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Protection of *Lactobacillus acidophilus* under *in vitro* gastrointestinal conditions employing binary microcapsules containing inulin

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In this research, microcapsules based on low acyl gellan (LAG) and sodium alginate (SA) containing inulin were developed in order to assess its protective effect on the viability of *Lactobacillus acidophilus* under *in vitro* gastrointestinal conditions.The results showed that microencapsulated cells display significantly (P<0.05) higher resistance to simulated gastrointestinal conditions (SGIC) than free cells. Besides, the incorporation of inulin into the wall matrix resulted in improved survival after 5 h incubation in SGIC. These results represent an alternative to vehiculate probiotics in food, especially in solid food due to the size of the microcapsules. Therefore, these microcapsules can contribute to possible industrial applications in the development of new alimentary products.

Key words: Inulin, microcapsules, probiotic, simulated gastrointestinal conditions, sodium alginate, low acyl gellan.

INTRODUCTION

Probiotics are defined as live microorganisms which when administered in appropriate concentrations provide health benefits to the host because they colonize the human gut in adequate amounts (10^6 CFU/mL) (Tripathi and Giri, 2014; WHO/FAO, 2002). These health benefits include therapeutic effects such as alleviating symptoms of lactose malabsorption, reducing the level of serum cholesterol, irritable bowel syndromes and colon cancer, besides enhancing resistance to gut infections (Kailasapathy and Chin, 2000; Sanders et al., 2013). All these effects are caused by inhibiting pathogen growth and stimulating the host's immune response (Figueroa et al., 2011). However, the incorporation and viability of these bacteria in food products still represent a technological challenge for researchers during the development of new probiotic products, because the viability of probiotics often decreases sharply during gastric transit due to the strong acidic conditions (Holkem et al., 2016).

One effective method to protect probiotic bacteria from

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the environmental factors encountered during the passage through the human gastrointestinal tract is the microencapsulation using various polysaccharides as wall material (that is, gellan gum and sodium alginate). Gellan gum is an anionic extracellular heteropolysaccharide produced by the bacterium *Sphingomonas paucimobilis* and consists of repeating units of a tetrasaccharide (1,3-β-D-glucose; 1,4-β-D-glucuronic acid; 1,4 β-Dglucose; and 1,4-α-L-rhamnose). It is available in two forms: High acyl gellan (HAG) and low acyl gellan (LAG). When HAG is exposed to strong alkali treatment at high temperature, the acyl groups are hydrolyzed and LAG is obtained. These structural differences between HAG and LAG allow great diversity of its textural properties. Therefore, HAG forms soft, elastic gels; while LAG gum forms strong gels (González et al., 2012). With regard to alginates, they are polysaccharides produced by brown algae (*Laminaria digitata, Laminaria hyperborea, Ascophyllum nodosum* and *Macrocystis pyrifera*). Alginates are widely used in the industry due to their non-toxic and gelling properties. Chemically, alginates are an anionic linear copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G) joined by β 1-4 links and structured in blocks that can be homopolymeric (M or G) or heteropolymeric (MG) (Rosas et al., 2013). Within the most important applications of alginates in biotechnology is the ability to create stable gels through the ionic interaction between two adjacent chains with monovalent or divalent cations, forming junction zones that stabilize the gel structure (Fabich et al., 2012; Tavassoli et al., 2016).

Different methods for probiotic microencapsulation have been reported, including spray-drying, ionic gelantion, extrusion and complex coacervation (Champagne and Fustier, 2007; Martín et al., 2015). Internal ionic gelation (IIG) has been used for microorganisms microencapsulation due to its low cost, mild formulation conditions and high cellular retention making this technique one of the most promising ones (Cook et al., 2012). The microencapsulation using IIG does not require specialized equipment, complex techniques or the use of expensive reagents; moreover, IIG protects the microencapsulated cells from the acidic condition facilitating the gradual cell release in the target place (Chavarri et al., 2010; Cook et al., 2011; Guerin et al., 2003; Kanmani et al., 2011). Therefore, the aim of this study was to evaluate the *Lactobacillus acidophilus* survival into microcapsules containing inulin as a prebiotic compound under simulated gastrointestinal conditions.

MATERIALS AND METHODS

Microencapsulation

Microcapsules were obtained using a technique based on the formation of a water–oil emulsion. The dispersion (aqueous phase) was prepared with a mixture of 25SA/75LAG at 0.8% w/v, incorporating 1 mL of the cell suspensión (*L. acidophilus*) and 30 mM of Ca^{++}. Then, the dispersion was added into the oil phase (sunflower oil and 0.1% v/v of surfactant) under constant agitation in a stirring plate followed by the incorporation of 1 mL of δ-gluconolactone up to pH 4 in order to start the internal ionic gelation process. The microcapsules were harvested by centrifugation at 5000 rpm for 5 min, and the pellets were washed twice with saline solution to remove the oil residues.

Microcapsule morphology and size

Twenty micro liter of the microcapsules were used to determine the diameter employing a Leica DM500 microscope with a digital camera. The samples were diluted in sterile saline prior to the optical analysis and the captured images were analyzed using the software Image Pro-Plus ver 5.1. The average size of microcapsules was evaluated by measuring 100 microcapsules.

Microencapsulation efficiency

The microcapsules suspension were centrifuged at 5000 rpm in order to separate the free cell from microencapsulated cells. Then, the bacterial concentration in the supernatant was determined and encapsulation efficiency (% EE) was calculated according to Equation 1 as proposed by Gonzalez et al. (2015).

$$EE\ (\%) = (A-B)/A \times 100 \quad (1)$$

In this equation, A is the total bacterial concentration in the suspension and B is the free bacterial concentration in the supernatant.

Viability of *L. acidophilus* microencapsulated

Since the encapsulation process may affect the viability of probiotics, in the present study, the viability of *L. acidophilus* was enumerated before being subjected to simulated gastrointestinal conditions. The microencapsulated bacteria were released from the microcapsules based on the method proposed by Sheu and Marshall (1993). The microcapsules (1 g) were suspended in 9 mL of phosphate buffer (pH 7, 0.1 M) and homogenized for 5 min at 14,000 rpm using a high-speed homogenizer (Ultra-Turrax, model T50) and the breaking of the microcapsules was confirmed by optical microscopy. The enumeration of the viable cells was carried out by the drop plate method after 48 h incubation at 37°C on MRS agar under anaerobic condition. After the incubation time, the viable probiotic cells were counted and expressed in log colony forming units per gram (log CFU g^{-1}).

Viability of free and microencapsulated *L. acidophilus* subjected to simulated gastric and intestinal juices

One gram of microcapsules was subjected during 1 h to simulated gastric juice (SGJ) which is prepared by adjusting the pH of 0.2% (w/v) NaCl solution to 3 through the addition of 1.0 M HCl solution in order to mimic the stomach condition (Cheow et al., 2014). Afterwards, the same microcapsules were also added to simulated intestinal juice (SIJ) (6.8 g of KH_2PO_4 in deionized water at pH 7.0) for 4 h, resulting in a total simulated gastrointestinal transit time of 5 h (Graff et al., 2001). It is interesting to mention that all the tests were performed at 37°C in order to simulate the body temperature and the solutions employed were prepared on the same analysis day. The survival of free and microencapsulated *Lactobacillus acidophilus* was conducted according to the aforementioned

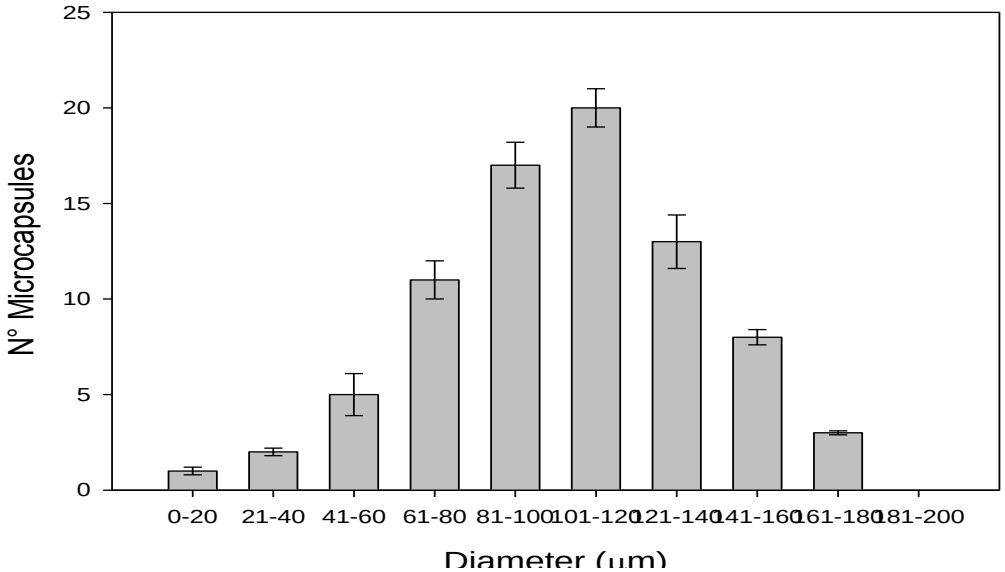

Figure 1. The size distributions of the microcapsules based on SA and LAG.

technique.

Statistical analysis

All the experimental data were subjected to analysis of variance (ANOVA- one way) using the software SPSS (ver. 17 for Windows) followed by Tukey's mean comparison test at a level of 5% significance. All the tests were carried out in triplicate and the data expressed as the mean ± standard deviation.

RESULTS

Microencapsulation

The microencapsulation method employed in this work is based on the emulsion between two phases, one hydrophobic and one hydrophilic containing the anionic polysaccharide, where by agitation, a great number of drops are originated which are gelled by acidification with δ-gluconolactone, since ion calcium is released from the calcium carbonate. The obtained microcapsules showed a unimodal behavior; which may be explained by the slow release of calcium ions from calcium carbonate because of the slow disruption of the gluconolactone.

Figure 1 depicts the number versus intervals of obtained microcapsules size. A unimodal behavior with particle sizes between 20 and 180 µm was observed. The microcapsule size is an important physical parameter since it can influence the sensorial attributes as aroma, texture and appearance when microcapsules are applied into food matrices. Microcapsules minor to 100 µm are desirable in liquid food, so as to avoid negative sensorial impact (Burgain et al., 2011).

Figure 2 shows the morphology of the obtained microcapsules with SA and LAG using calcium carbonate as a Ca^{2+} donor which was with spherical in shape and the outside surface with regular surfaces without the presence of deformations.

In order to determine the microencapsulation efficiency of the microencapsulation process, two counts were carried out. The initial count corresponds to the number of microorganisms added to the biopolymer dispersion (aqueous phase) and the second one was determined after the microcapsules were harvested. It is worth to mention that no negative effect was observed as there was no significant difference ($p < 0.05$) among the obtained CFU values before and after microencapsulation process; due to that, high averages of efficiency percentages were obtained (94.32 to 95.76%). Nonetheless, the encapsulation efficiency of the microcapsules was slightly improved when the prebiotic was incorporated into the microcapsule; thus, the loss of probiotic in microencapsulation process was reduced.

Viability of *L. acidophilus* microencapsulated

It was noted that there was no significant difference ($P < 0.05$) among efficiency and viability values of *L. acidophilus* encapsulated in binary microcapsules before incorporation to simulated gastrointestinal juices. It means that all the microencapsulated bacteria were able to grow, thereby yielding the beneficial effect associated with the probiotic intake. This also indicates that the microorganisms did not suffer pronounced damage during the microencapsulation process, showing that IIG is a feasible and adequate technique to produce microcapsules containing probiotics.

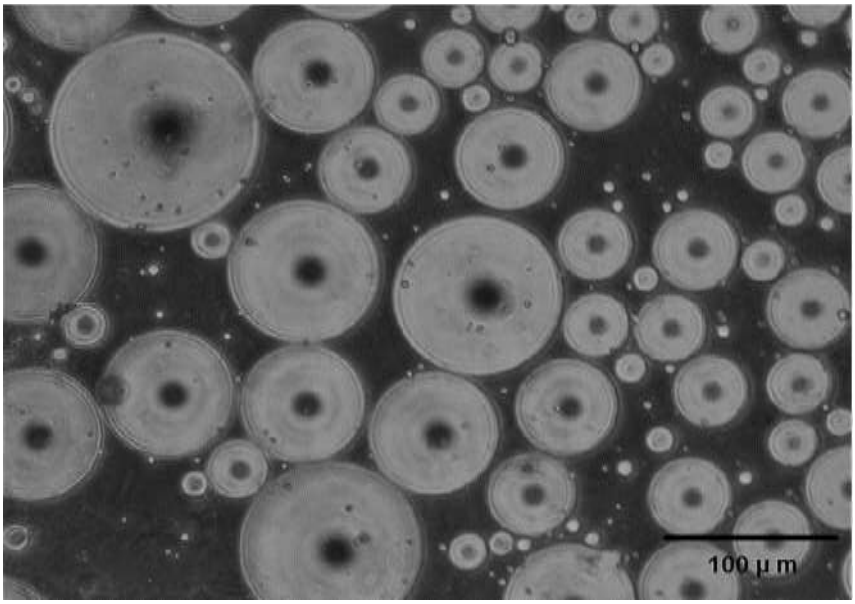

Figure 2. The optical micrographs at 10× of the binary microcapsules.

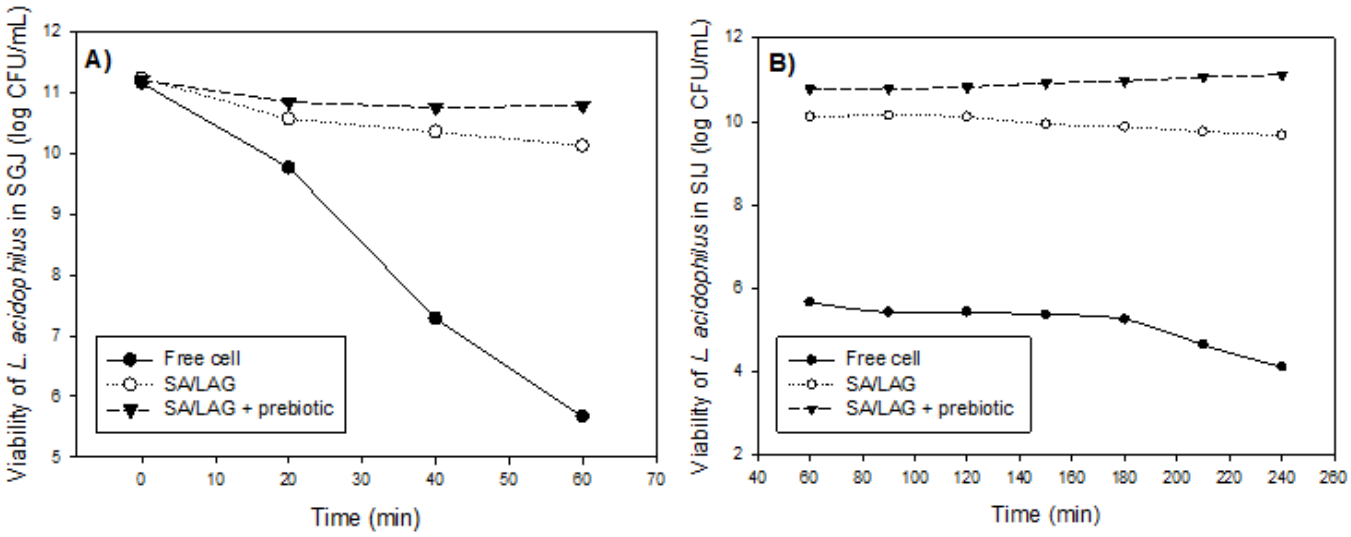

Figure 3. Viability of *L. acidophilus* free and microencapsulated in simulated gastrointestinal fluid (A: SGJ; B:SIJ).

Viability of free and microencapsulated *Lactobacillus acidophilus* subjected to gastric and intestinal conditions

Microcapsules containing *L. acidophilus* were initially exposed to SGJ for 1 h and then, the same microcapsules were transferred to SIJ for a further 4 h in order to mimic the gastrointestinal transit environment, equal procedure was realized for cells in free status. Figure 3 shows the results for the viability of *L.acidophilus* exposed to SGJ conditions for the free cells, microencapsulated and microencapsulated along with inulin. It was noted that the presence of inulin in the microcapsules provided the highest level of protection to the encapsulated cells, where 10.78 log CFU/ mL of the encapsulated cells survive to the SGJ during 1 h followed by cell microencapsulated alone with with 10.12 log CFU/mL, while free cells decreased sharply its viability until 5.67 log CFU/mL. Therefore, it is extremely important to protect *L. acidophilus* by microencapsulation These findings indicate that microcapsules based on mixture SA/LAG incorporated with inulin are stable under. acid solution likely by the interaction between biopolymer and the prebiotic. It should be clear that initial counts

before the incorporation to the SGJ were 11.23 log CFU/mL for the *L. acidophilus* microencapsulated, 11.15 log CFU/mL for free cells and 11.20 log CFU/mL for microencapsulated cell containig the prebiotic.

After immersion in SGJ, the difference between the viable number of *L. acidophilus* in free status, microencapsulated alone and microencapsulated along with inulin became highly significant ($P<0.05$) with longer incubation time.

At the end of the exposure time of *L. acidophilus* (free, microencapsulated and microencapsulated along with prebiotic) to SGJ conditions, the same probiotic bacteria was subjected to SIJ conditions at pH 7.0 as can be seen in Figure 3B. After submitting the *L. acidophilus* microencapsulated to SIJ conditions, they showed a significant decrease ($P< 0.05$) of 0.45 log CFU/mL when compared to the initial count, that is before the intestinal simulation. Most likely, some microcapsules were broken or there was a penetration of gastrointestinal juices into the microcapsules killing the probiotic. With regard *to L. acidophilus* microencapsulated along with inulin, an increase was observed ranging from 10.78 to 11.12 log CFU/mL; this is likely by a possible consumption of inulin by the probiotic or by a controlled release from microcapsules when the environmental pH rise. Conversely, the count of *L. acidophilus* in free status showed a reduction ($P< 0.05$) of 1.55 log CFU/mL when compared to the initial count before simulation.

In general terms, the number of the microencapsulated cells (both with and without inulin) that remain viable is approximately 6.27 log CFU/mL higher than free cells after being subjected to SIJ; which means that microcapsules protected from the acidic condition found in the gastrointestinal transited to *L. acidophilus* at the end of incubation period (5 h).

DISCUSSION

Characterization of microcapsules loaded with *L. acidophilus*

All the microcapsules revealed spherical shapes, as was displayed in Figure 2. The microcapsules had an average diameter of 102.82 µm being higher than those reported by Holkem et al. (2016) who reported mean diameters of 77.84 µm on microcapsules based on alginate. In the present study, smaller diameters were obtained than those obtained by Cai et al. (2014), who microencapsulated *L. acidophilus* with alginate by emulsification obtaining microcapsules with mean sizes ranging from 323 to 343 mm. Similar results were also published by Song et al. (2013), who studied microencapsulation of yeast by internal gelation and found microcapsule size with diameters between 35 and350 mm. Likewise, Wang et al. (2016) reported large size of microcapsules (1.5 mm) loaded with *Lactobacillus plantarum*, employing SA with or without inulin as inner layer and skim milk as outer layer. It should be noted that the diameters of microcapsules may affect the texture of the food products in which they are applied. For example, diameters about 100 µm are desired for most applications due to a better protection against acidic conditions as those found on the gastrointestinal transit (Arup et al., 2011; Champagne and Fustier, 2007).

The encapsulation efficiency (% EE) found in the present study had a mean value of 94.87%. These results are in agreement with those publised by Holkem et al. (2016) who found % EE values of 89.71% for Bifidobacterium BB-12 microencapsulated by IIG using alginate as a wall material. Likewise, Pitigraisorn et al. (2017) reported % EE values of 95.3% for *L. acidophilus* microencapsulated on non coated alginate beads. Nevertheless, in the current research, the efficiency was shown to be greater than that found in the studies of Zou et al. (2011) who microencapsulated *Bifidobacterium bifidum* F-35 obtaining values ranging from 43 to 50% using alginate microcapsules prepared by a similar technique of microencapsulation.

Viability of *L. acidophilus* microencapsulated

*L. acidophilus*is is a probiotic bacterium whose viability is significantly reduced at low pH values (Lee and Salminen, 2009). To overcome this problem, one objective of microencapsulation is to provide protection to probiotic cells during exposure at low pH (Çabuk and Harsa, 2015). However, microcapsules made of alginate tend to be highly porous leading to loss of core material. For this reason, blends of alginate and other polymers are employed in order to reduce wall porosity (Burgain et al., 2011).

Microcapsules loaded with *L. acidophilus* were initially exposed to SGJ at pH 3 for 1 h after which the same microcapsules were transferred to SIJ at pH 7 for a further 4 h. It should be noted that initial viable counts were in agreement with the recommended minimum values for the addition to a food probiotic product, as suggested by Aureli et al. (2011) and Salminen et al. (2011), who declared that the ingestion of probiotic cells should be around 8 to 9 log CFU/g to obtain beneficial effects on the health.

Various authors have reported that the microencapsulation with SA is effective for the survival of probiotics in acidic conditions (Ding and Shah, 2007; Doleyres and Lacroix, 2004). Maciel et al. (2014) reported an increase in the viability of *L. acidophilus* microencapsulated with sweet whey or skimmed milk by spray-drying during exposure to simulated gastrointestinal conditions at pH 2 to 7.

Etchepare et al. (2016) microencapsulated *L. acidophilus* in alginate beads with resistant starch (Hi-maize) and investigated the probiotic survival under

simulated gastrointestinal conditions. These authors reported that probiotic populations reduced to approximately 5.4 to 5.8 log CFU/g after exposure to simulated gastrointestinal fluids, which is similar to the values reported in the present study.

It was noticable that the barrier effect produced by microcapsules against acid conditions improve the probiotic viability because high count were obtained when *L. acidophilus* was microencapsulated along with inulin followed by the cells microencapsulated alone. Conversely, free cells showed a marked reduction in the population of *L. acidophilus*; these values demonstrate that the microorganism was fragile under acid conditions, which justify the microencapsulation to improve probiotic survival under gastric and intestinal conditions. Similar results were observed by Wang et al. (2016) who evaluated the viability of *L. plantarum* into microcapsules made of alginate containing inulin; these authors found that the microcapsules added with inulin resulting in reduction of the probiotic population to 0.4 log CFU/g, but when the inulin is absent, the probiotic population is reduced to 0.9 log CFU/g.

The enhancement of the *L. acidophilus* viability against gastrointestinal fluids could be due to the reduction of gastric fluid penetration into the microcapsule core, and the negative charges of the carboxylate groups that enhanced the buffer effect against infiltrated acid. Therefore, it could be a potentially effective matrix in protecting probiotics through the harsh environment it exists. It can be hypothesized that LAG formed the backbone of the microcapsules, while the SA is the factor governing the viability of encapsulated cells in SGJ due to alginate dissolution (Déat-Lainé et al., 2012). Alginate is converted into an insoluble layer of porous alginic acid, which at higher pH values dissolves and releases the active compounds (George and Abraham, 2006) in the desired location (intestine) (Park et al., 2014). Also, high obtained viability could be caused by SA particles dispersion which reduce the diffusion of oxygen into the microcapsules and thereby protect the microorganisms from oxygen exposure (Salminen et al., 2016).

In the present study, microcapsules based on LAG and SA loaded with *L. acidophilus* and inulin produced by IIG, represent an alternative to vehiculate probiotics in food, especially in solid food due to the size of the microcapsules.

Conflicts of Interests

The authors have not declared any conflict of interests.

REFERENCES

Arup NP, Kyoung H, Harjinder S (2011). Microencapsulation of probiotic bacteria using pH-induced gelation of sodium caseinate and gellan gum. Int. Dairy J. 21(4):247-253.

Aureli P, Capurso L, Castellazzi A, Clerici M, Giovannini M, Morelli L, Poli A, Pregliasco F, Salvini F, Zuccotti G (2011). Probiotics and health: an evidence-based review. Pharm. Res. 63(5):366-376.

Burgain J, Gaiani C, Linder M, Scher, J (2011). Encapsulation of probiotic living cells: from laboratory scale to industrial applications. J. Food Eng. 104(4):467-483.

Çabuk B, Harsa T (2015). Protection of *Lactobacillus acidophilus* NRRL-B 4495 under *in vitro* gastrointestinal conditions with whey protein/pullulan microcapsules. J. Bios. Bioeng. 120(6):650-656.

Cai S, Zhao M, Fang Y, Nishinari K, Phillips G, Jiang F (2014). Microencapsulation of *Lactobacillus acidophilus* CGMCC1.2686 via emulsification/internal gelation of alginate using Ca-EDTA and $CaCO_3$ as calcium sources. Food Hydrocol. 39(1):295-300.

Champagne C, Fustier P (2007). Microencapsulation for the improved delivery of bioactive compounds into foods. Curr. Opin. Biotechnol. 18(2):184-190.

Chavarri M, Maranon I, Ares R, Ibanez F, Marzo F, Villaran M (2010). Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. Int. J. Food Microbiol. 142(1-2):185-189.

Cheow W, Yi K, Hadinoto K (2014). Controlled release of *Lactobacillus rhamnosus* biofilm probiotics from alginate-locust bean gum microcapsules. Carbohydr. Polym. 103(1):587-595.

Cook M, Tzortzis G, Charalampopoulos D, Khutoryanskiy V (2011). Production and evaluation of dry alginate-chitosan microcapsules as an enteric delivery vehicle for probiotic bacteria. Biomacromolecules 12(7):2834-2840.

Cook M, Tzortzis G, Charalampopoulos D, Khutoryanskiy V (2012). Microencapsulation of probiotics for gastrointestinal delivery. J. Control. Release 162(1):56-67.

Déat-Lainé E, Hoffart V, Cardot J, Subirade M, Beyssac E (2012). Development and *in vitro* characterization of insulin loaded whey protein and alginate microparticles. Int. J. Pharm. 439(1-2):136-144.

Ding W, Shah N (2007). Acid, bile, and heat tolerance of free and microencapsulated probiotic bacteria. Food Microbiol. Saf. 72(9):M446-M450.

Doleyres Y, Lacroix C (2004). Technologies with free and immobilised cells for probiotic bifidobacteria production and protection. Int. Dairy J. 15(10):973-988.

Etchepare M, Raddatz G, Flores M, Zepka Q, Jacob E, Barin S, Ferreira C, Ragagnin C (2016). Effect of resistant starch and chitosan on survival of *Lactobacillus acidophilus* microencapsulated with sodium alginate. LWT – Food Sci. Technol. 65(1):511-517.

Fabich T, Vogt J, Sherick l, Seymour D, Brown R, Franklin J, Codd S (2012). Microbial and algal alginate gelation characterized by magnetic resonance. J. Biotechnol. 161(3):320-327.

Figueroa I, Quijano G, Ramirez G, Cruz A (2011). Probiotics and prebiotics – Perspectives and challenges. J. Sci. Food Agricul. 91(8):1341-1348.

George M, Abraham E (2006). Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan d a review. J. Control. Release 114(1):1-14.

González R, Ramos G, Cruz A, Salazar A (2012). Rheological characterization and activation energy values of binary mixtures of gellan. Eur Food Res. Technol. 234(2):305-313.

Graff J, Brinch K, Madsen J (2001). Gastrointestinal mean transit times in young and middle-aged healthy subjects. Clin. Physiol. 21(2):253-259.

Guerin D, Vuillemard J, Subirade M (2003). Protection of bifidobacteria encap-sulated in polysaccharide-protein gel beads against gastric juice and bile. J. Food Prot. 66(11):2076-2084.

Holkem T, Raddatz G, Nunes L, Cichoski A, Jacob E, Grosso R, Ragagnin C (2016). Development and characterization of alginate microcapsules containing Bifidobacterium BB-12 produced by emulsification/internal gelation followed by freeze drying. LWT - Food Sci. Technol. 71(1):302-308.

Kailasapathy K, Chin J (2000). Survival and therapeutic potential of probioticorganisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. Immunol. Cell Biol. 78(1):80-88.

Kanmani P, Kumar R, Yuvaraj N, Paari K, Pattukumar, V, Arul V (2011). Cryopreservation and microencapsulation of a probiotic in alginate-chitosancapsules improves survival in simulated gastrointestinal conditions. Biotechnol Biop. Eng. 16(6):1106-1114.

Lee Y, Salminen S (2009). Handbook of Probiotics and Prebiotic, second ed. John Wiley & Sons, Inc, New Jersey.
Maciel G, Chaves S, Grosso C, Gigante M (2014). Microencapsulation of *Lactobacillus acidophilus* La-5 by spray-drying using sweet whey and skim milk as encapsulating materials. J. Dairy Sci. 97(4):1991-1998.
Martín M, Lara F, Ruiz M, Morales M (2015). Microencapsulation of bacteria: a review of different technologies and their impact on the probiotic effects. Innov. Food Sci. Emerg. Technol. 27(1):15-25.
Park S, Ahn J, Choi S, Lee J, Lee H (2014). The effects of particle size on the physicochemical properties of optimized as taxanthin-rich Xanthophyllomyces dendrorhous-loaded microparticles. LWT - Food Sci. Technol. 55(2):638-644.
Pitigraisorn P, Srichaisupakit K, Wongpadungkiat N, Wongsasulak S (2017). Encapsulation of *Lactobacillus acidophilus* in moist-heat-resistant multilayered microcapsules. J. Food Eng. 192(1):11-18.
Rosas W, Ramos E, Salazar J (2013). Microencapsulation of *Lactobacillus helveticus* and *Lactobacillus delbrueckii* using alginate and gellan gum. Carbohydr. Polym. 98(1):1011-1017.
Salminen H, Gommel C, Leuenberger B, Weiss J (2016). Influence of encapsulated functional lipids on crystal structure and chemical stability in solid lipid nanoparticles: towards bioactive-based design of delivery systems. Food Chem. 190(1):928-937.
Salminen S, Kenifel W, Ouwehand A (2011). Probiotics, applications in dairy products. In J. W. Fuquay, P. F. Fox, & P. L. H. Mcsweeney (Eds.), Encyclopedia of dairy sciences. San Diego, California: Academic Press
Sanders M, Guarner F, Guerrant R, Holt P, Quigley E, Sartor R, Sherman P, Mayer E (2013). An update on the use and investigation of probiotics in healthand disease. Gut 62(5):787-796.
Sheu T, Marshall R (1993). Microentrapment of lactobacilli in calcium aginate gels. J. Food Sci. 54(3):557-561.
Song H, Yu W, Gao M, Liu X, Ma X (2013). Microencapsulated probiotics using emulsification technique coupled with internal or external gelation process. Carbohydr. Polym. 96(1):181-189.
Tavassoli E, Shekarchizadeh H, Masoudpour M (2016). Development of edible films and coatings from alginates and carrageenans. Carbohydr. Polym. 137(10):360-374.
Tripathi M, Giri S (2014). Probiotic functional foods: survival of probiotics during processing and storage. J. Funct. Foods 9(1):225-241.
Wang L, YuX, Xu H, Aguilar P, Wei H (2016). Effect of skim milk coated inulin-alginate encapsulation beads on viability and gene expression of *Lactobacillus plantarum* during freeze-drying. LWT – Food Sci. Technol. 68(1):8-13.
WHO/FAO (2002). Guidelines for the evaluation of probiotics in food. London: Author.
Zou Q, Zhao J, Liu X, Tian F, Zhang H, Zhang H, Zhang H, Chen W (2011). Microencapsulation of Bifidobacteriumbifidum F-35 in reinforced alginate microspheres prepared by emulsification/internal gelation. Int J. Food Sci. Technol. 46(8):1672-1678.