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Probiotic-loaded microcapsule system for human *in situ* folate production: Encapsulation and system validation

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ABSTRACT

This study focused on the use of a new system, an alginate|ε-poly-L-lysine|alginate|chitosan microcapsule (APACM), able to immobilize a folate-producing probiotic, *Lactococcus lactis* ssp. *cremoris* (LLC), which provides a new approach to the utilization of capsules and probiotics for *in situ* production of vitamins. LLC is able to produce 95.25 ± 26 μg·L^{-1} of folate, during 10 h, and was encapsulated in the APACM. APACM proved its capacity to protect LLC against the harsh conditions of a simulated digestion maintaining a viable concentration of 6 log CFU·mL^{-1}of LLC. A nutrients exchange capacity test, was performed using *Lactobacillus plantarum* UM7, a high lactic acid producer was used here to avoid false negative results. The production and release of 2 g·L^{-1} of lactic acid was achieved through encapsulation of *L. plantarum*, after 20 h. The adhesion of APACM to epithelial cells was also quantified, yielding 38% and 33% of capsules adhered to HT-29 cells and Caco-2 cells, respectively.

1. Introduction

Malnutrition is a problem that still affects one in three people in the world (International Food Policy Research Institute, 2015). There are different reasons explaining this problem, although the lack of some micronutrients, such as folate, can be associated with malnutrition in pregnant women, children, elderly people and in people consuming a limited diet (World Health Organization, 2012, 2014a, b). A possible reason for the occurrence of this problem in elderly people is the decline of the presence of bifidobacteria in their gastrointestinal system, that are responsible for the production of essential group of B vitamins, such as folate (vitamin B9) (LeBlanc et al., 2013). Folate is a vitamin with extreme importance because it is involved in cells' regulatory processes (Jacob, 2000). Considering its importance in the human diet, it has been introduced in food in its synthetic form, folic acid, in order to accomplish the recommended daily intake of 330 μg for adults and 600 μg for pregnant women (European Food Safety Authority, 2014). However, fortification with synthetic forms has been studied and some studies mention that there are some disadvantages in the utilization of this form (Bailey & Ayling, 2009; de Meer et al., 2005; Morris & Tangney, 2007). Considering this, it is clear that there is a need for fortification by a natural form of folate, so that these problems are avoided and the bioavailability of this vitamin is increased. Some probiotic bacteria, such as *Lactococcus lactis* ssp. *cremoris* (LLC), *Streptococcus thermophilus*, *Bifidobacterium lactis*, *Bifidobacterium breve*, *Bifidobacterium infantis* and *Bifidobacterium animalis* are capable of producing large amounts of natural folate (Crittenden, Martinez, & Playne, 2003; Sybesma, Starrenburg, Tijsseling, Hoefnagel, & Hugenholtz, 2003). These bacteria mainly produce the most common natural form that is 5-methyltetrahydrofolate (5-MeTHF), the form that is naturally assimilated by human cells (Scott, 1999). As mentioned before there are some bacteria capable of producing different folate forms, although other characteristics are important considering the direct utilization of these bacteria in the gut, such as their capacity to produce and excrete the vitamin. There are some bacteria able to produce high amounts of extracellular folate (>40 μg·L^{-1}) such as: *Lactobacillus amylovorus* CRL 887 (68.3 μg·L^{-1}) (Emiliano et al., 2014), *Lactobacillus plantarum* SM 39 (397 μg·L^{-1}) (Hugenschmidt, Schwenninger, Gnehm, & Lacroix, 2010), *Lactobacillus delbrueckii* ssp. *bulgaricus* 863 (86.2 μg·L^{-1}), *L. lactis* ssp. *cremoris* SK 110 (41 μg·L^{-1}), and *Propionibacterium freudenreichii* ssp. *shermanii* B365 (41 μg·L^{-1}). However, this production is influenced by the incubation conditions and is totally dependent of the species and strains (Lin & Young, 2000). Generally, these probiotics are used in a free

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state and mainly for industrial production of folate, that is conventionally produced by chemical processes (Hugenschmidt, Schwenninger, & Lacroix, 2011).

Probiotics, that are live microorganisms, which when administered in adequate amounts confer a health benefit to the host (Food and Agriculture Organization of the United Nations/World Health Organization, 2001) and have been used to improve human health but two problems have been identified regarding its utilization, which are: i) the low survival rate through passage in the stomach and ii) the low residence time in the gut (Gardiner et al., 2004; Gueimonde & Salminen, 2006; Klingberg & Budde, 2006). Regarding the first problem, several studies have indicated the efficiency of microcapsules on the protection of probiotics against these harsh conditions, where materials such as alginate (Chandramouli, Kailasapathy, Peiris, & Jones, 2004; Mokarram, Mortazavi, Najafi, & Shahidi, 2009) and chitosan (De Prisco, Maresca, Ongeng, & Mauriello, 2015; Graff, Hussain, Chaumeil, & Charrueau, 2008) can be used. The second problem is related with the relatively low residence time that probiotics have in the human gut. Probiotic colonization and consequent adhesion to the intestinal mucosa, a characteristic that has been better understood in recent times, indicating that a probiotic stays no longer than 5–8 days in the human gastrointestinal system (Rattanaprasert, Roos, Hutkins, & Walter, 2014; Wolf, Garleb, Ataya, & Casas, 1995), eventually with a residual activity in the latter stages of this period.

To overcome these two problems on probiotics utilization, encapsulation systems that are able to immobilize these microorganisms can be an interesting solution. These systems are able to protect probiotics, to promote the controlled release of some micronutrients and to adhere to the mucus layer (Brun-Graeppi, Richard, Bessodes, Scherman, & Merten, 2011; Takeuchi et al., 2005). They can be constituted by hydrophilic polymers that typically have muco-adhesive properties (Gombotz & Wee, 1998). These hydrophilic polymers have charged functional groups that are able to form hydrogen bonds with mucosal surfaces (Dhawan, Singla, & Sinha, 2004; Khutoryanskiy, 2011). Some of these materials are alginate, poly-L-lysine and chitosan, and have been used to increase the residence time of some microcapsules by adhesion to the intestinal mucus layer (Gombotz & Wee, 1998; Ma et al., 2015).

Probiotics encapsulation foresee the total release of these microbes into the gut, in order to achieve a direct contact of it with the epithelium. In the other hand, there are immobilization systems that have been used to encapsulate microorganisms, such as bacteria or yeast, in order to create a microreactor able to host and maintain the microorganism in a continuous active state (Callone, Campostrini, Carturan, Cavazza, & Guzzon, 2008). With this, a high diffusion rate through the microcapsule is necessary to ensure the adequate exchange rate of consumed nutrients and produced metabolites (Genisheva, Teixeira, & Oliveira, 2014). Immobilization systems are more frequently used in biomedical applications, more specifically for tissue reconstruction. These systems are usually used in gastrointestinal or intravenous administration (Uludag, De Vos, & Tresco, 2000). In both cases, an adhesion of the microcapsule to the correct site and continuous release of the encapsulated cells is expected (Zhang, Xie, Koh, & James Lee, 2009). However, these systems were not explored considering the *in situ* production of vitamins by probiotics.

In a first work, a microcapsule was developed and characterized to be applied in the present work. This structure is an alginate encapsulation system, smaller than 100 μm, with a rationally designed coating created through layer-by-layer assembly: an alginate|ε-poly-L-lysine|alginate|chitosan microcapsule (APACM) (Ramos et al., 2015). Unlike other works using alginate|poly-L-lysine|alginate (APA) microcapsules, APACM has another coating, such as chitosan, that has two main functions, the protection against the harsh conditions of the stomach and to promote the adhesion to the epithelial cells. Therefore, APACM is a new system with unique characteristics and functions (Ramos, Cerqueira, Vicente, & Teixeira, 2016), because of that no other system (*e.g.* APA) was used in this work to compare its performance. This research focused on the use of the developed system for the encapsulation of probiotics with a high capacity of producing extracellular folate. The aim of this work was to test this system in terms of its capacity to: i) host probiotic bacteria; ii) pass through the gastrointestinal system; iii) adhere to the intestinal mucus layer; and iv) exchange nutrients and products (probiotic activation) in the intestine, by changing the porosity of a membrane induced by pH, whilst retaining probiotics. This approach was aiming at: i) increasing the residence time of probiotics in the intestine; ii) avoiding, or at least reducing, possible inflammatory responses from the organism due to direct contact with new bacteria; iii) producing natural folate inside the gut where it will be assimilated by the human organism (*i.e.*, the intestine).

2. Materials and methods

2.1. Materials

Sodium alginate Protanal 8133, Protanal 8223 and Protanal LFR 5/60 were obtained from FMC BioPolymer (Brussels, Belgium). Calcium chloride ($CaCl_2$) was purchased from Panreac (Barcelona, Spain). ε-poly-L-lysine (ε-PLL, MW. 30 kDa) was purchased from Handary (Brussels, Belgium). Chitosan (MW. 5–10 kDa) was obtained from Golden-Shell Biochemical Co. Ltd. (Yuhuan, China) with a degree of deacetylation of 95%. Corn oil, Tween 80, rhodamine B isothiocynate (RITC), 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N,N*-dimethylformamide and folic acid were purchased from Sigma (St. Louis, USA). M17 and de Man, Rogosa and Sharpe (MRS) were purchased from Oxoid (Hampshire, England). M17 and MRS agar and glucose were purchased from Merck (Munich, Germany). potassium chloride (KCl), monopotassium phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$), ammonium carbonate ($(NH_4)_2CO_3$), calcium chloride dihydrate ($CaCl_2(H_2O)_2$), sulfuric acid (H_2SO_4), L-Lactic acid ($C_3H_6O_3$), hydrogen chloride (HCl), sodium citrate ($Na_3C_6H_5O_7$), phosphate-buffered saline (PBS), formic acid, acetonitrile and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, USA). To perform the gastrointestinal simulation pepsin (from porcine, car no. SLBL2143V, 3616 $U \cdot mg^{-1}$), pancreatin (from porcine, cat no. SLBL3953V, 6.1 $U \cdot mg^{-1}$) and bile salts (from porcine, cat n° SLBK9078V, 164 mM) were purchased from Sigma-Aldrich. Internal standard of ^{13}C folic acid and ^{13}C of 5-methyltetrahydrofolate (5-MeTHF), as the standards of the same forms, were purchased from Schircks (Switzerland), as the other folate forms such as tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-FoTHF), 10-methyltetrahydrofolate (10-MeTHF) and 10-formyltetrahydrofolate (10-FoTHF). To perform the adhesion tests with cell culture the following materials were used: fetal Bovine Serum (FBS), penicillin, Dulbecco's Modified Eagle Medium (DMEM), glutamax, Dulbecco's phosphate buffered saline (DPBS) and hank's balanced salt solution (HBSS), all from Invitrogen (Sydney, Australia). Fluorodish dishes (35 mm) were purchased from World Precision Instruments (Sydney, Australia). Caco-2 and HT-29 cells (sourced from American Type Culture Collection, Manassas, USA) were a gift from the Lowy Cancer Research Centre (University of New South Wales, Sydney, Australia). The probiotics used in this work were *Lactococcus lactis* ssp. *cremoris SK 110* obtained from Nizo (Nizo Food Research, Ede, The Netherlands) and *L. plantarum* UM7 isolated from cow milk and obtained from our private collection of lactic acid bacteria.

2.2. Bacterial growth and preparation of cell suspensions

Probiotic bacteria *Lactococcus lactis* ssp. *cremoris* (Nizo Food Research, Ede, The Netherlands). Probiotic LLC was cultured and propagated, overnight, in 200 mL of M17 broth at 30 °C (Certomat H, GCC, Singapore), in a 500 mL flask with a stopper, under anaerobic conditions. The cells of bacteria were collected by centrifugation at 2000*g*