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# Development of a probiotic delivery system based on gelation of water-in-oil emulsions

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ABSTRACT

The protection and delivery of probiotic cultures remain a challenge for the food industry. In this work, a new delivery system was developed based on gellan added to the aqueous phase of a water-in-oil emulsion. The concentration of polysaccharide was evaluated to control gel mechanical properties. Uniaxial compression tests were performed to characterize the mechanical properties of gels, and rheological oscillatory tests were conducted to evaluate gelation time. Different emulsion formulations were evaluated with the aim of producing small microgels. The resistance of microgels to digestion steps was evaluated by size measurements and ζ-potential analyses in *in vitro* tests. The microgels were stable to oral and gastric digestion, but they were destabilized in enteric fluid. *Lactobacillus rhamnosus* was encapsulated into the gelled and non-gelled emulsions, and its survival was evaluated after *in vitro* digestion. The probiotic viability was higher than 77% in gelled emulsion and decreased to 66% when the aqueous phase did not form gel. Our results show that emulsion gelation improved microbial resistance to *in vitro* digestion conditions.



## 1. Introduction

Probiotics have been gaining attention in the food industry due to the consumer demand for healthy products. However, to address this increased attention, some issues regarding the viability of probiotics in food need to be overcome. To act as a probiotic, bacteria must be metabolically stable and active in the product, and maintain their viability along the digestive tract (Coghetto, Brinques, & Ayub, 2016). Increased probiotic viability in food products can be achieved by probiotic encapsulation using different methods, but extrusion and spray-drying are the most common techniques. A number of studies have reported reductions in bacterial viability due to the severe conditions of spray-drying caused by high air temperature (Anal & Singh, 2007). Extrusion has the advantage of mild operation conditions, but large-sized beads (2–5 mm) are formed, limiting their application in food products due to the unpleasant sensory perception (Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011). Therefore, water-in-oil emulsions have emerged as a promising technique to incorporate microorganisms into food matrices due to the absence of harsh processing conditions, enabling the maintenance of cell viability.

Homogeneous carriers of reduced size can only be formed if the gelation of the aqueous phase occurs prior to droplet coalescence. In this sense, the viscosity of the dispersed phase plays an important role in microcapsule formation since this property defines the resistance of droplets to motion (enhancing or hindering flocculation and droplet collision) (Heeres, Picone, van der Wielen, Cunha, & Cuellar, 2014). Emulsions composed of a viscous continuous phase are usually more kinetically stable (Chen et al., 2016); however, the energy required for the homogenization process is also higher. The increase in the viscosity of the dispersed phase during gelation reduces the efficiency of homogenization, and large droplets are formed (Pandolfe, 1981). Therefore, to produce small and homogeneous carriers by emulsion gelation, a careful study of the gelation time of dispersed phase versus droplet coalescence time must be undertaken.

Gellan is an anionic polysaccharide that forms gels over a wide pH range and at a lower concentration than other polysaccharides commonly used in foods. Gellan gels are resistant to acidic conditions and are transparent, with no effect on the appearance of foods

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in which they are added (Lau, Tang, & Paulson, 2000). Rigid gels are formed, especially at low pH, and slow acidification rates can be obtained by the addition of glucono-δ-lactone (GDL) (Picone & Cunha, 2011). During the carrier formation, the acidification rate must be slow enough to provide gels with good mechanical properties.

Another challenge is to tune the carrier characteristics with respect to the delivery site of the bioactive material. The delivery system must protect the probiotics during product storage and digestion, providing safe release in the lower intestinal tract. This work aimed to develop a new probiotic delivery system based on the gelation of water-in-oil emulsions. Emulsions containing *L. rhamnosus* and gellan gum added in the dispersed phase were produced, varying the composition of the aqueous phase to change the gelation time and the coalescence rate of the emulsion droplets. Gellan gum was used in the aqueous phase, and its gelation was induced by slow acidification to pH 3.0 using GDL. The emulsified-gelled delivery system was evaluated for its resistance to degradation and the survival of microorganisms in digestion conditions through *in vitro* digestibility assays.

## 2. Material and methods

### 2.1. Material

Deacylated gellan gum (Kelcogel®) was kindly provided by CP Kelco (Atlanta, GA, USA). Polyglycerol polyricinoleate (Grindsted® PGPR) and the microorganism *L. rhamnosus* were donated by DuPont Nutrition & Health (Esteio, RS, Brazil). GDL, porcine pepsin and pancreatin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Refined soybean oil was acquired from Bunge Alimentos S.A. (Embu, SP, Brazil). The de Man, Rogosa and Sharpe (MRS) culture medium Difco™ Lactobacilli (BD Becton Dickinson and Company, Detroit, MI, USA) was used to activate and enumerate the probiotic cells. The other reagents were of analytical grade.

### 2.2. Methods

#### 2.2.1. Definition of gellan concentration

Gellan gum stock solutions (0.3 and 0.5 g/100 mL) were prepared by stirring the gellan powder in deionized water at 80 °C over 30 min in a jacketed vessel, followed by cooling to 10 °C in an ice bath. Different GDL/gellan ratios (1.0–2.5) were previously evaluated to obtain a final pH of 3.0 (ratio 1.2). Macrogels were produced to measure the mechanical properties of gellan gels in uniaxial compression tests. Aqueous gellan solutions were conditioned in cylindrical flasks 21 mm in diameter and 21 mm in height for 48 h at 25 °C. The time required for gel formation was determined from oscillatory rheological measurements.

The mechanical properties of the gellan macrogels were determined by uniaxial compression measurements in a TA-XT Plus Texture Analyser (Stable Microsystems Ltd., Surrey, England) equipped with a 60 mm diameter cylindrical acrylic plate lubricated with silicon oil to minimize friction between the sample and the probe. The gels were compressed to 80% of their original height at 25 °C, using a crosshead speed of 1 mm/s. Hencky stress ($\sigma$) and strain ($\varepsilon$) were calculated from the force-deformation data according to Steffe (1996).

The rheological behavior of the samples was evaluated in oscillatory shear measurements in a stress-controlled rheometer Physica MCR 301 (Anton-Paar GmbH, Graz, Austria) equipped with stainless steel cone-plate geometry that was 60 mm in diameter. The kinetics of gellan gelation was evaluated within the linear viscoelastic domain by time sweep using an oscillatory frequency of 0.1 Hz and a shear stress of 0.1 Pa. The gel point was determined from the crossover between the elastic ($G'$) and viscous ($G''$) moduli at 25 °C (Steffe, 1996).

#### 2.2.2. Emulsion production

Prior to microorganism encapsulation, the emulsion composition was defined, aiming to obtain small carriers. Emulsions were composed of 40 g/100 g of aqueous phase and 60 g/100 g of oil phase. The concentration of PGPR in the oily phase was fixed at 2.0 g/100 g, which was defined from preliminary tests that varied the PGPR concentration from 0.5 to 2.0 g/100 g. Aiming to increase the ratio of microgel production to oil, a secondary study was conducted, varying the ratio of aqueous:oil phase from 40:60 over 50:50 to 60:40; from the results, the ratio was fixed at 40:60. Gellan solution (0.5 g/100 mL) was pre-homogenized with the oily phase in a T-18 Ultra-Turrax homogenizer (IKA®, Staufen, Germany) for 4 min at 14,000 rpm. After that, this preliminary emulsion was homogenized at 30 MPa/5 MPa in a high-pressure two-stage Panda 2K NS1001L homogenizer (Niro Soavi, Parma, Italy). The emulsions were put in graduated glass vessels (50 mL) and stored at 25 °C for 24 h before size and morphological analyses.

The encapsulation and viability of *L. rhamnosus* were evaluated using non-activated and activated cultures in MRS broth. Freeze-dried *L. rhamnosus* culture (~0.08 g) was activated in 5 mL of MRS broth and incubated for 18 h at 37 °C. Thereafter, it was transferred to a flask of 45 mL of MRS broth and incubated for 18 h at 37 °C. The material obtained from this last stage was centrifuged at 2400 × *g* for 9 min at 10 °C, washed three times with 30 mL of sterile water, and then suspended in 0.5 g/100 mL gellan stock solution. All materials used during microbial activation were previously sterilized at 121 °C, 101 kPa for 15 min. The aqueous phase was prepared by the addition of activated or non-activated probiotic into the gellan solution added of GDL under stirring. The emulsion, composed of 2.0 g/100 g PGPR in the oily phase and oil: aqueous phase ratio of 40:60, was prepared as mentioned previously.

#### 2.2.3. In vitro digestion assay

The *in vitro* digestion assays were performed on three different systems: (i) gelled emulsions without microorganisms, (ii) non-gelled emulsions containing probiotic microorganisms and (iii) gelled emulsions containing probiotic microorganisms. The viable bacterial cells were enumerated before and after the digestion assays. As a control, the digestion of activated *L. rhamnosus* culture was also evaluated by simulating the *in vitro* digestion of its free form in suspension. *In vitro* digestibility tests were performed in duplicate by incubating emulsions (i, ii, iii) and the control in different media to simulate oral, gastric and enteric digestion (Mantovani, Cavallieri, Netto, & Cunha, 2013). Emulsions were mixed with a phosphate buffer (5 mmol/L, pH 6.9, 0.04% NaCl, 0.004 mol/L $CaCl_2$) at a ratio of 1 g of each sample to 4 mL of buffer. The gastric digestion was simulated by the addition of a simulated gastric fluid (SGF) consisting of porcine pepsin (40 mg/mL in 0.1 mol/L HCl) to the initial mixtures at a ratio of 0.5 g of pepsin per 100 g of sample. The pH value was adjusted to 2.0 with 6 mol/L HCl. After 1 h of incubation, the enteric digestion was performed. In this step, the resulting mixture of gastric digestion was adjusted to pH 5.3 with 0.9 mol/L sodium bicarbonate before the addition of 9 mL of simulated intestinal fluid (SIF) consisting of a mixture containing bile extract and pancreatin (2 mg/mL pancreatin, 12 mg/mL porcine bile extract and 0.1 mol/L sodium bicarbonate). The pH of the system was adjusted to 7.0 with 0.1 mol/L NaOH prior to incubation of the samples for 2 h. Incubation was performed in an ET-420 orbital shaking incubator (Tecnal, Piracicaba, SP, Brazil) at 100 rpm and 37 °C. Variations of the simulated fluid compositions (controls) were evaluated to identify the cause of the emulsion destabilization. To discriminate the role of pepsin, the gastric digestion was