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Soybean protein-based microparticles for oral delivery of probiotics with improved stability during storage and gut resistance

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ABSTRACT

The present work describes the encapsulation of probiotics using a by-product as wall material and a process feasible to be scaled-up: coacervation of soybean protein concentrate (SPC) by using calcium salts and spray-drying. SPC was extracted from soybean flour, produced during the processing of soybean milk, by alkaline extraction following isoelectric precipitation. Two probiotic strains were selected for encapsulation (*Lactobacillus plantarum* CECT 220 and *Lactobacillus casei* CECT 475) in order to evaluate the ability of SPC to encapsulate and protect bacteria from stress conditions. The viability of these encapsulated strains under *in vitro* gastrointestinal conditions and shelf-life during storage were compared with the most common forms commercialized nowadays. Results show that SPC is a feasible material for the development of probiotic microparticles with adequate physicochemical properties and enhanced significantly both probiotic viability and tolerance against simulated gastrointestinal fluids when compared to current available commercial forms.

1. Introduction

The intestinal microbiota of a healthy adult is relatively stable and contains various beneficial bacterial populations comprising primarily *Lactobacillus* and *Bifidobacterium* species that play an important role in host health. Other microbes such as bacteriophages, fungi, protozoa, archaea and viruses are also constituents of the gut microbiota. Recent studies have revealed that bacteriophages are as prevalent as bacteria in the gut (Miyoshi & Chang, 2017). The intestinal homeostasis can be altered by different factors: diet, environment conditions (i.e., stress) and antibiotics overuse (Sen et al., 2017). However, over 70% of the fecal microbiota is remarkably stable (Faith et al., 2013). An imbalance in the colonic microbiota may contribute to the development of different disorders including gastrointestinal tract infections, irritable syndromes, allergies, heart diseases and colon cancer (Hod & Ringel, 2016; Zoumpopoulou, Pot, Tsakalidou, & Papadimitriou, 2017). Nowadays, it is still unclear if intestinal bowel diseases-associated dysbiosis is causative, contributory, or consequential to the disease. This uncertainty is related to the limitations in technology, bioinformatics and clinical study design (Miyoshi & Chang, 2017). Despite these unresolved questions, probiotics are able to restore the intestinal microbial ecosystem balance and contribute to the health promotion of the host (Bron, van Baarlen, & Kleerebezem, 2012). The joint Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) Committee has recommended the use of probiotics to prevent these risks (FAO/WHO, 2001). The term probiotic defines the living microorganisms, which when consumed in adequate amount, may offer a healthy effect on the host (Guarner & Schaafsma, 1998). Among others, the most common probiotics that are used nowadays include bacteria such as lactic-acid bacteria (LAB) and *Escherichia coli* strains, as well as yeast species such as *Saccharomyces boulardii* (Verna & Lucak, 2010). In any case, a daily dose of at least 10^6–10^9 living cells has been suggested to assure health-relevant effects following the consumption of probiotic products (Lee & Salminen, 1995). The mechanisms by which probiotic bacteria confer such effects have been divided into three areas: (i) production of nutrients and co-factors, (ii) competition with pathogens and (iii) stimulating the host immune response (Saier & Mansour, 2005).

In general, four different ways for consuming probiotics may be distinguished: (i) as a concentrated culture added to a beverage (e.g., fruit juice, etc.), (ii) inoculated in prebiotic fibers, (iii) as a

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freeze-dried dietary supplement formulated in solid dosage forms (e.g., powder, capsules, tablets, etc.) and (iv) inoculated in milk-based foods.

However, many of these probiotics (e.g. *Lactobacillus* spp.) are faced to important challenges that seriously hamper their beneficial and healthy effects. Thus, the harsh pH conditions in the stomach as well as the presence of bile salts in the upper regions of the small intestine are the main barriers limiting the arrival of these microorganisms to the ileum and colon, where they have to compete with the endogenous microbiota for adhering to the mucosa (Vandenplas, Huys, & Daube, 2015). Another important factor limiting the efficacy of probiotics is their vulnerability. In fact, their viability may be compromised during processing, storage and consumption (Farnworth & Champagne, 2010).

To overcome these problems, microencapsulation is presented as one of the most efficient solutions not only to maintain the viability of probiotics during processing and storage, but also to ensure their activity within the gut (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012). Different materials have been recommended for the microencapsulation of probiotics, including alginate (Krasaekoopt, Bhandari, & Deeth, 2004), guar gum, locust beam gum (Ding & Shah, 2009) or waxy maize starch. Apart the use of carbohydrates, proteins have also been suggested for the design of biodegradable microparticles (O'Riordan, Andrews, Buckle, & Conway, 2001). In this context, microparticles based on soybean protein isolates (SPI) have been proposed for the oral delivery of *Bifidobacterium longum* (Dianawati, Mishra, & Shah, 2013). More recently, a combination between SPI and high methoxy pectin (HMP) as encapsulating material for probiotic bacteria has been described.

In spite of the benefits obtained with the microencapsulation of these microorganisms in biodegradable microparticles, there is still an important need of delivery systems offering both a superior protective capability against the physiological conditions of the gut and controlled release properties. In addition, these ideal microparticles should be prepared following simple preparative processes to be easily implemented at an industrial scale.

In this work, the production of encapsulated probiotics in soybean protein-based microparticles by a coacervation process in the presence of calcium salts followed by a drying step by spray-drying was studied. Soybean protein concentrate (SPC) was obtained from the soybean flour, a by-product from the processing of soybean milk. The work also describes the viability of the encapsulated bacteria during storage under controlled conditions (25 °C/60% RH) and the *in vitro* gastrointestinal resistance with respect to the most common forms commercialized nowadays.

2. Materials and methods

Soybean flours, obtained as by-products from the soybean milk processing, were kindly provided by Iparlat S.A. (Urnieta, Guipúzcoa, Spain). Solvents and reagents used for the extraction of soybean protein were of analytical grade and purchased from Panreac (Barcelona, Spain).

Probiotic bacteria strains *L. plantarum* CECT 220 and *L. casei* CECT 475, isolated from corn silage and cheese, respectively, were purchased from the *Colección Española de Cultivos Tipo* (CECT) – University of Valencia (Valencia, Spain). MRS broth and Buffer Peptone Water (BPW) broth were acquired from Merck KGaA (Darmstadt, Germany).

Maltodextrin (MD) Glucidex® 21 and the commercial blend of oligofructose-enriched inulin (OEI) Orafty® Synergy1 were kindly provided by Roquette Frères (Lestrem, France) and Beneo GmbH (Mannheim, Germany), respectively. Chromatography solvents were purchased from Sigma (Barcelona, Spain).

The enzymes and reagents used for *in vitro* gastrointestinal resistance assays were provided by Sigma (Barcelona, Spain) except KH_2PO_4 which was acquired from Panreac (Barcelona, Spain). Chips and analysis kit for microfluidic assays were purchased from Bio-Rad Laboratories Inc. Spain (Barcelona, Spain).

2.1. Characterization of composition of soybean flour

The soybean flour was characterised in relation to its nutritive value in order to determine the homogeneity among batches from the soybean milk production, mainly regarding total protein content. The following parameters were determined: humidity, protein content, fat content, total dietary fiber, ash, carbohydrate, energy value, sugar profile, fatty acid profile, and sodium content.

Briefly, the humidity was determined by gravimetric analysis to a constant weight at 105 ± 3 °C. Protein content was estimated from the total nitrogen content determined by Kjedhal method using a conversion factor (N × 6.25). Total lipid content was determined after extraction with a mixture of chloroform and methanol as described previously by Bligh and Dyer (1959), whereas the fatty acid profile was determined by Gas Chromatography-Flame Ionization Detector (GC-FID) according to the European Commission (EC, 2013). The total dietary fiber was analyzed following the method described by the A.O.A.C. (1992). Ash content was determined by gravimetric analysis after the incineration of samples at 550 ± 50 °C for at least 8 h. Sugar profile was analyzed by High Performance Liquid Chromatography-Refractive Index (HPLC-RI) following the method described by White and Kennedy (1983) and the carbohydrate content was calculated by difference. Finally, the energy value was calculated in accordance with the technique suggested by the European Commission (EC, 2011).

All the experiments were conducted in duplicate (except for sodium analysis) and three different samples were analysed, corresponding to different batches produced approximately over a year (April and November 2011, and March 2012). These samples are identified in Supplementary Table 1 as A, B and C, respectively.

2.2. Extraction of soybean protein from soybean flour

The extraction of proteins from soybean was carried out following a protocol described by Ma, Liu, Kwok, and Kwok (1996) with minor modifications. For this purpose, 100 g soybean flour was first defatted with hexane (mixture of isomers) using a soybean flour/hexane ratio (by weight) of 1/10. Once the solvent was removed by centrifugation (10 min, 15,317*g*) followed by evaporation at room temperature, the soybean flour was moistened with water and sterilized in a RF42J1 P rotary autoclave at 121 °C for 15 min (Ferlo, San Adrián, Spain). Afterwards, the protein was extracted using a carbonate buffer solution (by weight ratio soybean flour/buffer of 1/10) at 80 °C for 30 min and the mixture was centrifuged at 15,317*g* for 15 min. The protein was recovered from supernatants by isoelectric precipitation (pH 4.5) with a 10% HCl solution (v/v). Finally, the solution of proteins was filtered, dried by lyophilization and homogenized using a milling cutter. The process was optimized by evaluating the influence of pH and temperature conditions.

2.3. Characterization of the composition of soybean protein

The following parameters were determined: protein content, the molecular weight distribution and the amino acid profile.

Protein content was estimated from the total nitrogen content determined by Kjedhal method using a conversion factor (N × 6.25). Additionally, the amino acid profile was carried out by an external laboratory. All amino acids but tryptophan were