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Unpurified *Gelidium*-extracted carbohydrate-rich fractions improve probiotic protection during storage

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ABSTRACT

The objective of this work was to investigate whether the use of unpurified agar-based fractions extracted from the seaweed *Gelidium* as microencapsulation matrices has an impact on probiotic protection during storage. Therefore, unpurified and pure agar and agarose-based microcapsules were produced through emulsification/internal gelation for the protection of *Bifidobacterium pseudocatenulatum* CECT 7765. Initially, agarose-based formulations with other biopolymers were evaluated, given the excellent oxygen barrier properties of this polysaccharide. The optimal combination in terms of probiotic protection was selected for further experiments and this agarose-based formulation was compared with microcapsules produced using both pure and unpurified agar-based fractions. The presence of other compounds (mainly proteins and polyphenols) in the unpurified agar fractions significantly improved the viability of these sensitive probiotic bacteria both at ambient and refrigerated storage conditions. Furthermore, the presence of impurities allowed the increase of solids content in the formulation giving raise to stronger gel particles, which could contribute to limited oxygen diffusion, thus, partly explaining the improved protection. Therefore, this work demonstrates the potential of more cost-effective less purified carbohydrate-based fractions for probiotic protection.

1. Introduction

Food industry normally uses purified ingredients to formulate food products. However, this is impractical from a sustainability viewpoint as, apart from the increased processing costs associated to purification procedures, functional compounds originally present in the extracted matrices are lost during the extraction processes. This is, for instance, the case of phycocolloids. Seaweed industries use multistep procedures to obtain purified phycocolloids (like agar, agarose, carrageenan or alginates), which are mainly used as texturing agents and stabilizers in food products (Bixler & Porse, 2011; Tavares Estevam et al., 2018). The remaining algae residues contain a number of highly interesting compounds, like polyphenols, which could provide these food ingredients with functional properties (Michalak & Chojnacka, 2015). In this context, limiting the number of purification steps would be extremely beneficial since, apart from imparting added value to the obtained ingredients, processing costs and chemicals would also be reduced, thus constituting an excellent alternative in line with current circular economy policies.

Agar and agarose are structural polysaccharides present in different species of red seaweed (*Rhodophyceae*) which have a tremendous potential as encapsulation matrices for probiotic bacteria. Specifically, agarose has demonstrated that, even at low concentrations, is able to prevent the entry of oxygen into liquid media (Yokoyama, Kishida, Uchimura, & Ichinole, 2006). This fact is of tremendous relevance for oxygen-sensitive bifidobacteria. However, to the best of our knowledge, these materials have not been explored for probiotic protection, although agarose has demonstrated to be an excellent matrix to encapsulate bacterial cells for studying a variety of phenomena like antibiotic susceptibility (Akselband, Cabral, Shapiro, & McGrath, 2005), bacterial uptake of small molecules by electroporation (Gift & Weaver, 2000), enrichment of slow-growing bacteria (Manome et al., 2001) or

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the growth of "uncultured" bacteria for phylogenetic analyses (Zengler et al., 2002).

Microencapsulation techniques have been broadly developed for the preservation of biologically active ingredients in food systems including probiotic bacteria (Arslan-Tontul & Erbas, 2017; Liu et al., 2017). Amongst the existing microencapsulation processes for probiotics, the emulsification method has been widely used, as it is a cheap method with high cellular retention, mild formulation conditions and it does not require sophisticated and/or expensive equipment (Holkem et al., 2017; Takei, Yoshida, Hatate, Shiomori, & Kiyoyama, 2009). Microcapsule formation through this emulsification technique involves creating a water-in-oil (W/O) emulsion by homogenizing an aqueous biopolymeric solution containing the probiotic bacteria with an oil continuous phase (Sung, Xiao, Decker, & McClements, 2015). This process leads to the formation of biopolymer-rich water droplets dispersed in the oil phase, which are subsequently gelled by changing the system conditions in an appropriate manner (McClements, 2017). In the case of agar and agarose-based emulsions, gel microparticles can be easily obtained by cooling below a critical temperature, as they are cold-setting biopolymers. Moreover, agar and agarose gels exhibit thermal hysteresis (Fujii, Yano, Kumagai, & Miyawaki, 2000), implying that they would remain crosslinked at room or refrigerated temperatures.

Although a number of research works have demonstrated the potential of encapsulation to protect lactic acid bacteria (Chandramouli, Kailasapathy, Peiris, & Jones, 2004; Doherty et al., 2012; Gbassi, Vandamme, Ennahar, & Marchioni, 2009; Moumita et al., 2017) and/or commercial and relatively stable bifidobacteria (Holkem et al., 2017; López-Rubio, Sanchez, Sanz, & Lagaron, 2009; López-Rubio, Sanchez, Wilkanowicz, Sanz, & Lagaron, 2012), interesting *Bifidobacterium* species with proved beneficial health-related activities should be the target, although their inherent susceptibility to ambient conditions make their handling and subsequent protection a challenging task. This is, for instance, the case of *Bifidobacterium pseudocatenulatum* CECT 7765 isolated from stools of a breast-fed infant (Benítez-Páez, Moreno, Sanz, & Sanz, 2016), which has been reported to ameliorate metabolic and immunological alterations related to obesity in high-fat diet fed mice, reducing obesity-associated systemic inflammation and partially restoring microbiota alterations caused by these high fat diets (Gauffin, Santacruz, Trejo, & Sanz, 2013; Moya-Pérez, Neef, & Sanz, 2015). In order to make these sensitive health promoting strains commercially available, novel encapsulation strategies which create a suitable environment with low access to oxygen for the bacteria to remain viable need to be sought. A potentially interesting approach could be the incorporation of antioxidant molecules within the encapsulation structures. In fact, antioxidant molecules like polyphenols, widely present in seaweed biomass, have been seen to limit oxygen toxicity on specific oxygen-sensitive probiotic strains (Gaudreau, Champagne, Remondetto, Bazinet, & Subirade, 2013; Gaudreau et al., 2016) and exert a modulating effect on microbiota population improving gastrointestinal health (Kemperman et al., 2013). Moreover, it has been demonstrated that the survival of probiotic cultures in dairy products can be increased by ingredients that are rich in phenolic compounds (Ma, Gong, Liu, Ma, & Chen, 2015).

Therefore, the objective of the present work was to microencapsulate *Bifidobacterium pseudocatenulatum* CECT 7765 through an emulsion/gelation technique using pure commercial agarose and agar-based matrices (given their suitability in the handling of oxygen-sensitive bacteria), and to compare their performance in terms of probiotic protection during storage with counterpart samples prepared with unpurified agar-based fractions rich in polyphenolic compounds.

2. Materials and methods

2.1. Materials

Commercial agarose (type D2-LE) was kindly donated by Hispanagar (Spain). Whey protein concentrate (WPC), under the commercial name of Lacprodan® DI-8090 and with a w/w composition of ~80% protein, ~9% lactose and ~8% lipids, was provided by ARLA (ARLA Food Ingredients, Viby, Denmark), and was used without further purification. Type A gelatin from porcine skin (Gel), with reported gel strength of 175 g Bloom and buffer solution of pH 7.4 (phosphate buffered saline system, PBS) were obtained from Sigma-Aldrich. A commercial maize starch with 28% amylose content (CS) was obtained from Roquette Laisa (Spain).

2.2. Production of agar-based extract from Gelidium seaweed

Agar was extracted from raw *Gelidium sesquipedale* seaweed (kindly donated by Hispanagar, Spain) by applying a hot-water treatment. Briefly, 50 g of dry seaweed powder were immersed in 500 mL of distilled water and heated up to 90 °C for 2 h. After that, the agar-based solution was separated from the solid residue by filtration with muslin cloth. The filtrate was allowed to gel and frozen overnight. Subsequently, the material was subjected to two freeze-thaw cycles and the gel-like material was then freeze-dried. The obtained freeze-dried powder constitutes the unpurified agar-based material.

Additionally, a pre-treatment step was applied prior to the described hot water treatment to remove impurities from the raw seaweed and generate pure agar following a previously established protocol with slight modifications (Kumar & Fotedar, 2009). Briefly, the pre-treatment consisted of soaking 50 g of dry seaweed powder in 500 mL of 2.5M NaOH solution and heating up to 90 °C for 2 h. After that the solid material was filtered and washed repeatedly with distilled water using a muslin cloth, until the pH of the filtrate became neutral. The obtained solid material was then subjected to the hot water treatment described above, leading to the production of pure agar.

2.3. Quantification of protein and phenolic content from agar-based materials

The total protein content in the purified agar and the unpurified agar-based fraction was measured following the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) with some minor modifications. Briefly, 1 mL of the modified Lowry reagent was mixed with 0.2 mL of the agar-based materials (dissolved in distilled water at a concentration of 5 mg/mL) and incubated along for 10 min at room temperature. Then, 0.1 mL of Folin-Ciocalteau reagent (previously diluted 1:1 with distilled water) were added and vortexed, incubating the resulting solution for 30 min at room temperature. The absorbance values were read at 750 nm using a UV–Vis spectrophotometer (Agilent 8453 Spectroscopy System). A calibration curve was prepared with serial dilutions of bovine serum albumin (BSA) and the total protein content was expressed as mg BSA/g extract. The measurements were carried out in triplicate.

The total phenolic content was estimated by the Folin-Ciocalteau colorimetric assay (Singleton, Orthofer, & Lamuela-Raventós, 1999). Briefly, Folin-Ciocalteau reagent was diluted 1:10 with distilled water and 1 mL of the final dilution was mixed with 0.2 mL of the extract sample (previously dissolved in warm water). Subsequently, 0.8 mL of Na_2CO_3 (75 mg/mL) were added and the samples were heated up to 50 °C for 30 min. After that, the absorbance values at a wavelength of 750 nm were collected. A Calibration curve was built by using gallic acid as the standard, and the total phenolic content was expressed as mg of gallic acid (GA)/g extract. The measurements were carried out in triplicate.