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Survival of microencapsulated *Bifidobacterium longum* in Cheddar cheese during production and storage

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ABSTRACT

The aim of this study was to evaluate the effect of microencapsulation (ME) in alginate beads on the viability of *Bifidobacterium longum* 15708 in terms of their tolerance to freezing, storage in a frozen state, cheddar cheese manufacturing and storage as well as to a simulated gastro-intestinal environment. Two ME methods namely i) droplet extrusion method (ADE) and ii) emulsion method, involving two polymers (native (NA) and palmitoylated alginate (PA)) were compared. Results showed that ADE maintained higher viability of *B. longum* after 24 h freezing at −80 °C with no viability loss as compared to the emulsion process and free cells which lost approximately 0.8 and 1.5 log CFU/mL respectively. However, during a 4 weeks storage period at −80 °C, no significant difference ($P > 0.05$) was observed in the survival of free and immobilized *B. longum*, with no loss of viability. Cheddar cheeses supplemented with *B. longum* culture were prepared and analysed during storage at 4 °C. After 21 days of storage, Cheddar cheese containing encapsulated *B. longum* in NA and PA polymers produced with the emulsion process showed a good survival with 2 log CFU/mL reduction after 21 days, as compared to ADE-encapsulated *B. longum* and free cells with 3 and 4 log CFU/mL reductions respectively. The immobilized bacteria in both polymers were also more resistant than free cells to simulated gastric and intestinal environments by a factor of 30.

1. Introduction

Functional foods are defined as foods containing health-promoting components that extend beyond traditional nutrients (Halsted, 2003). Many ingredients serve to enrich the food matrix to develop such functional foods, and probiotics are amongst the most popular to this effect. Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit to the host (Araya et al., 2002). These microorganisms should remain viable in food until the time of consumption at a minimum level of 10^7 CFU/g to have positive effects on health (Chavarri et al., 2010; Heidebach, Först, & Kulozik, 2009). There is an increasing demand for dairy foods containing probiotics and many studies have been done in the incorporation of probiotics in yoghourt (Brinques & Ayub, 2011; Iyer & Kailasapathy, 2005; Sultana et al., 2000; Sun & Griffiths, 2000), ice-cream (Godward & Kailasapathy, 2003) and cheese (Bergamini, Hynes, Quiberoni, Suárez, & Zalazar, 2005; Ong & Shah, 2009). However, the low pH of fermented milks and the aerobic conditions of production and packaging are detrimental to their survival (Chan & Zhang, 2002; Reid, Champagne, Gardner, Fustier, & Vuillemard, 2007).

Cheese is an interesting food-based delivery vehicle of probiotics to the gastro-intestinal tract due to a higher pH, a higher fat content and a more solid consistency (Bergamini et al., 2005; Ozer, Uzun, & Kirmaci, 2008) than yoghurt, the most frequently used food matrix for probiotics. Cheddar cheese is the most widely produced and consumed hard cheese worldwide. However, this matrix can be detrimental to the viability of probiotic organisms due to the presence of many factors such as salt, oxygen and temperature (Fortin et al., 2011a; Ozer et al., 2008). It was reported that Cheddar cheese produced with added *Bifidobacterium longum* showed a decrease of probiotics during storage (Fortin et al., 2011b). Similar results were obtained by Godward and Kailasapathy (2003). On the

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other hand, some studies have reported more successful results using *Bifidobacterium bifidum* and *Lactobacillus paracasei* (Dinakar & Mistry, 1994; Gardiner et al., 2002). Therefore, some probiotic strains are not stable in Cheddar cheese and means to improve it are needed.

Microencapsulation (ME) of probiotic bacteria in biopolymers has received considerable research interest in order to prevent viability losses of probiotic bacteria in foods and during gastro-intestinal transit. Among ME methods, extrusion and emulsion methods are the most common techniques for probiotics (Albertini et al., 2010; Mortazavian, Razavi, Ehsani, & Sohrabvandi, 2007). These processes help limit the exposure of probiotic to oxygen (Talwalkar & Kailasapathy, 2003). ME by emulsion also reduces the particle size so as not to alter the sensory properties of food (Anal & Singh, 2007; McClements, Decker, & Weiss, 2007)

Alginate is the most commonly used biopolymer for the immobilization of probiotic bacteria due to its non-toxic nature, easy-handling, bioavailability, biocompatibility, low cost and easy bead-formation capacity by ionotropic gelation (Gombotz & Wee, 1998; Han, Guenier, Salmieri, & Lacroix, 2008; Le-Tien, Millette, Mateescu, & Lacroix, 2004). However, the effectiveness of alginate as a matrix for ME is limited due to its porous nature that can enable diffusion of water and other low molecular weight compounds into the matrix (Chandramouli, Kailasapathy, Peiris, & Jones, 2004; Cui, Goh, Kim, Choi, & Lee, 2000; Hansen, Allan-Wojtas, Jin, & Paulson, 2002). To overcome this problem, coatings of alginate particles were investigated. Most strategies involve coating with chitosan or poly-L-lysine (Burgain, Gaiani, Linder, & Scher, 2011). An outer oil coating has also proved effective in protecting cells towards acidity (Ding & Shah, 2009a), but the literature contains very few studies on the efficiency of modifying alginate itself by fatty acids.

Le-Tien et al. (2004) used *N*-palmitoylated alginate and succinylated alginate to increase the survival of *Lactobacillus rhamnosus* during passage 30 min into simulated gastric fluid (pH 1.5). Similar results were reported by Han et al. (2008) with *O*-palmitoylated alginate in order to protect the stability of vitamins during storage under high temperature and relative humidity. Therefore, alginate modification has improved its functionality in a gastric environment, but it is unknown if it also protects probiotic cells against some stressful conditions in a cheese matrix (salt, acid).

The objective of this study was to compare the effects of two ME techniques, using two polymers namely native alginate (NA) and palmitoylated (PA) alginate, on the viability of *B. longum* 15708 in Cheddar cheese after 21 days of storage. The effect of ME technique and alginate polymer on the protection of *B. longum* to simulated gastro-intestinal fluids was also examined.

2. Materials and methods

2.1. Materials

Raw milk was provided by Agropur (Granby, QC, Canada). Sodium alginate (Sigma, Oakville, Ontario, Canada, from brown algae, Mw 1.5×10^5 Da, $[\eta] = 620$ mL/g, containing approx. 67% of L-guluronate residues), calcium chloride, sodium citrate, sodium hydroxide, glycerol and Bacto-peptone were purchased from Sigma (St. Louis, MO, USA). MRS-grown and BHI were supplied from Difco (Detroit, MI, USA). Tween 80, palmitoyl chloride, acetone and methanol were purchased from Laboratoire Mat (Beauport, Quebec, Canada). Canola oil (100% pure canola oil) was purchased from a local supermarket.

2.2. Strains and cultures conditions

B. longum ATCC 15708 was purchased as freeze-dried cultures from the American Type Culture Collection (ATCC; Manassas, VA, USA). Stock cultures were obtained by mixing a MRS-grown (Difco/Becton-Dickinson, Detroit, MI, USA) cell suspensions with sterile BHI media (Difco) containing 15% (w/v) of glycerol (Sigma, St. Louis, MO, USA) in a 1:5 ratio, adding 1 mL of this cell suspension in cryovials (Nalgene; Rochester, NY, USA) and storing at −80 °C. *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *lactis* H-102 were obtained from CH-Hansen (Milwaukee, WI, USA) and used as primary starter for Cheddar cheese production.

B. longum were grown in MRS broth (Becton-Dickinson, Mississauga, ON, Canada) supplemented with 1% (v/v) of a sterile solution of 10% (w/v) ascorbic acid (Bioshop Canada Inc., Burlington, ON, Canada) and 5% (w/v) L-cysteine hydrochloride (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C until a pH 4.5 was reached. Cells were harvested by centrifugation at 6000 g for 10 min at 4 °C. The pellets were washed and resuspended at 1/20 in 0.1% peptone water. The cell suspensions were referred to as the free cells and were used either directly in assays or subjected to ME as described subsequently.

The starter (*L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* H-102) was prepared by inoculating 0.2% (w/w) of a thawed commercial culture of lactic acid bacteria into rehydrated skim milk (12% w/w), previously sterilized at 110 °C for 10 min. The culture was incubated to 21 °C for 15 h, in order to reach a pH of 4.5, and used within 2 h after this incubation time.

2.3. Production of *B. longum* microencapsulated culture

2.3.1. *O*-palmitoylation of alginate

Alginate *O*-palmitoylation was carried out following a modified method of Han et al. (2008). An amount of 5 g of sodium alginate was dissolved in 300 mL distilled water. The polymeric solution was heated at 70 °C and the pH was adjusted to 7.5. A volume of 20 mL of palmitoyl chloride was then slowly added, maintaining the pH at 7.2–7.5 with 0.5 M NaOH solution. After 2 h, the reaction medium was neutralized (pH 7.0) and the product was precipitated in 1 L ethanol, collected by filtration and washed at least three times with ethanol to eliminate free (non-reacted) fatty acid. Finally, purification was performed by washing with methanol heated at 50 °C, and the precipitate was dried at 40 °C to obtain the corresponding powder.

2.3.2. Microencapsulation of *B. longum* by co-axial air flow droplet extrusion

The conventional extrusion method was carried out using a nozzle system which is driven by co-axial air flow (Var J1 System, Nisco Engineering, Zurich, Switzerland). This system enables the production of smaller droplets than the traditional syringe methods and will be referred to as the co-axial air-flow droplet extrusion (ADE) process. A volume of 100 mL of 2% (v/w) NA or a blend of NA/PA in a 1:1 ratio was prepared in sterile 1 g/L peptone water. Prior to use, all polymers were sterilized at the Canadian Irradiation Centre (Laval, QC, Canada) by γ-irradiation at a dose of 10 kGy using a ^{60}Co source irradiator (γ-Cell 220, Nordion, Kanata, ON, Canada). The sterile alginate solution was mixed with 15 mL of the washed bacterial cell suspension which had 10^9 CFU/mL. The polymeric matrix solution was sprayed at a pressure of 90 psi using the nozzle system attached to a peristaltic pump and the pumping rate was 2 mL/min in a 30 g/L $CaCl_2$ solution supplemented with 1 g/L peptone (Difco/BD, Franklin Lakes, NJ, USA), 1 g/L tryptone (Difco/BD) and 0.5 g/L yeast extract (Difco/BD) under mild stirring. The pH of this calcium-based gelation solution was of 6.5. The microbeads were allowed to stand for 30 min for hardening, filtered with a Whatman #1 filter paper and then rinsed with sterile 1 g/L peptone water. All samples were resuspended (1:1 ratio) in a cryoprotective medium composed of 200 g/kg rehydrated skim

Table 1
Effect of freezing on viable counts of microencapsulated *Bifidobacterium longum* 15708.

Condition	Viable cell count (log CFU/mL or log CFU/g)				
	Free cells (control)	Microencapsulated			
		NA-ADE	PA-ADE	NA-emulsion	PA-emulsion
Fresh	9.21 ± 0.11^{aA}	9.30 ± 0.26^{aA}	9.28 ± 0.17^{aA}	9.10 ± 0.21^{aA}	9.31 ± 0.10^{aA}
Frozen	7.93 ± 0.36^{aB}	9.21 ± 0.22^{cA}	9.06 ± 0.14^{cA}	8.34 ± 0.15^{bB}	8.44 ± 0.10^{bB}

Values represent average Log CFU/mL ± standard deviation.
NA = native alginate; PA = palmitoylated alginate; ADE = co axial air-flow droplet extrusion.
[a,b,c] Means in the same row followed by different lower case are significantly different ($P \leq 0.05$).
[A,B] Means in the same column followed by different upper case are significantly different ($P \leq 0.05$).

milk powder (Agropur, Granby, Canada), poured in 15 mL plastic tubes and then frozen for 24 h at −80 °C. Viable counts of the frozen samples were ascertained during 30 days of storage. In assays on survival to freezing, the free-cell suspensions were suspended in 100 g/kg skim milk.

2.3.3. Microencapsulation of B. longum by emulsion

B. longum were also encapsulated using a modified method of Ding and Shah (2009c). Alginate solutions (100 mL) were prepared as described above and mixed with 15 mL of 10^9 CFU/mL probiotic organisms that had been washed and concentrated in sterile 0.1% peptone water. A volume of 300 mL of sterile canola oil containing 0.2% of sterile Tween 80 was also prepared in a glass beaker and stirred for 10 min at room temperature. A water-in-oil emulsion was made by mixing the bacterial alginate cell suspension with the canola oil under stirring with a magnetic bar. After 10 min stirring, a calcium chloride solution (0.1 M) supplemented with 1 g/L Bacto peptone (Difco/BD), 1 g/L tryptone (Difco/BD) and 0.5 g/L yeast extract (Difco/BD) was gently added down the side of the beaker until phase separation occurred. Microbeads were collected by draining the oil over a sterile Whatman #1 filter paper and then rinsed with sterile 0.1% peptone water. All samples were resuspended (1:1 ratio) in a cryoprotective medium composed of 20% (w/w) rehydrated skim milk powder (Agropur, Granby, Canada) and poured in plastic tubes then frozen for 24 h at −80 °C. Viable counts in these frozen *B. longum* cultures were followed during 30 days of storage.

2.4. Laboratory-scale Cheddar manufacturing system

Cheddar cheese production in 2 L milk batches was carried out as described by Fortin et al. (2011a). The frozen probiotic cultures were thawed at room temperature and added in milk in order to achieve a 10^7 CFU/mL level. In short, 2 L of milk at 30 °C was inoculated with a H102 starter (Chr Hansen, Denmark) at 10^7 CFU/mL as well as with the free or ME cultures, also at 10^7 CFU/mL, 0.5 mL rennet was added and, the curd was cut with a stainless steel mesh having 1 cm distance between strings. The traditional steps of cooking, stirring, whey drainage, cheddarization, curd cutting and salting were then carried out. Curds were incubated at room temperature (25 °C) for 24 h, which is allowed for this product by Quebec governmental legislation, and subsequently stored at 4 °C. Sampled were taken on the production day as well as at days 1, 7, 14 and 21 of storage for pH and viable counts.

2.5. Resistance to gastrointestinal media

2.5.1. Preparation of simulated gastric and intestinal fluids

The gastric and intestinal solutions were formulated according to the United States Pharmacopeia (USP) test solutions (TS). Simulated gastric fluid (SGF) was prepared by dissolving 2 g of NaCl and 3.2 g of porcine mucosa pepsin (1100 U/mg of protein; P-7000; Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) in 900 mL of water. The pH was then adjusted by HCl (1 N; Fisher Scientific Company, ON, Canada) to obtain a final pH of 2.0. The solution was completed with water for a final volume of 1000 mL.

Simulated intestinal fluid (SIF) was prepared by dissolving 6.8 g of KH_2PO_4 (Laboratoire Mat) in 250 mL of water. Then, 77 mL of NaOH (0.2 N) and 500 mL of water were added under mild stirring. A quantity of 1.25 g of pancreatin (Sigma–Aldrich) and 3 g of bile salts (Oxgall; Sigma–Aldrich) were added to complete the solution. The pH was adjusted to 6.8 ± 0.1 with NaOH (0.2 N) or HCl (0.2 N) and the SIF was completed by adding water to obtain a final volume of 1000 mL.

2.5.2. Treatment of B. longum in SGF and SIF

The SGF and SIF were pre-incubated at 37 °C for 60 min to simulate body temperature. A quantity of 1 g of frozen microspheres with entrapped bacteria or 1 mL of frozen free cell suspensions of *B. longum* were thawed at room temperature and then mixed in 25 mL of SGF in triplicate and incubated for 90 min at 37 °C with stirring (200 rpm) using an incubator-shaker (Environmental Shaker G24, New Brunswick Scientific Co. Inc.; Edison, NJ, USA) to simulate bowel movements. After incubation, 1 mL of SGF was transferred in 24 mL of SIF in triplicate. The intestinal suspensions were incubated at 37 °C for 180 min and sampled at the end of the incubation period. The determination of surviving bacteria concentrations was performed as described below. The experiment was repeated 3 times.

2.6. Determination of viable cells counts (VCC)

Total viable cells counts (VCC) of *B. longum* were determined by a pour plate method. A quantity of 2 g of free cells or microbeads were resuspended in 98 mL of a 20 g/L sodium citrate solution at 45 °C followed by homogenization in a stomacher (Stomacher 400, Seward Medical, West Sussex, UK) for 1 min. For milk, curd or whey, 10 g of product was added to 90 mL of a 20 g/L sodium citrate solution at 45 °C. To allow a good release of bacteria in frozen microbeads, this homogenate was incubated at room temperature for 15 min and further homogenized with Omni-Tips generator probes (Omni THQ Digital Tissue Homogenizer; Omni International, Kennesaw, GA, USA) for 30 s at 27,000 rpm. Appropriate dilutions from free cells and beads dissolved in sodium citrate solution were subsequently done in sterile peptone water (1 g/L) and poured on LP agar plates which contained, per L of medium: 35 g liver infusion, 10 g lactose, 10 g bacto-peptone, 2 g NaCl, 2 g LiCl, 3 g sodium propionate and 14 g agar (Fortin et al., 2011b). Plates were incubated for 48 h at 37 °C in an anaerobic (85% N_2/10%H_2/5%CO_2) atmosphere. VCC of bifidobacteria was carried out by Darkfield Quebec Colony Counter 3330 (American Optical Company, New York, USA), expressed as log CFU/g.

2.7. Statistical analysis

Each experiment was performed independently in triplicate ($n = 3$) in a completely randomized design. All analyses and enumerations of each independent assay were done in duplicate to adjust for intra-experimental errors. Data were subjected to one-way analysis of variance (ANOVA) using the software package PASW Statistics 18.0 (IBM Corporation, Somers, NY, USA) and multiple comparisons were performed by Duncan's test. $P \leq 0.05$ was considered statistically significant. Linear regression analyses between CFU values in curds and corresponding values in whey were carried out with SigmaPlot 12 (Systat Software, San Jose, CA, USA).

3. Results and discussion

3.1. Microencapsulation of B. longum: effect of freezing

Cheese manufacturers will not prepare microencapsulated probiotic bacteria on the plant site. Rather, they would purchase the cultures from specialized suppliers. Commercial probiotics are marketed in either a frozen or freeze-dried form. Therefore, in order to assess the technological challenge the suppliers of probiotics would encounter, as well as using industry-related experimental conditions, assays were carried out on the freezing of the cultures.

The VCC of free and microencapsulated cells after freezing are shown in Table 1. Results indicated that freezing reduced the VCC of *B. longum* by 1.28 log CFU/mL in free cells. The probiotic culture used in this study is known to be sensitive to oxygen, freezing and drying (Bolduc, Raymond, Fustier, Champagne, & Vuillemard, 2006; Fortin et al., 2011b). However, the drops in VCC were much lower in the microencapsulated cells with emulsion process and negligible with the ADE system (Table 1). The beneficial effect of ME on survival to freezing is well documented and these data confirm that of the literature (Shah & Ravula, 2000; Sheu, Marshall, & Heymann, 1993).

Preliminary studies comparing PA and NA alginate and with peptone as the freeze-drying medium instead of milk, showed much greater viability losses (Data not shown). These observations suggest that the use of skim milk with porous alginate matrix can have contributed to protect the probiotic bacteria against adverse environment such as freezing, confirming the usefulness of milk in freezing and drying of cultures (Albertini et al., 2010; Yu, Yi, Lee, & Heo, 2001). On the other hand, emulsion process led to a significant ($P \leq 0.05$) lower VCC after freezing in NA and PA microbeads as compared to ADE process (Table 1). It could be hypothesized that the addition of oil during ME limited the diffusion of cryoprotectants into the bead and protecting probiotic bacteria during freezing (Gbassi & Vandamme, 2012). Homayouni, Ehsani, Azizi, Yarmand, and Razavi (2007) also reported that Tween 80 used for emulsion process has detrimental effect on the viability of probiotic bacteria.

The ADE process generates beads of approximately 500 μm (Nisco Engineering AG, 2012) while the emulsion process results in smaller particles (Ding & Shah, 2009b). Preliminary data comparing survival of the same probiotic strain in alginate beads produced by two extrusion methods which generated beads of 2 mm or 200–250 μm sizes, revealed that smaller beads enabled greater survival to freeze-drying in a peptone medium. In the present study, however, a lower survival to freezing was registered with the smaller beads obtained by emulsion. This is in line with various data which report inferior survival of probiotics in smaller particles to freezing (Sheu et al., 1993) or to gastro-intestinal stresses (Lee & Heo, 2000; Muthukumarasamy, Allan-Wojtas, & Holley, 2006). In most cases where small beads are used, the emulsion technique served to manufacture them. Therefore, it is unknown if the inferior stability of the emulsion-generated beads is solely linked to their smaller size or if the production process is involved as well.

Evidently the use of milk instead of peptone as a protective medium improved the survival level, as compared to data in a previous study (Data not shown). The ME process strongly influences the subsequent survival of *B. longum* to freezing, but palmitoylation of the alginate had a negligible effect on survival to freezing (Table 1), in agreement with previous data (Data not shown).

Table 2
Influence of storage at −80 °C on the survival of microencapsulated *Bifidobacterium longum* 15708.

Storage time (days)	Viable cell count (log CFU/mL or log CFU/g)				
	Free cells (control)	Microencapsulated			
		NA-ADE	PA-ADE	NA-emulsion	PA-emulsion
1	6.93 ± 0.16[a]	7.04 ± 0.15[a]	6.92 ± 0.20[a]	6.90 ± 0.18[a]	6.91 ± 0.10[a]
7	6.78 ± 0.09[a]	6.90 ± 0.11[a]	6.68 ± 0.16[a]	6.73 ± 0.08[a]	6.72 ± 0.18[a]
14	6.79 ± 0.07[a]	6.74 ± 0.08[a]	6.72 ± 0.10[a]	6.76 ± 0.16[a]	6.76 ± 0.10[a]
30	6.85 ± 0.14[a]	6.86 ± 0.07[a]	6.85 ± 0.10[a]	6.69 ± 0.09[a]	6.81 ± 0.05[a]

Values represent average Log CFU/mL ± standard deviation.
NA = native alginate; PA = palmitoylated alginate; ADE = co axial air-flow droplet extrusion.
[a] Values which are followed by the same letter are not significantly different ($P > 0.05$).

3.2. VCC in microbeads during storage

Results showing the survival of *B. longum* 15708 under 30 days of storage at −80 °C are presented in Table 2. Data show that the probiotic cultures were rather stable during their storage at −80 °C. There was a mean reduction of only 0.15 Log/CFU over 30 days of storage at −80 °C. No statistically significant effect ($P > 0.05$) of ME or of the type of alginate was noted on this viability loss (data not shown). Such a minimal loss in viability may be due to the use of an appropriate freezing matrix and of a low storage temperature. The appropriateness of milk as a freezing matrix for lactic cultures has long been demonstrated (Wood & Kriel, 1978) and data from this study show that this observation also applies to bifidobacteria. Furthermore, a temperature of −80 °C for storage is recommendable for lactic cultures (Thunell, Sandine, & Bodyfelt, 1984). Commercial freezers at −20 °C are inappropriate for the storage of frozen starters and probiotics, and it is advisable to be lower than −40 °C (Champagne & Møllgaard, 2008). This recommendation was followed in the experimental design of this study. However, many small cheesemaking units might not have such facilities and data on higher storage temperatures in the frozen state might be of interest.

3.3. VCC during Cheddar cheese production

Frozen *B. longum* cultures were added during Cheddar cheese production in order to evaluate the interest of ME process in industrial conditions of manufacture. Results in Table 3 show VCC of *B. longum* 15708 during Cheddar at different steps of the cheese manufacturing process. Viable counts on the concentrates had been carried out and CFUs in milk before renneting should theoretically have been identical. However, the VCC of beads prepared with NA in the ADE process were higher than those prepared by emulsion. The reason for this small difference has not been established. There are two possibilities: variations in survival to thawing and variable loss of viability upon inoculation. Since the viability issues are only minor (Table 3), it is understandable that little is known on the interaction between thawing conditions and inoculation practices in milk (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011).

Table 3
Effect of microencapsulation on viable counts of *B. longum* 15708 during cheddar cheese production.

Cheddar production step or sample	Viable cell count (VCC expressed in log CFU/mL or log CFU/g)				
	Free cells (control)	Microencapsulated (ME)			
		NA-ADE	PA-ADE	NA-emulsion	PA-emulsion
Milk before renneting	6.85 ± 0.07^{ab}	6.93 ± 0.09^{a}	6.76 ± 0.14^{b}	6.73 ± 0.04^{b}	6.76 ± 0.05^{b}
Coagulum after cutting	7.18 ± 0.22^{a}	7.10 ± 0.18^{a}	6.97 ± 0.33^{ab}	6.65 ± 0.05^{b}	6.77 ± 0.06^{b}
Whey after cutting	6.53 ± 0.10^{a}	6.00 ± 0.22^{b}	5.98 ± 0.22^{b}	5.76 ± 0.15^{b}	5.91 ± 0.17^{b}
Curds after cheddarization (before salting)	7.98 ± 0.14^{a}	7.85 ± 0.23^{a}	7.90 ± 0.19^{a}	7.53 ± 0.14^{b}	7.54 ± 0.12^{b}
Salted curds	7.77 ± 0.20^{a}	7.76 ± 0.25^{a}	7.60 ± 0.14^{a}	7.36 ± 0.22^{b}	7.44 ± 0.12^{b}
Whey draw off before salting	6.63 ± 0.09^{b}	6.77 ± 0.16^{b}	6.70 ± 0.24^{b}	6.51 ± 0.29^{a}	6.35 ± 0.25^{a}

NA = native alginate; PA = palmitoylated alginate; ADE = co axial air-flow droplet extrusion.
[a,b] In a given row, values which are followed by the same letter are not significantly different ($P > 0.05$)

Cultures prepared by the emulsification method had the lowest viable counts in the coagulum, with no apparent growth after 30 min of coagulation and cutting (Table 3). This would suggest that ME by emulsification reduced the retention of probiotics in curds, but it was not the case. Indeed, a lower recovery level in the curd would result in greater losses of cells in whey. This was not the case, since low VCC counts in whey were also observed with ME-emulsion treatment (Table 3). Increases in VCC were obtained in all cheese curds after cutting and cheddaring and before salting. This could be related to either/both growth in the curd and physical concentration of the cells in the matrix due to gel contraction. The VCC in the post-cheddarization curds were lower with the ME by emulsion (Table 3) but this was due to the lower initial VCC in the post-cutting curd. A significant correlation ($R^2 = 0.88$; $P = 0.02$) was calculated between post-cutting and post cheddaring VCC values.

Salting reduced the viability of *B. longum* and ME did not prevent this. These data confirmed the detrimental effect of salting on probiotics noted in a previous study under the same free-cell conditions (Fortin et al., 2011a). Higher VCC levels were noted in the whey draws off at salting as that after cutting the coagulum. This would presumably be due to a higher VCC level in the curd from which the whey was obtained. Again, lower VCC counts were obtained in the whey of curds containing the probiotic ME by the emulsion technology. This would suggest a link between VCC in post-cheddaring curds and VCC in whey-of during salting. However, a linear regression analysis did not find the relationship to be quite statistically significant ($R^2 = 0.62$; $P = 0.10$). Therefore, cell release in whey at salting was not solely linked to viable counts in the curd prior to salting. Presumably, the lower CFUs in wheys obtained from curds having the emulsion-based beads were not solely linked to lower CFUs in curds but might actually be associated a higher retention level in the curd of the emulsion-produced ME particles. It remains to be ascertained if this is linked to the small size of the emulsion-made particles or to the properties of the beads resulting from this encapsulation process.

3.4. Stability of VCC during storage of Cheddar cheese

The survival profiles of free and microencapsulated *B. longum* 15708 in Cheddar cheese are presented in Fig. 1. The counts of *B. longum* in all fresh curds ranged from 7.44 to 7.77 log CFU/g (Table 3). Curds were incubated at room temperature for 24 h which is allowed for this product by governmental legislation (1 day at 25 °C and subsequently at 4 °C) (Québec, 2013, chapitre P-29). After one day of storage at room temperature, plate counts for probiotic bacteria decreased significantly ($P \leq 0.05$) for cheese formulated with ADE NA and PA respectively (Fig. 1). At 7 days of storage, VCC for all cheeses was reduced by 1.5–2 log CFU/g respectively as compared to day 0. Fortin et al. (2011a) reported that high temperature (in first 24 h storage) and salting could affect the viability of *B. longum* during the first days of storage. Further reductions in VCCs were noted after 14 days of storage for all formulations (Fig. 1). Indeed, some studies showed that during exposure to continuous acidification of cheeses during storage, low values of pH would be responsible for low stability of probiotic during this period (Fritzen-Freire, Mueller, Laurindo, Amboni, & Prudêncio, 2010). However, in our study, after 21 days of storage, maximum viability was found in cheese produced with emulsified PA and NA beads, with only 2 log CFU/g reduction as compared to ADE formulation and free cells showing 3 and 4 log CFU/g reduction in cheese respectively. It could be hypothesized that lipid content in the alginate and/or residual oil from the emulsion process could be involved in the protection of probiotic bacteria.

3.5. Resistance to gastrointestinal media

Fig. 2 presents viable cell populations of the cultures during simulated gastrointestinal (GI) transit. To determine the survival of bacteria under GI conditions, the assessment of their survival was performed at the initial time ($t = 0$) and at the end of intestinal treatment. The differences between the two CFU values were analysed. A significantly higher loss in viability ($P \leq 0.05$) was observed in free cells as compared to those which were microencapsulated (Fig. 2). This may be attributed to the fact that the highly sensitive bacteria used in this study were more extensively exposed to harmful media of gastric fluid and bile salts as free cells than the microencapsulated cells. The protective effect of alginate

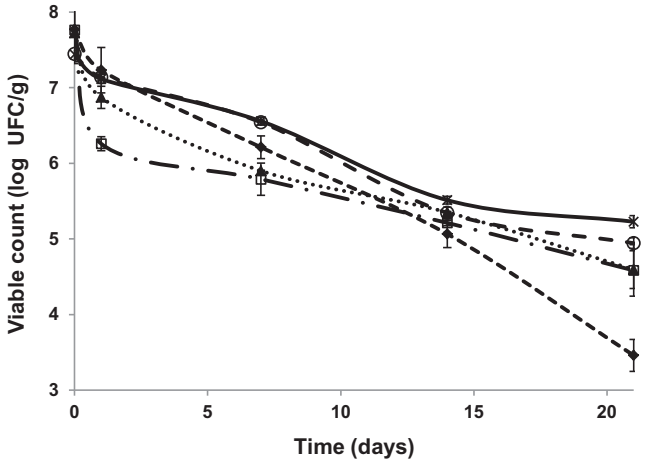

Fig. 1. Viable counts of *B. longum* 15708 in model cheese throughout storage (1 day at 22 °C and 20 days at 4 °C). (◆ – – – – – –) free cells; (□ — • —) NA-ADE; (▲ ••••••) PA-ADE; (○ — — —) NA-Emulsion; (X ————) PA-emulsion. Error bars represent standard deviation of the means.

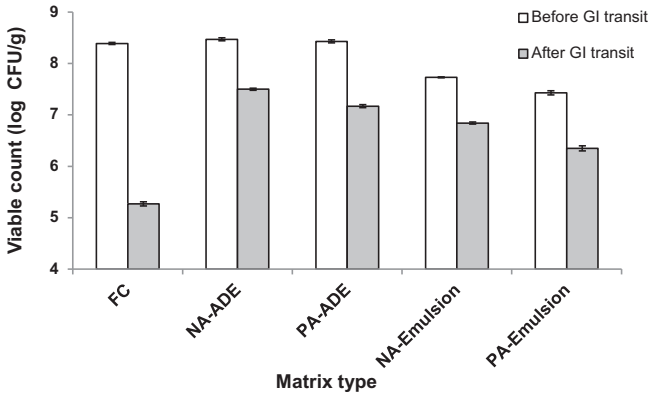

Fig. 2. Survival of free and microencapsulated *B. longum* 15708 after 1 h 30 min incubation in simulated gastric fluid (pH 2.0) and 3 h in simulated intestinal fluid (pH 6.8). Error bars represent standard deviation of the means.

ME on survival of probiotics to gastric stresses is well documented (Guerin, Vuillemard, &Subirade, 2003; Heidebach, Forst, & Kulozik, 2012; Lee & Heo, 2000) and data from this study are in line with these results. Furthermore, there was a significantly higher viability ($P \leq 0.05$) of bacteria after GI passage in NA microbeads than those prepared with the PA matrix, and for both bead production processes, with less than 1 log reduction. It seems that the presence of fatty acids in functionalized polymers (PA) did not have any influence on the protection of *B. longum* during the GI transit. Presumably, the minor losses of probiotics noted with the ME cultures could be assigned to cells remaining on or close to bead surface that are more accessible to acidic medium and bile salts. Therefore, these results suggest that ME of bacteria limited the diffusion of enzymatic and acidic environments, resulting in a higher survival rate of the cultures in alginate matrix as compared to free cells. Several studies have reported that the GI survival of probiotic bacteria increased when the probiotics were encapsulated in alginate particles (Champagne & Kailasapathy, 2008). In addition, some studies have shown that probiotic strains survived better to stressful GI conditions in a milk matrix (Lo Curto et al., 2011; Siro, Kapolna, Kapolna, & Lugasi, 2008; Tompkins, Mainville, & Arcand, 2011). These results could be related to the buffering effect of milk which could protect the strains against harmful effect of gastric and duodenal environment.

4. Conclusions

Numerous studies have examined the benefits of microencapsulation in the protection of probiotics. This study is original by the fact that the effect of two encapsulation methods and two types of alginate are compared: co-axial air flow droplet extrusion and emulsion involving two alginate polymers NA and PA. This work also has novelty in scope, which not only examined the gastro-intestinal protection of probiotics but also at two industrial applications: culture freezing for suppliers and stability in cheese or food processors.

The results generated 10 contributions to science. A first series consist on confirmation of data previously published: 1) ME improves survival of probiotics to freezing, 2) salting of the curd has a negative effect on the viability of *B. longum*, 3) ME in alginate beads improves survival of probiotics to simulated gastro-intestinal conditions.

Original information was also generated in this study: 1) ME by using ADE-produced beads enabled a higher survival of *B. longum* to freezing than those produced by the emulsion technique, 2) *O*-palmitoylation of alginate does not improve survival to freezing of the microencapsulated probiotic, 3) there is no significant difference between free and ME *B. longum* during storage of the cultures at −80 °C, 4) more extensive growth of *B. longum* occurs in the initial steps of Cheddar cheesemaking with free cells that with those in alginate capsules, 5) cell release in whey at salting is not solely linked to viable counts in the curd prior to salting, which suggests an effect of ME, 6) *B. longum* encapsulated in the PA-emulsion were more stable during storage of Cheddar than were free cells 7) there is higher viability of bacteria after passage through the in-vitro GI in NA microbeads than those prepared with the PA matrix.

From the present study it can be concluded that the use of ME is a promising strategy for maintaining viability of *B. longum* in cheese and during the gastro-intestinal transit because it benefits three areas of the sequence of use of probiotics by consumers: culture processing sector, food manufacturing sector and in the consumer itself. Alginate modification appears to be a novel approach and this polymer could be use for preserving the viability of some probiotics in food matrix. However, further research on these issues still need to be carried out in order to develop probiotic-containing cheese with highly sensitive cultures such as *B. longum* 15708. Indeed, although there were about 100 times lower viability losses with the best ME culture during the four technological steps that lead to consumption, there was still a loss of over two logs in CFU after the 21 day storage. This is still too high for commercial use which should presumably not be greater that 1 log CFU.

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