated during 30 days of storage at 4 °C. Samples were taken at 0, 1, 8, 15 and 30 days. The bacterial count was made with 1 g of each microencapsulated were blended in a stomacher with 9 mL of sterile peptone water for 10 min to dissolve. The samples were serially diluted into 0.1% peptone water and 0.1 mL was spread plated on MRS agar under aerobic conditions at 37 °C for 48 h. The results were expressed as log CFU g^{-1} of microcapsules.

2.5. Survival of microencapsulated L. plantarum to simulated gastrointestinal conditions

For the study of the storage times (0, 15 and 30 days) of the microencapsulated samples on the survival during the gastrointestinal digestion lasting 60 min. The EM and IM microcapsules were made using higher *L. plantarum* concentrations of 0.5:1 (culture solution:Na-alginate solution). The simulated gastrointestinal digestion of the microencapsulated *L. plantarum* was made according to previously described methods (Anal & Singh, 2007; Nazzaro et al., 2009)

The simulated gastric juices (SGJ) were prepared with MRS broth (Oxoid; Madrid, Spain) and 3 g/L of pepsin (Farma-química; Málaga, Spain). The pH was adjusted to 3 with HCl 0.1N (Panreac; Barcelona, Spain). The simulated intestinal juice (SIJ), was prepared with 1 g/L of pancreatin (Sigma; Madrid, Spain) and 4.5 g/L of bile salts (Sigma; Madrid, Spain). The pH of MRS broth was adjusted to pH 7 with NaOH 0.1 N (Panreac; Barcelona, Spain). Both solutions were sterile-filtered through a membrane (0.45 μm, Millipore; Spain).

The microcapsules (1 g) were homogenized for 2 min in a vortex (Selecta; Barcelona, Spain) with 9 mL of SJG at 37 °C and were incubated during 60 min at 37 °C. The enzymatic reaction was stopped by neutralization with 1 N NaOH, to pH 7. The SIJ (9 mL) and MRS broth were then added to the suspension up to a volume of 20 and was incubated for 60 min at 37 °C. The viable count in both SGJ and SIJ was determined by the plate count method in MRS agar and expressed as log CFU g^{-1}.

2.6. Statistical analysis

All experiments and analysis were made in triplicate. The results were expressed as mean ± standard deviation. The analysis of variance followed by a Duncan test ($p < 0.05$) was used for the mean comparison, using SPSS v21.0 software package (SPSS Inc., Chicago-Illinois-USA).

3. Results and discussion

3.1. Size and structure of probiotic beads

Fig. 1 shows the average sizes obtained for each of the types of the beads. There was a big variation in beads size depending on