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Characterization of freezing effect upon stability of, probiotic loaded, calcium-alginate microparticles

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ABSTRACT

Microencapsulation, utilizing different techniques and polymers, has been studied with the objective of maintaining probiotic viability in food matrices, protecting the cells from their detrimental environment, storage conditions and the passage of gastrointestinal tract (GIT). The main objective of this study was to assess the effect of freezing at −20 °C upon probiotic alginate-calcium microparticles' integrity and functionality through parameters such as size, morphology and structure of microparticles as well as to assess cell resistance to simulated gastrointestinal tract conditions upon storage. In order to study the effect of freezing upon the stability of the microparticles, calcium-alginate microparticles, with or without probiotic cells (*Lactobacillus casei*-01, *Lactobacillus paracasei* L26, *Lactobacillus acidophilus* KI and *Bifidobacterium animalis* BB-12), were characterized at production time and after 60 days storage at −20 °C. An increase in particle size, loss of the spherical shape and porous net damages were observed after 60 days of storage at −20 °C. In accordance, encapsulation in alginate was not able to exert protection to the encapsulated probiotic cells stored at −20 °C for 60 days, especially from acid and particularly bile salts. *B. animalis* BB-12 revealed to be the most resistant probiotic strain, to both the microencapsulation process and to GIT simulated conditions.

Keywords: Calcium-alginate; Encapsulation; Microparticles; Probiotic; Freezing; Gastrointestinal resistance

1. Introduction

The market of functional food products has been growing for the past years, and many products have been made available to the consumers. Functional foods promote beneficial effects on one or more functions of the human organism, besides a basic nutritional impact, and food products containing probiotics are among the most popular (Sousa et al., 2012; Rodrigues et al., 2012a). According to the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (FAO/WHO, 2001) probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Consequently, it has been recommended that, in order to produce the desired health benefits, probiotic bacteria must be present at minimum level of 10^6 colony forming units per gram (CFU/g) of food product or 10^7 CFU/g at point of delivery or the total daily intake must be of 10^8 CFU (Chávarri et al., 2010). These requirements have a significant impact on the technological challenges of incorporating probiotics in different food products (Sousa et al., 2012). As the food matrices can represent an adverse environment for the maintenance of probiotic viability, most of the available functional food products containing probiotics are dairy or dried products.

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Besides the challenge presented by the food matrices themselves, there is also the concern regarding the ability of the probiotics to resist the passage through the gastrointestinal tract (GIT), in order to be effective in the host. Encapsulation of probiotics, using different techniques (extrusion, emulsion, spray-drying) and different polymers (alginate, carrageenan, cellulose, chitosan, inulin, sweet whey, whey protein concentrate), has been utilized as a means to protect probiotic bacteria against the previously mentioned detrimental conditions (Cook et al., 2012; Rodrigues et al., 2011a, 2012b; Doherty et al., 2011; Chávarri et al., 2010; Nazzaro et al., 2009; Pimentel-González et al., 2009). Regarding the different encapsulation techniques and polymers, an important concern is the particle size, as it can have direct implications upon the protective effect of encapsulation (Anal and Singh, 2007; Hansen et al., 2002), as well as on the sensorial properties of the food product to which they are added (Nag et al., 2011). Dong et al. (2013) reviewed recently the encapsulation methods of probiotics involving alginate and protein based materials as well as their application in food industry. In turn, Rathore et al. (2013) discussed the conventional methods to microencapsulate microbial cells presenting their most recent advances.

Besides the previously mentioned concerns regarding probiotic viability, storage temperature also plays an important role on the stability of the probiotic cells, and it has been previously reported (Sousa et al., 2012; Rodrigues et al., 2011b) that encapsulation could have a positive protective effect, preventing the loss of viability, especially at low freezing temperatures. However, the freezing process can have detrimental effects upon particles and therefore affect microparticles size, morphology and integrity (Mortazavian et al., 2007). These effects can compromise the protective function of the microparticles upon probiotic viability, during the passage through the GIT. In this study calcium-alginate, probiotic loaded, microparticles were characterized regarding several parameters including size, morphology, structure and gastrointestinal resistance. These parameters were analysed at time of production, and after 60 days of storage, at −20 °C. The main objective of this study was to assess the effect of freezing at −20 °C upon microparticles integrity and functionality in order to understand why the protective effect of encapsulation in calcium alginate at this temperature over this storage period was variable and strain dependent (Sousa et al., 2012). As far as we are aware of, the assessment of the effect of freezing temperature of −20 °C on calcium-alginate probiotic microparticles' integrity and functionality has not yet been reported.

2. Experimental

2.1. Materials

Sodium alginate was provided by FMC Biopolymer (Ireland). Probiotic strains were obtained from different companies. *Lactobacillus paracasei* LAFTI® L26 from DSM Food Specialties (The Netherlands), *Lactobacillus acidophilus* Ki from CSK (The Netherlands) and *Bifidobacterium animalis* BB-12® and *Lactobacillus casei* -01 from Christian Hansen (Denmark).

2.2. Microencapsulation procedure

Probiotic cultures were prepared in de Man–Rogosa–Sharpe (MRS) medium (supplemented with filter-sterilized 0.05% (w/v) of L-cysteine·HCl, in the case of *B. animalis* BB-12® and *L. acidophilus* Ki) and were centrifuged at 4000 rpm for 20 min, at 4 °C. The supernatant was discarded and the pellet was resuspended in a 0.85% (w/v) NaCl solution.

Microencapsulation procedure was based on Sousa et al. (2012) with some modifications. Each probiotic suspension was added at 10% (v/v) to a 2% (w/v) sodium alginate solution, and the resulting alginate-culture mixtures (50 mL) were extruded using a Nisco Var J30 (Nisco Engineering AG) microencapsulation unit with a 0.5 mm orifice and a nitrogen pressure of 1.4 bar. The extrusion rate was 4.0 mL min^{-1} and the flow rate was controlled using a syringe pump (Genie Plus). The mixtures were extruded into 200 mL of $CaCl_2$ solution 4% (w/v), stirred at 200 rpm. The resulting microparticles were left in contact with the $CaCl_2$ solution for 30 min at room temperature to ensure complete solidification. Afterwards the $CaCl_2$ solution was removed through decantation, and the microparticles were suspended in Ringer solution. The microparticles were recovered (separated from the Ringer solution) by gravity filtration, using a glass filter funnel (porosity 1; Schott Duran®). Microparticles without probiotic bacteria (loaded with NaCl 0.85% (w/v) solution), were also prepared so that the effect of the microorganism upon the microparticle morphology could be analysed. Microparticles were weighed into 25 mL sterile tubes and suspended in Ringer solution in a 1:9 (g mL^{-1}) ratio and were stored for 60 days, at −20 °C.

The selection of encapsulation conditions were based on previous assays (data not shown) that were made in order to identify those conditions which at a high enough flow rate enabled, the production of the highest amount of microparticles with the smallest possible size taking into consideration the extruder possibilities/limitations.

2.3. Characterization

The effect of freezing upon particle size, morphology and gastrointestinal resistance of the microparticles was assessed, at the beginning and after 60 days of storage in two replicates, derived from one experiment.

2.3.1. Particle size analysis

Microparticles' size was determined with a particle size analyser Mastersizer 2000 (Malvern) using Mastersizer 2000 Version 5.60 software (Malvern), and the results were an average of 6 measurements. Particle size distribution, *d* (0.1, 0.5 and 0.9) – size in which 10, 50 and 90% of the particles are under, surface area moment, D[3,2], and volume moment mean, D[4,3], were determined.

2.3.2. Morphology

Microparticles' shape was observed using an optical microscope (Nikon Eclipse E400; Nikon) coupled with a camera (Nikon Coolpix 950), and a cryo scanning electron microscope with microanalysis by X-rays (FE-CryoSEM/EDS – JEOL JSM 6301F; JEOL Ltd./Oxford INCA Energy 350; Oxford Instruments/Gatan Alto 2500; Gatan Inc.). For cryo-SEM analysis, samples were plunge-frozen in liquid nitrogen and transferred to the cryo-chamber, where they were fractured and subsequently sputter-coated with gold/palladium (Au/Pd). Observation was performed with the cryo-SEM operated at 15 kV. An X-rays microanalysis of the microparticles was also performed via energy-dispersive X-ray spectroscopy (EDS).

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