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.

Keywords

Internal gelation; encapsulation; probiotics; agarose; whey protein concentrate

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 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 (Takei, Yoshida, Hatate, Shiomori, & Kiyoyama, 2009; Holkem et al., 2017). 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 2h. 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 & Foteman, 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 2h. 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 minutes at room temperature. Then, 0.1 mL of Folin-Ciocalteu reagent (previously diluted 1:1 with distilled water) were added and vortexed, incubating the resulting solution for 30 minutes 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-Ciocalteu colorimetric assay (Singleton, Orthofer, & Lamuela-Raventós, 1999). Briefly, Folin-Ciocalteu 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 Na₂CO₃ (75 mg/mL) were added and the samples were heated up to 50°C for 30 minutes. 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.

2.4 Preparation of probiotic cells suspensions

Bifidobacterium pseudocatenulatum CECT 7765 was isolated from stools of a breast-fed infant and identified by 16S rRNA gene and whole-genome sequencing as described previously (Benítez-Páez et al., 2016). The bacterial strain was routinely grown in Man, Rogosa and Sharpe (MRS) broth (Scharlau, Barcelona, Spain) supplemented with cysteine (0.05% w/v) for 24 h at 37°C in microaerophilic conditions (AneroGen; Oxoid, Basingstoke, UK). The bifidobacterial cells were then collected by centrifugation at 13000 rpm for 10 min using an Eppendorf Centrifuge 5415R, obtaining a pellet that was subsequently washed twice with phosphate buffer saline (PBS) supplemented with cysteine (0.05%) and re-suspended in the biopolymeric dispersions.

2.5 Preparation of biopolymeric solutions for encapsulation

Purified agar (Agar 0.5 wt.%) and agarose (Agarose 0.5 wt.%) solutions were prepared by dissolving 0.005 g/mL of the carbohydrate polymers in PBS and heating the solutions at ~90°C under constant stirring. Non-purified agar solutions (NP-Agar 3 wt.%) were prepared like the previous ones, but greater concentrations were used in this case (0.03 g/mL) as the presence of impurities prevented the gelling of the solutions at 40°C. WPC dispersions (WPC 5 wt.%) were prepared by mixing the protein concentrate with distilled water under magnetic stirring at room temperature to achieve a concentration of 0.05 g/mL. Gelatin solutions were prepared at 0.01 (Gel 1 wt.%) and 0.05 g/mL (Gel 5 wt.%) by dispersing the protein powder in distilled water under magnetic stirring at 50°C until complete dissolution. For preparing the starch solutions

(Starch 1 wt.%), 0.01 g/mL of granular maize starch were dispersed in water and heated in a warm bath until complete gelatinisation. All the solutions were kept at 40°C in a water bath until use. The different formulations tested along this work were obtained by blending the aforementioned biopolymer solutions according to the compositions compiled in Table 1.

Table 1. Biopolymer compositions used during the emulsification/internal gelation process used for *B. pseudocatenulatum* encapsulation. UPAgar stands for unpurified agar fraction, while Agar refers to purified agar. WPC stands for whey protein concentrate and Gel for gelatin.

Sample Name	Agarose/Agar concentration (% w/v)	Blended biopolymer concentration (% w/v)	Volume Ratio Agarose/Agar: blended biopolymer	Total solids content (% w/v)
Agarose:WPC5	0.5	5	1:2	3.5
Agarose:Gel1	0.5	1	1:2	0.8
Agarose:Gel5	0.5	5	1:2	3.5
Agarose:Starch1	0.5	1	1:2	0.8
UPAgar:WPC5	3	5	1:2	4.3
UPAgar:Gel1	3	1	1:2	1.7
UPAgar:Gel5	3	5	1:2	4.3
UPAgar:Starch1	3	1	1:2	1.7
Agar:WPC5	0.5	5	1:2	3.5

2.6 Rheological properties of agarose and agar-based solutions

The gelation curves of the agarose and agar-based solutions were monitored using an AR-G2 rheometer (TA Instruments, USA) with cone-plate geometry (40 mm diameter, 2° steel cone, and gap 54 μ m) following the method of Wang et al. (2018) with slight modifications. The temperature dependency of the storage (G') and the loss (G'') modulus was examined by lowering the temperature from 60 to 10 °C at a cooling rate of 1°C/min with constant frequency $f = 1$ Hz and strain $\gamma = 0.001$ (Russ, Zielbauer, Koynov, & Vilgis, 2013). To avoid evaporation of water in the solution, the exposed surface of the sample was covered with a layer of paraffin oil. Rheological measurements were done in triplicate on all samples.

2.7 Preparation of probiotic-containing capsules through emulsion/internal gelation

Figure 1 shows a scheme of the process for obtaining the microencapsulation structures. This encapsulation method was adapted from a previous study with slight modifications (Holkem et al., 2017). Briefly, 3 mL of the different biopolymeric solutions containing the probiotic bacteria were added dropwise to falcon tubes containing 17 mL of soybean oil and ultraturrax (11000 rpm during 1 min) was applied to make the W/O emulsions. The falcon tubes were immediately immersed in an ice bath where they were kept for 10 minutes to allow the gelation of the microcapsules. Then the tubes were centrifuged at 4000 rpm during 10 min, the upper oil layer was removed and replaced by PBS. Subsequently, the tubes were centrifuged at 4000 rpm during 10 min at 20°C and the PBS solution was discarded.

FIGURE 1. Scheme of the probiotic-containing capsule preparation protocol.

2.8 Morphological characterization of the capsules

Optical microscopy images of the capsules were taken using a digital microscopy system (Nikon Eclipse 90i) fitted with a 12 V, 100 W halogen lamp and equipped with a digital imaging head which integrates an epifluorescence illuminator. A digital camera head (Nikon DS-5Mc) was attached to the microscope. Nis Elements software was used for image capturing.

In order to confirm the presence of the probiotic bacteria within the capsules, the cells were stained using SYBR Green I (10,000x concentrate in DMSO, Invitrogen) diluted 100 times in PBS prior to capsule preparation.

2.9 Viability of encapsulated and non-encapsulated bifidobacteria during storage

The viability of *B. pseudocatenulatum* CECT 7765 was evaluated by plate counting at different times during storage (up to 15 days) at refrigerated (4°C, stored in falcon tubes

inside a fridge) and ambient conditions (25°C, 80%RH). Samples were subjected to 10-fold serial dilutions in sterile phosphate buffer saline solution (PBS, 130 mM sodium chloride, 10mM sodium phosphate, pH 7.4) supplemented with cysteine (0.05% w/v) and plated on MRS agar supplemented with cysteine (0.05% w/v). After 24-48 h incubation at 37°C, the number of colony-forming units (CFU) was determined. Cell viability was evaluated for the probiotics-containing biopolymeric structures by resuspension of a precise amount of the capsules (which corresponded to 100 µL of bacterial suspension) in 1 mL of PBS+cysteine. Tests were made in triplicate.

2.10 Statistical analysis

Data analysis was carried out using IBM SPSS Statistics software (version 23, IBM Corp., USA). One-way analysis of variance (ANOVA) was done to determine the significant differences between sample means, at a significance level of $\alpha=0.05$. Mean comparisons were performed by the Duncan's Test.

3 RESULTS AND DISCUSSION

3.1 Pre-optimization of the starting formulations

Agarose was initially selected as encapsulation matrix given the excellent oxygen barrier properties of this carbohydrate biopolymer which could contribute to maintaining the viability of the sensitive *B. pseudocatenulatum* used in this study. For processing purposes, agarose needs to be first heated up to its melting point and then cooled down before incorporating the probiotic bacteria. The objective in this case, was to have a liquid solution at around 40°C so as to be able to prepare the emulsions by blending the biopolymeric solutions with oil and obtain the subsequently gelled particles upon cooling them (cf. Figure 1). The maximum agarose concentration which

269 did not gel at 40°C was 0.25 wt.%. However, high biopolymer concentrations are
270 usually desired for encapsulation purposes since they yield denser gel networks which
271 provide greater barrier effects (Gómez-Mascaraque, Llavata-Cabrero, Martínez-Sanz,
272 Fabra, & López-Rubio, 2018). Thus, different blends of agarose with various
273 biopolymers were tested to increase biopolymer concentration while avoiding gelation
274 at 40°C. In fact, blends of agarose with other biopolymers have previously shown
275 smaller gelation temperature (T_{gel}) values than pure agarose (Russ et al., 2013).
276 Specifically, whey protein concentrate (WPC), gelatin (Gel) and starch solutions were
277 prepared at two different concentrations (1 and 5 wt.%) and blended with agarose
278 solutions at different concentrations (0.25-2 wt.%) in different ratios (1:2, 1:1 and 2:1
279 agarose:biopolymer). With this strategy, the agarose concentration used in the blends
280 could be increased up to 0.5 wt.% and the ratio 1:2 agarose:biopolymer was found to be
281 the most adequate as it prevented the gelling of the blended solutions. The blends which
282 fulfilled these requirements and gave raise to gelled particles upon cooling, as observed
283 by optical microscopy, were selected for subsequent incorporation of *B.*
284 *pseudocatenulatum*. The selected formulations are compiled in Table 1 and Figure 2
285 shows the microscopy images of the gelled particles obtained for the various agarose-
286 based systems. From Table 1 it can also be observed that the total solids concentration
287 was efficiently increased through blending the agarose solutions with the other
288 biopolymers.

FIGURE 2. Microscopy images of the various hydrogel particle formulations: (A) Agarose:Gel1, (B) Agarore:Gel5, (C) Agarose:WPC5, (D) Agarose:Starch1, (E) UP Agar:Gel1, (F) UP Agar:Gel5, (G) UP Agar:WPC5, and (H) UP Agar:Starch1. UP Agar stands for unpurified agar fraction. Scale bars correspond to 50 μm .

Figure 2 shows that depending on the blend composition, different morphologies of gelled particles were obtained. While blending agarose with WPC gave rise to almost spherical hydrogel particles, much more heterogeneous structures were obtained when blending the agarose with gelatin or starch. From the microscopy images, it can also be clearly observed that oil droplets were trapped within the biopolymer structures during the emulsification/internal gelation process, having the agarose-based formulations containing gelatin or WPC more affinity for the oil, as less oil droplets were observed in the formulation containing starch.

Unpurified agar-based fractions extracted from *Gelidium* species were also used for developing the encapsulation structures using the same formulations as selected with the agarose. The protein and polyphenol content of these unpurified fractions were found to be 136 ± 9 mg BSA/g sample and 30 ± 2 mg GA/g sample, respectively (in contrast with the pure agar, which contained only 16 ± 3 mg BSA/g sample and 3 ± 0.2 mg GA/g sample). The presence of these compounds in the unpurified agar-based material, implied that greater concentrations could be used to prepare solutions that did not gel at 40°C. Therefore, while the particles were produced using 0.5 wt.% agarose solutions for the various formulations developed, 3 wt.% solutions could be used to develop the unpurified agar-containing formulations. The increase in the total solids content (cf. Table 1) resulted in denser hydrogel particles (as observed in Figure 2). Moreover, it should be highlighted that a greater oil droplet accumulation was observed for the unpurified agar-based formulations containing gelatin or WPC, suggesting a greater affinity for oil of these unpurified fractions, fact which could also have an impact on the subsequent protection of the encapsulated probiotic bacteria, by limiting water access to the interior of the encapsulation structures. High humidity conditions are known to be detrimental for probiotic viability and, thus, generating structures with a more

hydrophobic character which can limit water vapour diffusion could have an advantage in probiotic stabilization as observed when co-encapsulating oils and probiotics (Eratte, Wang, Dowling, Barrow, & Adhikari, 2016). In contrast, less dense hydrogel particles were obtained for the unpurified agar-based formulation containing starch, which again showed, the lower affinity for oil droplets, which remained outside the heterogeneous particles (see Figure 2).

Figure 3 compiles the viability of the probiotic bacteria in the agarose and unpurified agar-based formulations in comparison with the bacteria in PBS with cysteine (the common media used to maintain the bacteria viable during manipulation). The data has been normalized to account for an initial viability around 10^8 CFU/mL to facilitate comparison.

FIGURE 3. Viability of *B. pseudocatenulatum* in the different agarose-based and unpurified agar-based hydrogel particles during refrigerated storage in comparison with the probiotic bacteria in PBS with cysteine.

As observed from Figure 3, this probiotic species is very sensitive and a quick viability drop was observed independently of the system employed for its protection.

Interestingly, while the blends containing WPC suffered from a significant viability drop after only two days of storage at refrigeration temperature, the viability was then better maintained than in the other systems with gelatin or starch. It has been previously reported that microencapsulation itself can affect cell viability (Rathore, Desai, Liew, Chan, & Heng, 2013). In fact, in a recent study it was shown that encapsulation through emulsification can cause bacterial cells damage, as demonstrated by flow cytometry (Bernucci et al., 2017), which could explain the initial viability drop observed. However, the improved protection of the WPC formulation with time could be due to a combination of factors. First of all, the greater amount of total solids contents in these specific capsules (if compared with the formulations with 1% gelatin or starch). It has been previously demonstrated that increasing the amount of biopolymers in the encapsulation structures improves cell protection (Bosnea, Moschakis, Nigam, & Biliaderis, 2017). Additionally, biopolymers in the encapsulation matrices may also be used by the bacteria as nutrient (carbon) sources (Bosnea et al., 2017) and whey proteins are especially interesting sources for bifidobacteria (Janer, Pelaez, & Requena, 2004). When the statistical analysis was carried out comparing between the three different groups (i.e. agarose-based, non-purified agar-based and PBS+cysteine), a significant improvement ($p<0.05$) in probiotic viability was observed for the non-purified agar-based formulations, thus suggesting that the increased concentration of biopolymer and/or the presence of other compounds like proteins and polyphenols may be having a positive effect on *B. pseudocatenulatum* protection. Previous studies have suggested positive effects of both proteins and polyphenols on probiotic viability (Dave & Shah, 1998; Pacheco-Ordaz et al., 2017). In the case of proteins, being a nitrogen source for the bacteria, they seem to positively impact bifidobacteria viability (Dave & Shah, 1998), but the protective effect may also be due to either modified diffusion properties

of the encapsulating matrix, or oxygen scavenging effects provided by the presence of antioxidant molecules like polyphenols (Gaudreau, Champagne, Remondetto, Bazinet, & Subirade, 2013). In fact, chemical deteriorative reactions, particularly oxidation of lipid membranes, have been suggested to be a major cause for *Bifidobacterium* viability loss when exposed to air, high temperature and high humidity, being common practice the addition of antioxidants to partially counteract them (Chen, Wang, Liu, & Gong, 2017). Moreover, when comparing between the different hydrogel particles and the bacteria in PBS with cysteine, a significant viability improvement (i.e. a significantly lower viability drop) was observed in all cases. Regarding the impact of biopolymer concentration on bacterial viability, the results were not conclusive as, while for the agarose-based formulations increasing the gelatin concentration seemed to have a positive effect on bacterial protection, no significant viability differences were observed after 15 days of storage for the unpurified agar-based formulations. These results seem to point out that the presence of impurities in the agar material (mainly proteins and polyphenols) is also contributing to the improved probiotic viability observed. Recent studies have shown that phenolic compounds selectively allow the growth of probiotic microorganisms, while inhibiting pathogenic bacteria (Pacheco-Ordaz et al., 2017). The formulation which showed a lower viability drop at the end of the storage period was the one with WPC and, moreover, it also displayed a better morphology than the other agarose and unpurified agar-based particles with gelatin or starch. Furthermore, whey protein concentrate (WPC) is known to be an excellent matrix for probiotic protection (Dianawati et al., 2013; López-Rubio et al., 2012) and, thus, the formulation of unpurified agar with 5 wt.% WPC was selected for further experiments. However, as rather heterogeneous structures were formed, the influence of microcapsule morphology on probiotic viability could not be ascertained. Specific interactions between the capsule

components were neither expected to influence probiotic protection, but rather the network structure and composition. In fact, in a previous study of gels from agarose and whey protein concentrate, as deduced from the DSC results, it appeared that there were no specific interactions between both components, consisting the network on a mixture of the individual preparations (Katopo, Kasapis, & Hemar, 2012). From the microscopy images in Figure 2, it seems that oil droplets were trapped within this network.

3.2 Survival of encapsulated *B. pseudocatenulatum* during storage

In order to have a fairer comparison and ascertain if the presence of proteins and polyphenols in the unpurified agar-based fractions had an influence on the viability of encapsulated bacteria, the selected formulation containing 5 wt.% of WPC was compared with a counterpart material prepared with pure agar. In this case, the samples were stored not only at refrigerated conditions, but also at room temperature, in which the viability loss for the samples in PBS with cysteine was really quick as observed in Figure 4.

FIGURE 4. Viability of *B. pseudocatenulatum* in pure agar (Agar) and unpurified agar (UPAgar) hydrogel particles containing WPC during storage at 5°C and 25°C in comparison with the probiotic bacteria in PBS with cysteine.

While in the case of the probiotic in PBS with cysteine a complete viability loss was observed after only 4 days of storage, the agar-based capsules prepared by emulsion/internal gelation were effective in protecting the bacteria and, some bacterial counts remained during the 15 days of storage at room temperature, which for such a sensitive bacterial strain is a promising result. Upon storage at a greater temperature, gas diffusion will increase, thus drastically reducing the bacterial viability when no protective matrix was present. In contrast, increasing the temperature for gelling materials implies greater matrix mobility. In a recent study it was reported that when the biopolymers involved in an encapsulation structure are stiff, the structural rearrangements are lessened and the protective effect of the matrix environment to the entrapped cells is reduced (Bosnea, Moschakis, & Biliaderis, 2014). This would explain the greater improvement observed at room temperature for the studied materials. Moreover, when comparing the pure agar and non-purified agar-based formulations, the latter ones again displayed improved protection, thus again suggesting that the presence of other compounds in this extracted fraction had a positive effect on probiotic protection. As previously explained, the presence of proteins and polyphenolic compounds in the unpurified agar samples allows, on one hand, to increase the biopolymer concentration in the formulations, as gelling is somehow prevented and, moreover, the inherent antioxidant properties of polyphenols may also be exerting a favorable action, limiting oxygen access, but also positively interacting with the

bacteria, as previously reported (Kemperman et al., 2013; Ma, Gong, Liu, Ma, & Chen, 2015).

3.3 Rheological characterisation of agarose and unpurified agar solutions

Knowing that the physical properties of the capsules may have an influence on probiotic protection, the rheological behavior of the formulations containing 5% WPC was analyzed. The experiments were carried out by temperature-dependent oscillation measurements in the linear viscoelastic region. For that means, the hot solutions were placed on preheated plates and subsequently cooled down to until strong gels were formed.

FIGURE 5. Oscillatory measurements of G' and G'' during cooling for (A) Agarose:WPC5 solution and (B) UPAgar:WPC5 solution.

Figure 5 shows the storage modulus G' and the loss modulus G'' as a function of temperature during the cooling processes for the agarose and unpurified agar-based solutions. Agarose was chosen instead of pure agar, as agarose is the gelling fraction of agar (Armisen, 1991) and, thus, stronger gels were expected to be obtained from this biopolymer. The samples were initially prepared as specified in section 2.5 and they were poured onto the rheometer plate, which was set at 60°C. At the initial temperature of 60 °C the samples did not show a true solution behavior, but instead presented a behavior typical from weak gels, with low G' and G'' values and with $G' > G''$. After the initial stage, at which G' and G'' remained almost constant, both moduli increased sharply when lowering the temperature below a certain point. The temperature at which this abrupt rise in G' and G'' was observed, which is normally ascribed to the transition from weak to strong gels, was taken as the apparent gelling point T_{gel} (Russ et al., 2013) and it was ca. 36 °C and 32 °C for the UPagar-WPC and agarose-WPC formulations, respectively. Moreover, in contrast with pure agarose, the mixed solutions of agarose and unpurified agar with WPC, showed a gradual transition, taking place much more slowly and giving raise to gels significantly weaker than those obtained from pure agarose (Wang et al., 2018). This can be explained by the WPC chains hindering proper agarose fibers network formation via hydrogen bonding. Similar effects have been observed upon addition of other biopolymers like xanthan (Nordqvist & Vilgis, 2011). Comparing the graphs from both systems, one can clearly observe that, most likely due to the increased biopolymer concentration in the capsules containing the unpurified agar fractions, stronger gels were formed, with significantly greater elastic modulus values at 10 °C ($G'=258$ Pa) than the agarose-WPC system ($G'=100$ Pa). This difference in physical properties of the gelled particles could also have an influence on probiotic

467 stabilization through restricted molecular diffusion of solutes, as polymer chain mobility
468 is an important factor governing solute movement within hydrogels (Amsden, 1998). A
469 recent study trying to understand the effect of gel strength on probiotic protection,
470 showed that cell survival was positively correlated with the mechanical strength of the
471 microcapsules, explained by the lower permeability coefficients observed using
472 different probes (Zhao et al., 2017).

474 **3.4 Fluorescence microscopy of the hydrogel particles**

475 Finally, the presence and distribution of labeled *B. pseudocatenulatum* cells within the
476 agarose and unpurified agar formulations with WPC was observed by fluorescence
477 microscopy. After staining the bacterial solution with SYBR Green I, the microgel
478 particles were prepared, filtered, spread on a microscope slide and taken directly for
479 observation under a microscope with a fluorescence source. Figure 6 shows the
480 microphotographs of the agarose-based (Figures 6A and 6B) and unpurified agar-based
481 gel particles (Figures 6C and 6D).

FIGURE 6. Optical micrographs of *B. pseudocatenulatum* encapsulated in hydrogel particles of (A,B) Agarose:WPC5 and (C,D) UPAgar:WPC5. Images B and C were taken using a fluorescence source.

In Figure 6, it is observed that the labeled *Bifidobacterium* cells were successfully encapsulated in the hydrogel particles and were evenly distributed. The presence of individual cells or cell agglomerates was more clearly observed in the agarose-based particles (cf. Figures 6A and 6B). In contrast, the unpurified agar-based particles containing WPC, had an intrinsic fluorescence which complicate spotting individual bacteria, although brighter spots, corresponding to the labelled cells can be distinguished in the image. The intrinsic fluorescence of some polyphenols (Gómez-Mascaraque et al., 2017), could be the cause of such an intense fluorescent signal in the

particles made with the unpurified agar, which moreover, appeared more fused after filtration.

4 CONCLUSIONS

The present work shows the suitability of using more cost-effective and environmentally friendly unpurified agar fractions as encapsulation matrices for probiotic protection. Hydrogel particles were made from various agarose, pure agar and unpurified agar-based formulations using a simple emulsion/internal gelation process. All the particles developed were successful in prolonging the viability of encapsulated *Bifidobacterium pseudocatenulatum* CECT 7765 during refrigerated storage. Although no specific interactions between the components were expected, the combination of agar and agarose with WPC significantly improved probiotic protection even at ambient conditions. The improvement was greater when unpurified agar was used in the formulations, fact which could be attributed to the presence of proteins and polyphenols (which could be used as nutrient sources and provide antioxidant activity, respectively) and/or to the greater biopolymer concentration that could be used in this case, which gave raise to stronger gel particles and, thus, to limited solute diffusion.

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