Viability of Bifidobacterium longum in cheddar cheese curd during manufacture and storage: effect of microencapsulation and point of inoculation

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ORIGINAL PAPER

Viability of *Bifidobacterium longum* in cheddar cheese curd during manufacture and storage: effect of microencapsulation and point of inoculation

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Abstract The goal of this study was to assess the effect of methods of inoculation on the viability of probiotic bacteria during cheddar cheese manufacture as well as their stability during storage. *Bifidobacterium longum* ATCC 15708 was freeze-dried and microencapsulated by spray-coating. The effect of inoculation of free whole cell or microencapsulated cells at three points during manufacture (milk before renneting, at cheddaring or at salting) on the viable counts in cheese and whey was investigated. Microencapsulation had no effect on viable counts, chemical parameters (lactose, lactic acid, total nitrogen, nitrogen soluble in TCA, moisture) or sensory properties during manufacturing or storage of the fresh cheeses for 14 days. Inoculation of the bifidobacteria in milk before renneting resulted in higher viable counts in comparison to other points of inoculation. Bifidobacteria added at the salting step, which survived pressing, were subsequently more stable during storage than those inoculated in milk. The stability of *B. longum* 15708 during storage was greater in the pressed cheeses that in the free curds. The results of this study provides technological data for cheese makers on the optimum point of inoculation as well as

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the benefit of pressing the curds in order to ensure high levels of probiotics in fresh cheddar cheese.

微胶囊和接种时段对长双歧杆菌 (*Bifidobacterium longum*) 在切达干酪中活菌数的影响

摘要 本文主要目的是分析益生菌的接种方法对其在新鲜切达干酪中活菌数以及对益生菌贮藏稳定性的影响。以冷冻干燥和喷雾涂布微胶囊化的长双歧杆菌ATCC 15708 (Bifidobacterium longum) 为目标菌株，将游离细胞和微胶囊的长双歧杆菌ATCC 15708分别在干酪加工的三个阶段 (凝乳前、凝块切割和加盐) 进行接种， 研究了不同的接种过程对长双歧杆菌ATCC 15708在干酪和乳清中存活数的影响。干酪的加工过程以及新鲜干酪贮藏 (14d) 对微胶囊化长双歧杆菌ATCC 15708在干酪中活菌数没有产生影响， 并且对干酪的化学成分 (乳糖、乳酸、总氮、三氯乙酸可溶性氮和水分) 以及感官特性也没有影响。比较了长双歧杆菌分别在凝乳前、凝块切割和加盐过程接种的三组干酪，在凝乳前接种的干酪中长双歧杆菌的活菌数最高。而在加盐过程中接种的干酪， 由于长双歧杆菌经历了高盐渗透压的作用， 使得这些长双歧杆菌在贮藏期间的稳定性较高。长双歧杆菌ATCC 15708在压榨后干酪中的稳定性比在鲜凝块中高。该研究可为干酪生产者在实际生产过程中有效的添加益生菌提供了技术支持。

Keywords Probiotic bacteria · Stability · Microencapsulation · Cheddar cheese manufacturing · Inoculation point · Storage

关键词 益生菌· 干酪加工· 接种时间· 微胶囊· 稳定性· 贮藏

1 Introduction

The market for products containing probiotic bacteria is in expansion throughout the world principally because of the growing number of clinical studies concluding that these microorganisms may be linked to beneficial health effects (Ouwehand et al. 2003). There is a growing consumer interest for functional foods enriched with these bacteria, in preference to powder supplements or pills (Bruhn et al. 2002). Although yoghurt has been the main food matrix to which probiotics have been incorporated, cheese is increasingly considered as a valuable delivery vehicle (Gomes da Cruz et al. 2009).

Cheddar cheese is most commonly consumed in the form of pressed blocks of ripened cheese. A niche market in Canada is non-pressed salted cheddar curds obtained after milling. Immediately after manufacturing, these curds are placed in bags and shipped to retailing points, which are allowed to place the product on the shelf at room temperature for 24 h. Subsequently, the cheese curds must be refrigerated. This type of product is typically consumed within 1 week. In some cases, the curds are pressed and also sold immediately under the same conditions. It was our hypothesis that such a fresh product would constitute a good matrix for highly sensitive probiotic bacteria which otherwise loose viability during the ripening period of cheddar. This hypothesis constitutes the rationale for the manufacturing and storage conditions used in this study as well as for the selection of a highly sensitive probiotic strain.

In cheddar cheese, many studies report severe declines in viable counts of probiotics during storage (Godward and Kailasapathy 2003; Lynch et al. 1996; Sharp et al. 2008), but some studies report more successful results (Daigle et al. 1999;

Stanton et al. 1998). Viability losses seem to be mostly related to the probiotic strain (McBrearty et al. 2001; Ong et al. 2007). In the past, manufacturers used to select probiotic cultures which were stable in their particular product. However, strain selection is increasingly based on purported health benefits, and a desired culture might show viability problems during manufacture and storage. Thus, there is still a need to develop technological strategies to protect probiotic bacteria in cheddar cheese.

Technologies can be adapted to prevent viability losses of probiotic bacteria in foods. In yoghurt, such adaptations include carrying out lactose hydrolysis, modifying the starter strains and inoculation levels, adding antioxidants, packaging in anaerobic environments and microencapsulating the probiotic cultures (Champagne et al. 2005; Stanton et al. 2005). Much less has been done in this area in cheese, but selection of compatible starter, probiotic inoculation practices, microencapsulation and packaging (Gomes da Cruz et al. 2009) have been proposed. In most studies, only one treatment has been applied at a time, and little data are available on combined treatments. There is a need to examine interactions between technological adaptations on the survival of probiotics in cheese. In this study, it was hypothesized that targeting a fresh cheese, using a novel microencapsulation technology and carrying out novel inoculation points, would provide such technological tools. Furthermore, the combined effects of microencapsulation and inoculation practices were examined.

Microencapsulation has been suggested as a potential solution to losses in viability due to salting or extended storage (Gomes da Cruz et al. 2009). In most cases, the microencapsulation technology was based on microentrapment in gel particles, particularly alginate. Contrary to yoghurt, microencapsulation of probiotics in alginate beads does not seem to improve their stability during cheese storage or ripening (Gobbetti et al. 1998; Godward and Kailasapathy 2003). Although microencapsulation by spray-coating is the main technology used by the industry, only one study has examined the benefits of this microencapsulation methodology on bacterial stability during storage. There is no data on the benefits of microencapsulation by spray-coating on stability of probiotics during cheese processing or on cell retention in the curds.

Inoculation practices are an important means of adapting a milk fermentation process to enhance probiotic survival. Most data on the benefits of inoculation strategies are found in yoghurt manufacture, and little is known in cheesemaking. In cottage cheese, it was suggested to add the probiotic culture in the cream dressing rather than in the curds (Blanchette et al. 1996). Other studies have examined inoculation at the cheddaring step or at salting (Dinakar and Mistry 1994; Fortin et al. 2011; Gomes da Cruz et al. 2009). With free cells, these inoculation practices raise the concern of cell losses in whey. However, no study has examined the effect of microencapsulation by spray-coating on cell recovery and stability in the cheese curds under various inoculation procedures.

The goal of this study was to examine the combined effects of microencapsulation of *Bifidobacterium longum* by spray-coating and the processing step chosen for inoculation on the viable counts of the bifidobacteria in cheddar cheese curds during production and storage. The effects of these technological adaptations on cheese composition and sensory properties were also examined.

2 Materials and methods

2.1 Cultures

B. longum ATCC 15708 was purchased from the American Type Culture Collection. Stock cultures were obtained by mixing MRS-grown (Difco, 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 the cell suspension in Cryovials (Nalgene, Rochester, NY, USA) and freezing at −80 °C.

The starter used for pilot-scale cheese manufacture was prepared by inoculating 0.2% (*w/w*) of a thawed commercial culture of lactic acid bacteria (*Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* H-102 from CH-Hansen, Milwaukee, WI, USA) 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 and used within 2 h after this incubation time.

2.2 Production of freeze-dried and microencapsulated probiotic cultures

B. longum was grown in MRS broth (Becton, Dickinson and Company, France) 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). The culture was concentrated by centrifugation at $6,000 \times g$ for 20 min at 4 °C. The cell pellet was resuspended in one tenth of its original volume in a cryoprotective medium composed of 20% (*w/w*) rehydrated skim milk powder (Agropur, Granby, Canada) and 5% (*w/v*) of sucrose to which 2% (*v/v*) of an ascorbic acid solution (17.5% *w/v*) was added. The cell suspension was poured in metal trays and frozen at −20 °C for 18 h. Trays were placed in a freeze-dryer (FTS Systems, Stone Ridge, N.Y., USA) to lyophilise under the following programme: −40 °C for 4 h under atmospheric pressure, 16 h at 0 °C and 100 mTorr vacuum, 16 h at 20 °C and 100 mTorr vacuum, 60 h at 20 °C and 10 mTorr vacuum. The powder was grinded using a Ultra Centrifugal Mill ZM-1 unit (Retsch Inc. Newtown, PA, US) equipped with a sieve size of 1 mm and then filtered in stainless steel mesh (W.S. Tyler Canada Ltd, St. Catharines, Ontario, Canada) to only retain the particles sizes varying between 53 and 250 μm. The powders were subsequently placed in hermetic glass bottles and kept at 4 °C The final powder had a concentration of 2.2×10^9 CFU.g^{-1} and this product was referred to as the "free-cell culture".

Microencapsulation was carried out by spray-coating as described by Durand and Panes (Durand et al. 2003) in a STREA-1 fluid bed system (GEA, Columbia, MD, USA) equipped with a bottom-coating Würster vessel assembly. The air used for fluidization was previously dried (relative humidity of approximately 5%) and injected at room temperature. The spraying air was injected at a velocity of 30 L. min^{-1}. The fat used for coating was the DP108 blend of fractionated palm kernel oil and palm oil from Aarhus United (Port Newark, NJ, USA). The fat was heated at 80 °C and distributed at 10 mL.min^{-1} over 400 g of the free-cell culture. In all, 160 g of fat was sprayed over the 400 g of powder. The resulting spray-coated product of *B. longum* ATCC 15708 will be referred to as the "ME culture". The viable count was of 1.1×10^9 CFU.g^{-1}. This was half the bacterial density of the free-cell culture

(per g of powder) because of the addition of fat and because a small loss of viability occurred during the spray-coating process. As a result, a greater quantity of ME culture powder was required to carry out the same CFU inoculation level as the free cell culture.

2.3 Cheese production

Cheese vats having a 270-L capacity equipped with double-wall, adjustable outlet and stirrer blades (Kusel Equipment Co., Watertown, WIS, USA) were used. Raw milk was provided by Agropur (Granby, QC, Canada). On each production day, milk was pasteurized using a continuous flow plate exchanger at 73 °C for 16 s.

The starter was inoculated at 1.5% (*w/w*). Addition of 0.026% (*v/v*) of a 30% (*w/v*) CaCl$_2$ solution and 0.01% (*v/v*) double force rennet Maxiren (Danisco, Copenhague, Danemark) was done 1 h after starter addition. The coagulum was cut after 30 min and curds were cooked at 38 °C until pH reached 6.0 to 6.1. After whey drainage, curds were piled in order to carry out the cheddaring step, until the pH dropped to 5.2±0.1. Finally, the cheese was cut in small pieces and salted at 1.8% (*w/v*) with iodine-free salt, which resulted in a salt-in-humidity level of 4.5%. Some free curds were kept separately, and the rest of the salted cheddar curds were then put in moulds and pressed under 0.205 MPa for 1 h. During cheese making, temperature, pH and titratable acidity were followed at defined intervals for ascertain reproducibility of the fermentation. The packaged blocks were then stored at 4 °C for up to 14 days. At days 1, 4, 7 and 14, bags were opened and 500 g portions were taken for chemical, microbiological or sensory analyses.

2.4 Incorporation of probiotics during cheese manufacturing

Since three cheese vats were available and since seven treatments were carried out, two series of productions were carried out. In the first series, the following treatments were done: vat nos. (1) inoculation of 20 g free cells in 150 L of milk (0.13%), just before rennet addition, (2) inoculation of 40 g ME cells in 150 L of milk (0.27%), before rennet addition, (3) no inoculation in milk nor at the cheddaring step; the unsalted curds obtained after milling were divided in three; at the salting step, one third was kept as control, one third was inoculated with 6.7 g of free cells and one third was inoculated with 13.4 g of the ME culture. In the second series of productions, the same pattern was carried out except that for vats #1 and #2, instead of inoculation in milk prior to rennet addition, the free (20 g) or ME (40 g) cultures were blended into the small grains obtained after the first whey drainage prior to cheddarization.

These inoculation levels were expected to result in curd viable counts around 10^6 CFU.g^{-1}, which is rather low since the laboratory-made culture obtained had only 2.2×10^9 CFU.g^{-1}. Such a viable count in a powder is much lower than commercial products, which typically have between 10^{10} and 10^{11} CFU.g^{-1} of powder. In the attempt to duplicate industrial conditions, it was decided to add the quantity of culture powder that would apply under commercial conditions. Thus, the quantity of bifidobacteria-containing powder added at the various steps followed the quantities of freeze-dried powder that would currently be inoculated in industry. It was considered that the quantity of powder added would affect distribution

properties in the curds as well as texture and that applying industrial parameters was critical to the value of the experimentation. Although viable counts in cheese were lower than what would be expected or commercially recommended, it still enabled the evaluation of the hypotheses.

In each assay, the pasteurized milk was distributed in three cheese vats. In experimental treatments based on inoculation in milk or at cheddaring step, vats filled with 150 L of milk were inoculated with free cell or ME *B. longum*. For the treatment of addition at salting step, a vat was filled with 270 L of milk, and fabrication was followed normally without inoculation of bifidobacteria until salting step. Curds were salted all together and then divided in three equal masses: one third was inoculated with free bacteria, one third with ME bacteria and the other was not inoculated and represented the control treatment. Three independent repetitions were done using three separate culture batches and milk lots.

One of the aims of this study was to examine the effect of air on *B. longum* viability in products having free and ME cells. Thus, individual salted curds and pressed cheese blocks were analyzed separately, except for inoculation at the salting step where only blocks were analyzed. In this latter treatment, the decision was taken not to test curds, because powder particles might not adhere to the surface, and cells would be lost in the packaging.

The individual curds (portions of approximately 250 g) were kept in sealed plastic bags in an air atmosphere. They were placed at 23 °C for 24 h and then stored at 4 °C for up to 14 days. A portion of the curds was pressed in 10 kg blocks that were subsequently cut and vacuum-wrapped in 500 g portions and kept for 16 h at 23 °C.

2.5 Analyses

Milk, curds and whey were analyzed for viable counts throughout cheese manufacturing as well as at days 1, 4, 7 and 14 of storage. For microbiological analyses, homogenization of the casein matrix was carried out by adding 4 g of curds to 36 g of a 2% sodium citrate solution (Anachemia, Montreal, Canada) kept at 45 °C and blending them with sterilized Polytron-type high-shear generator probes (OMNI TH International, Model TH-115, Marietta, GA, USA) for 30 s at high speed (20,000 rpm). When liquid samples were analyzed, 1 mL of milk or whey were mixed with 9 mL of sodium citrate solution and homogenized with Omni-Tips generator probes (Omni TH unit; Marietta GA, USA) at high speed for 30 s. For both liquid and solid samples, serial decimal dilutions were done in 0.1% peptone water tubes (*w/v*) (Becton Dickinson, Mississauga, ON, Canada). Viable counts of *B. longum* were determined by plating on LP medium pH 6.7 since this medium was found to effectively select *B. longum* from lactic acid bacteria (Lapierre et al. 1992). The LP medium contained, per L of medium: 35 g liver infusion, 10 g lactose, 10 g bacto-peptone, 2 g NaCl, 2 g LiCl and 3 g sodium propionate. The appropriateness of LP medium for selective counts of *B. longum* 15708 in whey and curds, in the presence of the cheese starter cultures, was confirmed in a previous study (Fortin et al. 2011). Colonies of bifidobacteria were counted after a 48 h incubation period at 37 °C in an anaerobic incubator ($85\%N_2/10\%H_2/5\%CO_2$). Lactococci populations from the starter were obtained on M17 medium (Becton, Dickinson and Company, France) following 48 h incubation at 30 °C under aerobic conditions.

Carbohydrates and organic acids of interest (acetic acid, lactic acid, glucose, lactose) were analyzed at days 1 and 14 by ion exchange HPLC (Dionex, Model DX-500; Oakville, ON, Canada), following the procedure of St-Gelais et al. (1991). Total protein and soluble nitrogen in trichloroacetic acid (TCA-SN) were analyzed at days 1, 7 and 14 with the Kjeldhal technique (Christensen et al. 1991; Turcot et al. 2002), using a Kjeltec 1030 distillation unit (Foss, Eden Prairie, MN, USA). For practical reasons, not all analyses could be done on day 1. Since cheeses were packaged in sealed bags, some parameters were not expected to change throughout the 14-day storage period. Thus, total lipid concentrations were analyzed at day 7 by the Mojonnier technique (Mojonnier Bros. Co., Chicago, IL, USA) (Atherthon et al. 1977), salinity was ascertained at day 6 with a Chloride Analyzer 326 unit from Corning (Nelson-Jameson Inc., Marshfield, WI, USA) and humidity was measured at day 6 by gravimetry following drying at 100 °C for 24 h (Fortin et al. 2011).

A sensory analysis was also carried with 20 panellists at the 4th day of storage. The sensory analysis was delayed a few days in order to carry out microbiological analyses (coliforms, *Staphylococcus aureus*) ensuring safety for the tasters. This was a requirement from the ethics committee. Tasters had to smell and taste pressed cheese only and compare it to the control cheese in order to monitor any difference in appearance (particles of ME cultures), texture, mouth-feel and flavour between the various pressed cheeses produced. Free curds were not tested by the sensory panel.

2.6 Calculations

On average, from 100 L of milk, we obtained 83 L of whey at the first drainage, 5 L of whey during cheddarization and 1 L of whey during pressing. Final cheese curd mass varied between 10 and 11 kg. The CFU were measured at every processing step, which enabled the estimation of total bacterial populations in the whey and curd products.

The following equations were used to evaluate cheese yield and bacterial balances.

Equation 1.   Yields for cheese production:

$$[(\text{Pressed cheese final mass} + \text{Curds final mass}) \div (\text{Milk mass})] \times 100$$

Equation 2.   Ratio of cells recovered to those inoculated (ratio R/I)

$$\left[\left(\text{g of final curd mass} \times \text{CFU.g}^{-1} \right) + \left(\text{g of whey recovered} \times \text{CFU.g}^{-1} \right) \right]$$
$$\div \left[\left(\text{g of culture added} \times \text{CFU.g}^{-1} \right) \right]$$

Equation 3.   Distribution of probiotics between curds and whey:

$$\left[\left[(\text{Final curds mass}) \times (\text{CFU.g}^{-1} \text{of probiotics in curds}) \right] \right.$$
$$\div \left[((\text{Final curds mass}) \times (\text{CFU.g}^{-1} \text{of probiotics in curds})) \right.$$
$$\left. + ((\text{Total whey mass}) \times (\text{CFU.g}^{-1} \text{of probiotics in whey})) \right]\right] \times 100$$

It must also be kept in mind that the distribution of cells between curds and whey (Equation 3) is only an indicator of a percentage or recovery in curds; indeed CFU

values in whey and cheese at the end of processing are the result of cell recovery (or loss in whey) as well as potential growth or viability losses in each matrix.

2.7 Statistical analyses

Statistical analyses were done with InStat (GraphPad, La Jolla, CA) on Log10 values of viable counts. When comparing multiple treatments, the ANOVA test was carried out. In some instances where the specific effects of ME or pressing were examined, paired *t* test were used. Differences between means were considered significant when *P* value was lower or equal to 0.05.

3 Results and discussion

A preliminary study had been carried out on a laboratory scale (2 L) where inoculation points of probiotics in milk and at cheddaring had been compared (Fortin et al. 2011). This study expanded the preliminary work by verifying the observations on a 100-fold larger scale, by including a microencapsulated culture, by adding the cells at salting and by examining the effect of curds pressing.

3.1 Effect of ME on viable counts during production

Spray-coating efficiency in delaying the release of probiotic cells was studied in a previous work (Champagne et al. 2010), and its application to this condition was first examined. This was carried out by evaluating the rates and levels of solids released in water after addition of the powder. Commercial powders of ME cultures obtained from spray-coating technology show ~50% of their non-fat solids released after 15 min dispersion, while the ME powders prepared in our lab released 85% of their non-fat solids (Champagne et al. 2010). Therefore, the ME culture prepared in this study was not as well coated as that of commercial products. It is well known that spray-coating is a difficult technology to master and that the products are only partially microencapsulated. It was tested nevertheless since viability losses can reach four logs during storage (Fortin et al. 2011) and partial ME could still have a significant benefit, especially since ME protects cells against oxygen (Talwalkar and Kailasapathy 2004), and *B. longum* 15708 is sensitive to oxygen (Bolduc et al. 2006; Fortin et al. 2011).

When ME cultures were incorporated into milk before renneting, greater CFU losses in whey were observed at most steps during the production cycle than with the free-cell cultures (Table 1). A greater loss of cells in whey would suggest that lower CFU values would be obtained in the curds ultimately resulting from milk inoculation with the ME culture. However, viable counts in curds were not significantly affected by the form of the probiotic inoculated (Table 1). This is explained by the observation that between 64% and 72% of the cells inoculated in milk are recovered in curds (Table 2). Consequently, the fraction that ends up in whey is only a minor portion of the cells added; as a result, the different cell losses in whey did not ultimately affect the viable counts in the curds. When the ME culture was inoculated in milk, it was observed that particles were floating at the surface of

Table 1 Effect of microencapsulation (ME) and of the point of inoculation on viable counts of *B. longum* 15708 (Log CFU.mL^{-1}or .g^{-1}) during cheddar cheese production

Cheddar production step or sample	Point of inoculation					
	Milk		Cheddaring		Salting	
	Free cells	ME cells	Free cells	ME cells	Free cells	ME cells
Milk before renneting	5.3 [a]	5.2 [a]	–	–	–	–
Coagulum before cutting	5.1 [a]	5.9 [a]	–	–	–	–
Whey after cutting	4.8 [a]	6.2 [b]	–	–	–	–
Coagulum after 30 min cooking	5.6 [a]	5.8 [a]	–	–	–	–
Whey after 30 mincooking	4.9 [a]	5.3 [a]	–	–	–	–
Curds after drawing off whey	6.3 [a]	6.2 [a]	–	–	–	–
Whey draw off	4.7 [a]	5.4 [b]	–	–	–	–
Curds after inoculation of probiotics	–	–	5.6 [a]	5.6 [a]	–	–
Curds after 30 min cheddaring	6.3 [a]	6.6 [a]	5.9 [a]	5.6 [a]	–	–
Residual whey at cheddaring	3.9 [a]	4.0 [a]	6.6 [b]	6.2 [b]	–	–
Curds before salting	6.8 [a]	6.7 [a]	5.9 [b]	5.8 [b]	–	–
Salted curds	6.6 [a]	6.8 [a]	5.4 [b]	5.6 [b]	5.3 [b]	5.6 [b]
Residual whey after pressing	4.6 [a]	4.9 [ab]	4.7 [ab]	5.3 [bc]	5.7 [c]	5.9 [c]
Cheese after pressing	6.6 [a]	6.7 [a]	5.6 [b]	5.0 [b]	5.0 [b]	5.2 [b]

In a given row, values which are followed by the same letter are not significantly different ($P \geq 0.05$)

milk and oil at the surface of whey. This could partially explain the higher losses of the ME cells in whey. There was no further effect of ME on viable counts in whey or curds when the inoculation was carried out at cheddaring or at salting (Table 1).

Irrespective of the distribution of the viable cells between curds and whey, calculations were made to examine the evolution of total bacterial populations during the processing steps. As the cells recovered to those inoculated (Ratio R/I) show, in many cases, there were lower CFU counts in whey and curds at the end of

Table 2 Effect of the point of inoculation on cell distribution and on the evolution of the ratio of *B. longum* 15708 cells recovered to those inoculated (Ratio R/I) during Cheddar cheese production

Production parameter		Viable counts	
Curds	Point of inoculation	Distribution (% of probiotics in curds)[a]	Ratio (R/I)[b]
Pressed cheese	Milk	72 [a]	1.7 [a]
	Cheddaring	18 [b]	1.1 [ab]
	Salting	83 [a]	0.12 [b]
Free curds	Milk	64 [a]	1.4 [a]
	Cheddaring	12 [b]	0.98 [ab]

For a given column, means that are followed by the same letter are not significantly different ($P \geq 0.05$)

[a] Calculated with Equation 3

[b] Calculated with Equation 2

the cheddar manufacture than there were bifidobacteria cells inoculated (Table 2). This would suggest viability losses during production. There was no statistically significant effect of ME on the growth or mortality of the probiotic bacteria during cheddar manufacture ($P < 0.05$).

3.2 Effect of point of inoculation on viable counts during cheddar manufacturing

When *B. longum* cultures were inoculated in milk, higher viable counts were obtained in cheese curds than when inoculation was carried out at cheddaring (Table 1). These data confirmed the results from a previous study (Fortin et al. 2011).

In addition to the viability losses, which occurred immediately at the moment of inoculation, there might also be continued viability losses during the final processing steps. Indeed, when the probiotic bacteria were added in milk, there was an increase in curds CFU counts of about 0.2 log between the "30 min after cheddaring" and in the "salted curds" (Table 1). No such increase was noted when the bifidobacteria were added at cheddaring, and in fact, a 0.5 log decrease was even noted in one instance (free cells; Table 1). Since curds expel whey and contract during cheddaring, an increase in CFU would normally occur due to gel contraction. All these data point to a loss of viability of the cells inoculated at cheddaring during the final processing steps and particularly at salting (Table 1). The detrimental effect of salting on viability had already been observed (Fortin et al. 2011), but this is the first observation on the effect of inoculation point on subsequent viability during processing. Apparently, the potential benefit of reducing exposure to oxygen by adding the cells at a later processing stage was outweighed by the detrimental effects of rehydration in more acidic and salty environments. The literature reports variable situations with respect to viable counts of bifidobacteria added during cheddar cheese making. In one instance, no growth was noted (Daigle et al. 1999), as in this study, while in other instances, significant CFU increases occurred (Ong et al. 2007). Evidently, this is strain-related, and the fact that we selected an oxygen-sensitive culture (Bolduc et al. 2006; Fortin et al. 2011) might explain our results.

Little information is available on the recovery level of probiotics in curds. The more cells are entrapped in the curd, the less is lost in the whey. Ong et al. (2007) observed differences between CFUs in whey and those in curds ranging from 0.6 and 4.6 log with an average of 1.8 log CFU. Data from this study tend towards a 2 log CFU difference (Table 1), which is therefore in line with the literature.

Data show that the highest proportion of cells in curds was observed when they were added at salting (Table 2). This would erroneously suggest that the best inoculation point for high cell recovery in curds is at salting. This is not the case. High losses in viability occur at this inoculation point as evidenced by the lowest R/I ratio (Table 2). The high % of viable cells in pressed curd is presumably due to the very small quantity of whey produced during pressing (about 1% of original milk volume) as well as higher loss of viability of the probiotic culture during rehydration in this salted and acid whey.

3.3 Viable counts during storage

Microencapsulation in alginate beads was shown to reduce viability losses due to oxygen in yoghurt (Talwalkar and Kailasapathy 2004), and it was hoped that ME by

spray-coating would also reduce viability losses during storage by reducing the exposure to both acidity and oxygen. Paired *t* tests between comparative viability data during storage showed that there was no significant difference ($P=0.08$) in viability patterns between products inoculated with free or ME cultures. This was in line with the data of Belvis et al. (2006). The limited effect of ME might be due to the low encapsulation level of the product used in this study, which was estimated to be less than 10% (Champagne et al. 2010). Further studies are required to determine the benefits of using encapsulated cultures similar to those currently marketed by industry, which have up to 50% coating efficiency.

In the province of Québec (Canada), a fraction of cheddar cheese is marketed as fresh curds. The cheese curds are packed in plastic bags and marketed fresh, instead of being pressed and sold in blocks. Viable counts of these two products during storage showed important viability losses of *B. longum* 15708 (Fig. 1). They were much higher when curds were not pressed into typical cheddar blocks but packaged in plastic bags as free curds; paired *t* tests showed this difference to be statistically significant ($P=0.002$). A lower oxygen level is expected in pressed curds, which were subsequently packaged under vacuum than in the free curds packed in plastic bags. The probiotic strain used in the present study is sensitive to oxygen (Bolduc et al. 2006; Fortin et al. 2011), and it is presumed that exposure to oxygen was much lower in pressed cheese. In addition, the level of salt in moisture was higher in the free curds than in the corresponding pressed cheese ($P\leq0.01$ for both inoculation points) (Table 3). It has been shown that the stability of this strain was lower in salted cheese than in unsalted curds (Fortin et al. 2011). It must be kept in mind that the salt distribution in the cheese matrix varies initially. During the first hours following salting, it is high at the surface of the grains and it gradually decreases as the minerals diffuse towards the core. It could be argued that cells at the surface of the grains suffer a salt shock. It is unknown if the effect of salt on viability which was observed is linked to these initial differences in salt distribution throughout the food matrix.

The effect of the point of inoculation on subsequent stability during storage is not clear. A reduction of 0.4 log CFU.g^{-1} during pressing was noted in products inoculated at salting while no such loss in viability was noted in cheeses inoculated

Fig. 1 Effect of pressing of curds on the subsequent viability of *B. longum* 15708 during storage. *Unfilled circles* Free cheese curds; *filled circles* pressed cheese. Results obtained from free cell and ME cultures added to milk or during cheddarization were averaged. *Error bars* represent standard error of the means
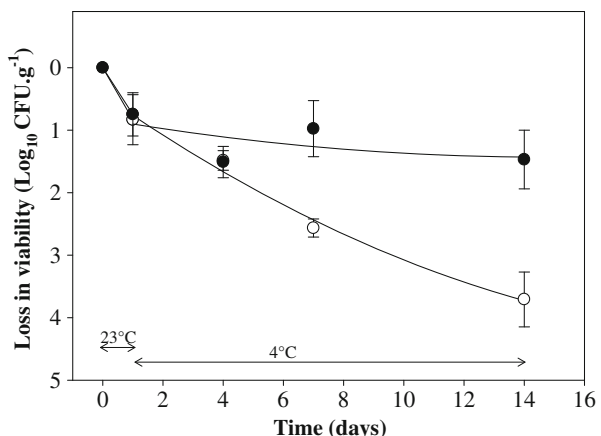
Table 3 Effect of the point of inoculation of *B. longum* 15708, the pressing of curds as well as storage time on some cheese chemical characteristics
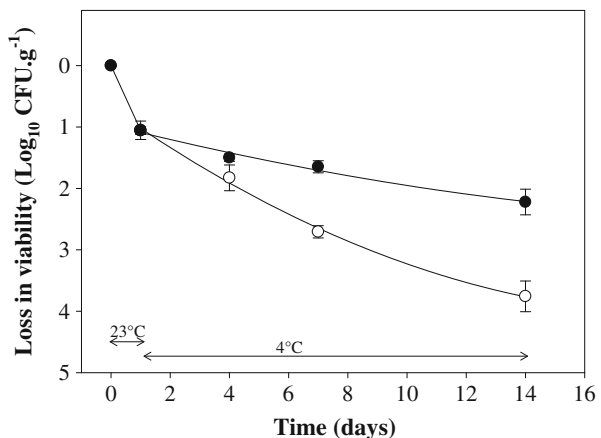
%	Day	Pressed cheese Control	Pressed cheese inoculated at			Free curds inoculated at	
			Milk	Cheddaring	Salting	Milk	Cheddaring
WSN/TN	1	8.0 [a]	7.9 [a]	7.4 [a]	7.4 [a]	9.2 [a]	8.1 [a]
	7	9.7 [a]	9.9 [a]	9.5 [a]	9.3 [a]	10.6 [a]	10.8 [a]
	14	12.7 [a]	12.4 [a]	11.5 [a]	12.4 [a]	13.7 [a]	14.0 [a]
TCA-SN/TN	1	4.6 [a]	4.5 [a]	4.1 [a]	4.1 [a]	4.7 [a]	4.3 [a]
	7	5.3 [a]	5.3 [a]	5.6 [a]	5.3 [a]	5.7 [a]	5.7 [a]
	14	6.4 [a]	6.0 [a]	6.7 [a]	6.5 [a]	6.2 [a]	6.8 [a]
TN	7	23.8 [a]	23.1 [b]	24.0 [a]	23.8 [a]	21.7 [c]	22.1 [c]
Moisture	6	37.9 [a]	38.8 [a]	37.4 [a]	38.2 [a]	42.0 [b]	41.4 [b]
Salt in moisture	6	4.0 [a]	3.6 [a]	3.9 [a]	4.1 [ab]	4.4 [b]	4.6 [b]
Total lipids	7	32.8 [a]	31.4 [a]	32.7 [a]	32.6 [a]	28.6 [b]	29.8 [b]
Lactose	1	0.46 [a]	0.38 [a]	0.41 [a]	0.40 [a]	0.45 [a]	0.43 [a]
	14	0.40 [a]	0.29 [a]	0.29 [a]	0.27 [a]	0.31 [a]	0.34 [a]
Lactic acid	1	0.99 [a]	1.05 [a]	1.07 [a]	1.00 [a]	0.98 [a]	0.99 [a]
	14	1.20 [a]	1.16 [a]	1.18 [a]	1.13 [a]	1.08 [a]	1.09 [a]

WSN water-soluble nitrogen, *TN* total nitrogen, *TCA-SN* nitrogen soluble in trichloroacetic acid

For a given row, means that are followed by the same letter are not significantly different ($P \geq 0.05$) using ANOVA. The average values presented are from the combined data of free and ME cultures

in milk (Table 1). However, during storage, the products inoculated at salting were more stable than those inoculated in milk (Fig. 2), and paired *t* tests showed this difference was statistically significant ($P=0.03$). A previous study had also shown that cells added at salting were stable during storage (Dinakar and Mistry 1994), but no comparison with other points of inoculation had unfortunately been carried out. The cultures inoculated at the cheddaring step basically had the same pattern as

Fig. 2 Effect of the point of inoculation of *B. longum* 15708 during manufacture on the culture's stability during cheese storage. *Unfilled circles* Inoculation in milk before renneting; *filled circles* inoculation at salting, prior to pressing. Data are the average of results obtained with free cell and ME cultures, as well as those from curds and pressed cheeses. *Error bars* represent standard error of the means

those inoculated at salting (data not shown). Therefore, different patterns in viability losses were noted as a function of inoculation point and storage period. More data are needed to clarify these observations.

A common observation was the high loss of viability during the first day of storage (Figs. 1 and 2) likely due to the higher temperature (23 °C). It has frequently been observed that refrigeration improves the stability of probiotics during storage (Champagne et al. 2005). This distribution and marketing practice is carried out to obtain desirable texture and flavour of the curds. Clearly, however, the 24-h room temperature storage practice provides a challenge in maintaining probiotics viability. Interestingly, other studies without this particular storage pattern also show initial viability losses, which are then followed by stabilization (Daigle et al. 1999) or even growth (Ong et al. 2007). Therefore, although the high storage temperature on day 1 might have accelerated the initial loss in viability of the bifidobacteria, high rates of viability losses during the first few days or weeks of ripening/storage seem to be a common occurrence in cheddar.

The fresh curds are typically consumed within 1 week, while the pressed cheese can be stored and marketed over many weeks. Whatever the product, fresh or pressed, this high initial viability loss limits the application of probiotics to cheese with such a strain. The Canadian Food Inspection Agency (2009) requires that one billion (10^9) viable cells per portion be present in the product when consumed in order to allow a general non-strain-specific claim. Even considering a cheese portion to be 50 g, none of the experimental conditions used in this study (ME, inoculation point) enabled reaching this population level. Therefore, strain selection, inoculation in milk and high inoculation level still appear to be the best methods of achieving high viable counts of probiotics in cheddar.

3.4 Effect of inoculation methods on the chemical composition of cheeses

Yields in cheese varied between 10.3 and 10.7 kg cheese per 100 L of milk, but these differences were not found to be statistically significant. Cheddar cheese productions typically give yields around 9.5% (Mahaut et al. 2000). Since our humidity levels (~39%) were higher than those typically obtained for cheddar cheese undergoing ripening (~37%) (Mahaut et al. 2000), these yield values were anticipated. There was a relationship between yields and moisture in cheese ($R=0.6$). It must also be kept in mind that both curds and pressed cheese were used in calculating the overall yield in this study while the typical calculation is based on pressed cheese.

Cheese composition is presented in Table 3. Paired *t* tests showed that the cheeses produced with the ME culture did not significantly differ from those produced with free-cell cultures (data not shown). In all cheeses, glucose and acetic acid concentrations were below 0.1 g.L^{-1} (data not shown). A high acetic acid level would have indicated fermentative activity by the bifidobacteria (Ong and Shah 2009). However, since the population in probiotic cultures was only around one million cells per gram of cheese (Table 1), it was presumably insufficient to generate significant changes in the chemical composition.

Statistical analysis showed no significant effect of the point of inoculation of *B. longum* on the chemical parameters in both products tested: pressed cheese and free curds (Table 3). However, moisture and salt-in-moisture readings were significantly

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higher in the free curds than in the pressed cheese while the opposite was observed for TN and fat (Table 3). A higher moisture level in the free curds indicates higher whey content. As a result, higher levels of compounds mainly found in whey (soluble nitrogenous fractions, lactose and lactic acid) were expected to be detected in the free curds. When individually comparing all six treatments in the ANOVA analysis, the higher levels of lactose, lactic acid and soluble nitrogen in free curds were not found to be statistically significant from those in the pressed cheese (Table 3), but paired t tests did detect the difference. As a rule the higher WSN, TCA-SN, lactose and lactic acid values observed in free curds, as compared to pressed cheese, were in correlation with the curds' higher moisture content.

Chemical composition of the cheeses was altered during storage. Thus, an increase in lactic acid concentration during storage ($P<0.05$) was accompanied by a decrease in lactose (Table 3). There were also increases in various soluble nitrogenous fractions during the 14-day storage period (Table 3) ($P<0.05$) as previously reported for cheese ripening (Lacroix et al. 2010). This is attributed to proteinase and peptidase activities of the mesophilic starter culture (Lane and Fox 1997). The population in lactococci was around 10^9 CFU.g^{-1} of cheese, which was at least 100 times higher than that of the bifidobacteria. Therefore, the changes in nitrogenous fractions, lactose and lactic acid were probably due to the action of the starter culture.

3.5 Sensory analyses

The panel did not find any significant difference in texture between any of the pressed cheese samples. This suggested that the addition of ME particles at a 0.27 g/L of milk did not influence sensory properties, whatever its addition time. This is noteworthy because the ME cultures average particle size was above 250 μm (Champagne et al. 2010). The addition of alginate-encapsulated cultures with particle size above 100 μm was shown to affect sensory properties of ice cream (Sheu et al. 1993), and it was a concern that cheese made with the ME culture would suffer the same defect. The low level of powder used during cheese making might explain the absence of defect. It should be kept in mind that such a low level of powder addition is typical of that encountered in industrial practice.

The addition of probiotics had no effect on flavour attributes of the pressed cheeses, which was in line with the absence of a significant effect on cheese composition (Table 3). This observation was expected because the concentration of probiotics inoculated was low. At such CFU levels, another study also showed that bifidobacteria do not influence the sensory properties of cheddar (Ong and Shah 2009). It can then be concluded that addition of free or ME $B.$ $longum$ 15708 to cheese does not alter its sensory qualities, still keeping in mind that further experiments with higher bacterial concentrations need to be done.

4 Conclusion

This study confirmed various data in the literature with respect to the addition of probiotics to cheese: (1) the highest CFU levels in cheese curds were obtained when

inoculation was carried out in milk rather that at the cheddaring step, (2) when milk was inoculated with bifidobacteria before renneting, the viable cells recovered at the end of cheesemaking increased, suggesting that growth occurred during processing; however, the opposite was noted when inoculation was carried out at the cheddaring step and (3) salting of the cheddar curds is detrimental to the viability of bifidobacteria.

In addition, many new observations were made: (1) there were increased cell losses in whey when ME cultures of the bifidobacteria were added before renneting, (2) inoculation with probiotics at the salting stage resulted in lower CFU counts in pressed cheese than when they were added to milk prior to renneting, (3) ME did not affect cell recovery in curds, (4) the 1-day storage period at room temperature of fresh cheddar cheese is highly detrimental to the viability of *B. longum*, (5) viability losses during a 14-day storage were lower when the curds were pressed and vacuum-packed than when marketed as free curds in bags, (6) bifidobacteria added at the salting step which survived pressing were subsequently more stable during storage than those inoculated in milk and showed similar behaviour to bifidobacteria added at the cheddaring step, (7) addition of probiotics did not significantly affect pressed cheese composition, (8) addition of ME culture particles did not affect sensory properties of pressed cheese.

In the past, probiotic cultures were selected for their ability to survive in the food product. Today, strain selection is mostly based on demonstrated clinical effects. Data from this study show that the fresh cheddar cheese environment can be highly detrimental to probiotics viability, particularly during storage. As a result, CFU decreased more than 1 log, which arguably, is the limit for commercial acceptability. The point of inoculation is an important technological parameter to ensure recovery and stability of the cultures in cheese. However, for some strains, improvements in microencapsulation or additional technological means to prevent viability losses still need to be developed.

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