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Effect of different calcium salts and methods for triggering gelation on the characteristics of microencapsulated *Lactobacillus plantarum* LIP-1

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Abstract Probiotic *Lactobacillus plantarum* isolate LIP-1 was microencapsulated in milk protein matrices by means of rennet-induced gelation combined with an emulsification technique. The effect of different calcium salts ($CaCO_3$ and $CaCl_2$) and different methods for triggering gelation (acid-triggered versus temperature-triggered) on the characteristics of microencapsulated *Lactobacillus plantarum* LIP-1 were investigated. Results indicated that microcapsules prepared by $CaCO_3$ with acid-triggered gelation showed significantly better qualities than microcapsules prepared by $CaCl_2$ with temperature-triggered gelation. Specifically: microencapsulation efficiency (ME), particle size and size distribution (as observed by optical microscopy), the surface morphology and the microstructure (as observed by scanning electron microscopy), resistance to passage through simulated gastric fluid (SGF), release characteristic in simulated intestinal fluid (SIF) and viability during 4 weeks storage at different temperatures (4°C, 20°C and 37°C) were all better in the microcapsules prepared by $CaCO_3$ with acid-triggered gelation. For these reasons we propose that $CaCO_3$ with acid-triggered gelation might be a better choice for crosslinking casein to form microcapsules.

Keywords: Microencapsulation; Viability; $CaCO_3$; Acid-triggered gelation; $CaCl_2$; Temperature-triggered gelation

Introduction

Probiotics are defined as living microorganisms that, when consumed in adequate quantities, confer a health benefit on the host.[1] (Homayouni *et al*., 2007) Consistent with increasing demand, probiotics have been used widely in the production of functional foods, especially dairy foods such as yoghurt and cheese, which represent about 65% of the global functional food market.[2] (Agrawal, 2005)

It is generally believed that the therapeutic minimum level of probiotics should be at least 10^7cfu/g or ml of live cells in the product[3] (Jankovic *et al*., 2010), but the actual levels detected in probiotic products are often much lower due to adverse conditions during product storage, transportation, marketing and consumption. Factors that influence the stability of probiotics include food composition (e.g. pH, moisture content, hydrogen peroxide concentration, molecular oxygen availability and additives), strains of probiotic bacteria, microbial interactions, heat treatment during the production process, storage temperature and host's own digestive system (e.g. gastric acid, bile salts, enzymes).[4,5,6] (Malmo *et al*.,2013; Akalin *et al*.,2004; Ross *et al*.,2005)

Studies have shown that microencapsulation techniques can provide protection for probiotics as they represent a physical barrier against adverse environmental conditions, increasing the viability of probiotics in food matrices, increasing stability during storage and protecting against the host's digestive system.[7,8] (Anal & Singh, 2007; Kailasapathy, 2002). There are various types of coatings and mixtures of effective biopolymers that have been used for microencapsulation of probiotics, such as alginate, gellan-gum, xanthan and κ-carrageenan.[9] (Heidebach *et al*., 2010). However, they are all of non-dairy origin and, therefore, undesirable or prohibited from use in dairy products in some countries.[10] (Picot & Lacroix,2004)

Milk proteins as matrix materials possess well-known physico-chemical properties that could make them appropriate for use as encapsulating agents in food production. Milk proteins are generally recognized as safe (GRAS) raw materials with high nutritional value and good sensory properties.[11] (Livney, 2010) They also have the

properties required for the delivery of functional ingredients. For example, they can bind small molecules and ions; they have self-assembly properties; superb gelation properties; pH-responsive gel-swelling behaviour; they interact with other polymers to form complexes; are biocompatible and biodegradable; and have an excellent buffering capacity against the harsh acid environment of the stomach.[12,13](El-salam and El-shibiny, 2012; Doherty *et al*.,2012) These attributes mean they are capable of controlling the bioaccessibility of bioactive substances. Furthermore, they have good emulsification properties, are highly soluble and, as they have a bland flavour they do not interfere with the sensory properties of the product.[9,14] (Heidebach *et al*., 2010; Heidebach *et al*., 2009b) Another potential benefit associated with protein encapsulation matrices involves hydrolysis of milk proteins by digestive enzymes potentially generating bioactive peptides that may exert a number of physiological effects in vivo.[15,16] (Kilara & Panyam, 2003; Korhonen & Pihlanto, 2003). These properties indicate that dairy proteins are good candidates for encapsulating probiotics, and as such they are attracting more research attention.[2] (Agrawal, 2005)

For example, Heidebach *et al*. (2009b)[14] used rennet-induced gelation of milk proteins in reconstituted skimmed milk powder with emulsification to encapsulate probiotics. In their experiment a soluble calcium salt, $CaCl_2$, was added to the suspension of cold milk protein and probiotic cells during renneting, and the mixture dispersed in cold oil resulting in a water-in-oil-emulsion (W/O) that was further emulsified until a sufficiently small droplet size was achieved (<5 °C). Subsequently the temperature was raised to 40°C for 15min to complete the encapsulation reaction. The method was based on a temperature-triggered instant gelation effect; although the κ-casein was cleaved because the renneting was conducted at low temperatures, the resulting micelles did not coagulate, until the temperature was raised to above 18~20°C, where a gel was formed instantly.[17] (Bansal *et al*., 2007). However, during the rise in temperature, disruption of the emulsion system equilibrium may sometimes cause significant clumping of the microcapsules before they become properly hardened, leading to reduced encapsulation efficiency and stability. The step

involving raising the temperature from 5℃ to 40℃ also requires a lot of energy and can cause microbial contamination during industrial production.

Based on the method of Heidebach *et al*.(2009b)[14], the objective of this study was to develop a modified encapsulation process based on emulsification and subsequent acid-triggered gelation by means of cold renneting of skim-milk concentrates and the insoluble calcium salt, $CaCO_3$, for the production of dairy-based microcapsules of the probiotic *Lactobacillus plantarum* LIP-1. Furthermore, the characteristics of microencapsules prepared using two different calcium salts and different methods for triggering gelation were compared. Characteristics included microencapsulation efficiency, particle size and size distribution, surface morphology and microstructure, and their ability to protect probiotic cells during subsequent exposure to different environments.

Experimental

Materials

Lactobacillus plantarum LIP-1 originated from the culture collection of the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education PRC, Inner Mongolia Agricultural University. *Lactobacillus plantarum* LIP-1 was identified as a probiotic isolate during screening of lactic acid bacteria from koumiss samples collected in Inner Mongolia; our research showed that *L. plantarum* LIP-1 had cholesterol-reducing health benefits.[18] (Wang *et al*.,2012).

Skimmed milk powder was obtained from Fonterra Ltd. (Auckland, New Zealand) and the rennet, with a declared activity of 1070 IMCU mL^{-1}, from Shanghai Aijie Biological Technology Co. Ltd. (Shanghai, China). A fresh stock solution of rennet was prepared daily by diluting 1 g rennet preparation in 10g double-distilled water. Sunflower oil was purchased from JingLongYu Ltd. (Chengdu, China). $CaCl_2$, $CaCO_3$, glacial acetic acid and other chemicals used in this study were all of analytical grade and purchased from Sinopharm Chemical Reagent Co.,Ltd. (Shanghai,China).

Lactobacillus plantarum LIP-1 culture and production of cell suspension

Lactobacillus plantarum LIP-1 was grown in Man, Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C for 18 h in an incubator (SANYO Electric Co., Ltd.,Japan). The bacteria were subcultured three times prior to being used for the preparation of cell suspensions. They were then centrifuged using a centrifuge (Anhui USTC Zonkia Scientific Instruments Co.,Ltd.,China) at 1000 g for 10 min. The supernatant was discarded and the cell precipitate was washed thoroughly with sterilized 0.85% NaCl solution. This washing process was repeated two times and the final cell slurry was made up to 2 mL by adding sterilized 0.85% NaCl solution and mixing thoroughly. For enumeration of bacteria, the number of colony forming units (cfu) was determined on MRS agar using the plate count method at 37°C for 48 h.[19 20] (Grosso *et al.*, 2004; Pedroso *et al*., 2012)

Preparation of the milk-*L.plantarum*-cell suspension

All glassware used were sterilized at 121°C for 30 min. Skimmed milk powder was dispersed in double-distilled water to obtain a 30% (w/w) solution and stirred overnight at 4°C. 2.0 g of the concentrated LIP-1 cell suspension was mixed with 28.0 g of the skimmed milk to achieve a culture with a concentration of approximately 10^9~10^{10} cfu/mL of probiotic culture.

Microencapsulation

Two microencapsulation methods were compared: In the first method microcapsules were prepared using $CaCl_2$ according to previously described methods.[14] (Heidebach *et al*. 2009b) Firstly, 30g of the *L. plantarum* cell suspension was cooled to 4°C with 400 µL rennet stock-solution (100 IMCU/mL), and incubated at 4°C for 60 min until cleavage of κ-casein had finished (renneting). Then 60 µL 10% (w/v) $CaCl_2$ solution was added to 10g of the cold-renneted suspension, and the entire mixture added to 100 g of tempered (4°C) vegetable oil to initiate the encapsulation process. To achieve emulsification the mixture was stirred for 15 min using a magnetic stirring apparatus. The temperature was maintained at 4°C throughout the process to prevent premature gelling. Following the emulsification process at 4°C, the temperature was raised to 40°C over a period of 15 min during which time it was stirred continuously. When the temperature exceeded 18~20°C the

emulsified droplets turned into gel particles instantly. Subsequently, the gelatinized microcapsules were separated from the oil by gentle centrifugation (500 g, 1 min). The supernatant was removed and the sediment was diluted with approximately twice its volume of double-distilled water, shaken for 1 min and then centrifuged again under the same conditions. The supernatant, consisting of water and residual oil was removed. The virtually oil-free aqueous microcapsule-slurry was stored at 4°C before experimentation.

The second method was a modified encapsulation process using $CaCO_3$ as follows: After 60 min incubation (4°C), 1.5g $CaCO_3$ was added to 10g of the cold-renneted suspension. This was then added to 100 g vegetable oil and stirred at 800 rpm for 15 min to emulsify the mixture into the oil. To this 100μL of glacial acetic acid was added to achieve dissociation of the Ca^{2+} and for the κ-casein to become cross-linked by Ca^{2+} within the emulsion droplets. After allowing the mixture to stand for 30 min, the microcapsules were generated. All steps were done at room temperature except the cleavage of the κ-casein. Subsequent centrifugation and dilution was the same as in the first method，and the resulting microcapsule-slurry was stored at 4°C prior to experimentation.

Microencapsulation efficiency

Cell viability within both types of microcapsules was determined by counting the number of colony forming units (cfu/g) developing on MRS agar using a pour-plate technique described previously. Plates were incubated at 37°C for 48 h before the counts were taken. Microencapsulation efficiency (ME) was calculated using Eq. (1):

$$ME\ (\%) = N/N_0 \times 100\% \qquad (1)$$

where N is the number of microencapsulated cells released from the microcapsules (log cfu/g microcapsule) and N_0 is the number of free cells added to the *L. plantarum* LIP-1 suspension during the production of the microcapsules (log cfu/g).

Effect of freeze-drying

The microcapsule slurries produced in the two different ways were each frozen for 24h at −80°C before drying under 90 mTorr vacuum in a freeze dryer (SANYO

Electric Co., Ltd., JAPAN)[21](Amine *et al.*,2014), with the cell-free protection agent (10% skimmed milk and 0.1% L- monosodium glutamate) as the control. The survival of encapsulated LIP-1 after freeze-drying was expressed using Eq. (2):

$$\text{Cell survival (\%)} = N_2/N_1 \times 100\% \quad (2)$$

where N_1 is the initial number of microencapsulated cells before freeze-drying (log cfu/g microcapsule); and N_2 represents the number of viable cells after the freeze-drying (log cfu/g microcapsule).

Analysis of microcapsule morphology and particle size

The morphology of the microcapsules produced using different calcium salts was observed by scanning electron microscope (SEM) (SEM-SU8010, Hitachi, Tokyo, Japan). The samples were fixed to double sided conductive tape on top of SEM stub and then sputter-treated in a metallizer (Agar Sputter Coater) with gold-palladium to reach a thickness of coating of 100 A and then observed in high vacuum mode.

The average particle size and size distribution of the microcapsules were determined by Leica Confocal Laser Scanning Microscope (TCS SP-2, Leica Inc., Wetzlar, Germany). To calculate their diameter, at least 50 particles from each of the different formulations of microcapsules were measured at a magnification of 20×.[22] (Krasaekoopt, Bhandari, &Deeth, 2004)

Release of encapsulated LIP-1 under simulated intestinal conditions

The release of encapsulated LIP-1 under simulated intestinal pH conditions was investigated according to previously described methods[23,24] (Mokarram *et al.*,2009; Mandal *et al.*,2006), with some modifications. To prepare simulated intestinal fluid (SIF), the pH of a 0.85% NaCl solution was adjusted to 8.0 using NaOH (1 M), then sterilized by autoclaving at 121°C for 15 min. Trypsin（1:250 u/mg, High Purity Grade; Biotopped Co.,Ltd., Beijing, China）was then suspended in the solution to achieve a final concentration of 0.1% (w/v) and the solution was sterilized by filtration (0.22 mm). Samples (0.5g) of the two kinds of freeze-dried microcapsules were each suspended in 4.5 mL of sterile SIF and incubated at 37°C with constant agitation at 100 rpm. Aliquots of 1.0 mL were taken at 0, 30, 60, 90, 120 and 150 min and the survival of encapsulated LIP-1 released in to the SIF was determined using

the pour-plate technique as described previously.

Survival of encapsulated and free cells in simulated gastric fluid

Simulated gastric fluid (SGF) were prepared and survival of encapsulated cells in SGF was determined according to previously described methods[25,26,27] (Dolly *et al.*, 2011; Rajam *et al.*, 2012;Sohail *et al.*,2011), with slight modification. To prepare simulated gastric fluid (SGF), the pH of a 0.85% NaCl solution was adjusted to 2.0 using 1 M HCl and sterilized by autoclaving at 121°C for 15 min. pepsin (1:3000 NFU/mg, High Purity Grade; Biotopped Co.,Ltd., Beijing, China) was then suspended in the solution to achieve a final concentration of 0.3% (w/v). Samples (0.5g) of the two kinds of freeze-dried microcapsules were each suspended in 4.5 mL of sterile SGF and incubated at 37°C with constant agitation at 100 rpm. The control was 0.5g of the *L. plantarum* suspension (free cells). Aliquots of 0.5 mL were taken at 30, 60, 90, 120 and 150 min. The survival of encapsulated *L. plantarum* released in to SGF was determined using the pour-plate technique as described previously and compared with the free cells control.

Survival of microencapsulated LIP-1 during storage

Samples of each of the two types of freeze-dried microcapsules were placed in sealed glass vials and stored at 4°C, 20°C and 37°C for 28d. At weekly intervals, the survival of bacterial cells was determined by enumeration on MRS agar, using the pour-plate technique as described previously.

Statistical analysis

All experiments were carried out in triplicate in a completely randomized design and the data were analyzed using SPSS Statistics software,version 22.0 (IBM SPSS Statistics 22). The results were expressed as means ± S.D. Data comparisons were made using the Studente Newman Keuls test to determine whether there were significant differences ($p<0.05$) between treatment means.

Results and discussion

Microencapsulation efficiency

Microencapsulation efficiency (ME) is an important index for microcapsule evaluation, because it is crucial to maintain high viability within the microcapsules to achieve the full benefits of LIP-1. The ME for viable cells in microcapsules prepared by $CaCO_3$ (75.6 ± 5.1 %) was significantly greater ($p<0.05$) than for viable cells in microcapsules prepared by $CaCl_2$ (58.4 ± 11.3 %) (Table 1). The high ME may be attributed to the fact that the entire process involved no detrimental steps such as heat treatment or high shear forces. The ME appeared to be in agreement with the results of Heidebach *et al.* (2009b)[14], who used a similar process with a sodium caseinate emulsion. Shi *et al.* (2013)[28] encapsulated *L. bulgaricus* in skimmed milk-alginate and an extrusion method, and the ME was around 60%. In the present study, a slight loss of cells may be because the cells present on the droplet surface were lost into the oil phase or drained with the washing water.

Although the microcapsules produced by insoluble $CaCO_3$ and acid-triggered gelation had a higher ME than those produced by $CaCl_2$ and temperature-triggered gelation, the specific mechanism is not entirely clear. It may be because $CaCl_2$ microcapsules produced by emulsification are formed instantly when the temperature is raised and Ca^{2+} crosslinks rapidly with casein, potentially resulting in some premature gelling that could limit the number of viable cells embedding in the microcapsules. In contrast $CaCO_3$ releases Ca^{2+} for crosslinking with casein within W/O gelation when the glacial acetic acid is added; in this way the dense structure of the microcapsule enables the immobilized *L. plantarum* to remain entrapped, thereby increasing the ME.

Table 1: The microencapsulation efficiency (ME) and size of microcapsules produced in two different ways

The type of microcapsules	Microencapsulation efficiency (%)	Microsphere size (μm;n=50)
$CaCl_2$	58.4 ± 11.3^{A}	109.05 ± 19.53^{A}
$CaCO_3$	75.6 ± 5.1^{B}	143.57 ± 29.18^{B}

AB means in the same column followed by a different upper case letter are significantly different ($P < 0.05$).

Particle size and distribution

Particle size and its distribution are important physical properties that directly affect the use of microcapsules into food formulations. The results showed that the mean diameters of microspheres prepared by $CaCO_3$ (109.05 ± 19.53 μm) were significantly smaller than by $CaCl_2$ (143.57 ± 29.18 μm) ($p<0.05$) (Table 1). Fig. 2 indicated that the beads produced by the two methods exhibited nearly unimodal and normal particle-size distribution, and the size distribution of two microcapsules was significantly different. Microspheres prepared by $CaCl_2$ exhibited a wide range of sizes varying from 107.86 μm to 172.75 μm diameter, and 78% of the particles were between 120 and 160 μm. While microcapsules prepared by $CaCO_3$ showed a narrower size distribution ranging from 89.52 μm to 143.84 μm, and 82% of the microspheres were between 90 and 120 μm (Fig. 1 and Fig. 2).

It has been known that the size and size distribution of the microspheres could be influenced by the concentration of skimmed milk, the ratio of water to oil (v/v), the stirring speed, and so on.[29] (Lin *et al.*, 2007) In this study, the microspheres were prepared to ensure the comparability between both methods at given conditions, that is, the same batch of skimmed milk, stirring speed of 700 rpm and the ratio of water to oil of 1:10. Therefore the variations in particle size and distribution between two microspheres might be due to the different Ca^{2+} used for encapsulation. For microspheres producted by $CaCl_2$, Ca^{2+} was distributed in the emulsion system before temperature raised, and the larger mean size was attributed to the collision of the dispersed Ca^{2+} and skimmed milk droplets. The collision could involve two or more droplets, which cause the aggregation of microcapsules.[30] (Silva *et al.*, 2006) While $CaCO_3$ releases Ca^{2+} for crosslinking with casein within W/O gelation when the glacial acetic acid is added, so the size distribution of microspheres prepared by $CaCO_3$ depends on the size of the emulsion droplets, which is determined by a balance between the dispersive and the surface tension forces. So the latter tends to well-dispersed and the former causes coalescence.[31] (Chan, Lee, & Heng, 2002)

Several researchers reported that the optimum microcapsule size is a compromise between the effectiveness of encapsulation and the sensory properties.[32] (Arup *et al.*, 2011) A minimum diameter of 100 μm has been suggested to offer better protection in

gastric juice, and an optimum range of 100-200 µm has been proposed.[33,32](McMaster *et al.*, 2005; Nag *et al*., 2011) The mean diameter of our two microcapsules were both within this suggested range.

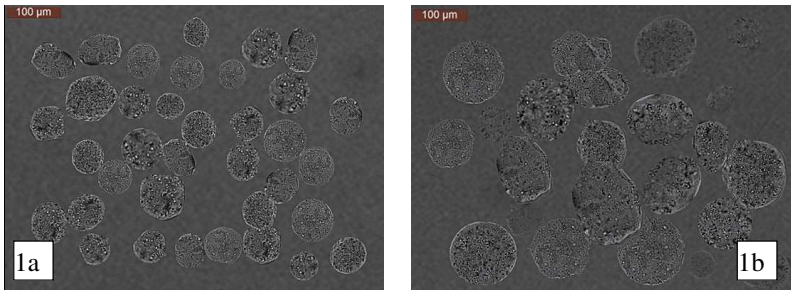

Figure 1: Optical images of two different microencapsulations: (a) prepared by $CaCO_3$; (b) prepared by $CaCl_2$ (bar = 100µm).

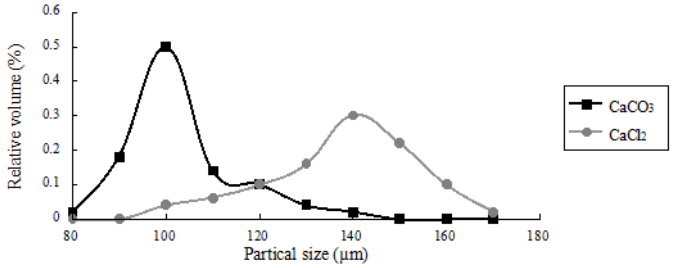

Figure 2: Particle mean size distributions of two different microencapsulations

Effect of freeze-drying

Probiotic cells should be capable of being dried so that they can serve as a functional ingredient in food products but also be easily transported. Otero *et al.* (2007)[34] suggested that freeze-drying might be appropriate for this as it is a well-documented technique used to obtain stable cultures in terms of viability and functional activity. However, either freezing or freeze-drying of LIP-1 could reduce its viability due to deterioration of the physiological state of the cells.[35,36] (Chavarri *et al*., 2010; Crittenden, *et al*., 2006). De Giulio *et al*. (2005)[37] reported that, during freeze-drying, bacteria were subjected to adverse and potentially lethal conditions, such as water crystallization, protein denaturation and bacterial membrane injury. The bead manufacturing process had the most influence on subsequent viability following freeze-drying, but microencapsulated bacteria were also more stable than free cells.

In our study, however, encapsulated LIP-1 cells prepared by either $CaCO_3$ or $CaCl_2$ retained significantly greater ($P<0.05$) viability than free cells (Table 2). The survival of encapsulated LIP-1 were above 60% while free cell was about 8% after freeze drying for 24h. There was no significant difference（$P>0.05$）in survival after freeze-drying of encapsulated *L. plantarum* prepared either using $CaCO_3$ or $CaCl_2$.

The beneficial effect of microencapsulation prior to freeze-drying has often been reported[38,39,40] (Homayouni *et al*., 2008; Shah and Ravula, 2000; Sheu and Marshall, 1993), and so the data from this study are in line with the existing literature.

Overall, cell mortality during the freeze-drying process is known to be mostly due to injuries to the membranes of cells.[41,42] (Carvalho *et al*.,2003; Saarela *et al*., 2005) These injuries include structural changes to sensitive proteins resulting from intracellular ice formation, the physical status of membrane lipids, osmotic shock and recrystallization. Consequently, complete protection of the membrane of cells via coating techniques, as we saw in this study (Table 2), could be very effectual. Shah & Ravula (2000)[39] also reported that microencapsulation of probiotic bacteria prior to freeze-drying for incorporation into frozen fermented dairy desserts was very effective for improving cell stability during the freeze-drying process. They noted that encapsulation protected the cells from cold shock induced by the process.

Skimmed milk proteins have been reported widely as very effective cell protectants during freeze-drying, a function that has been attributed to the presence of lactose; lactose has the ability to interact with cell membranes retaining their structural integrity.[43] (Corcoran *et al*.,2004) Moreover, as a disaccharide, lactose can depress the gel to liquid crystalline state transition temperature of the lipid bilayer of cell membranes, aiding cells to retain their natural structural conformation.[44] (Fu & Chen,2011) This mechanism could also be extended to other milk protein formulations with similar compositional elements such as whey protein concentrates milk protein concentrates or caseinates. Another mechanism that could also potentially affect the survival rate of LIP-1 during the freeze-drying process, is the ability of proteins to bind water through hydrogen bonds[45] (Kinsella and Morr, 1984), which can protect cellular membranes from dehydration. From our data, it seems that

a synergism between these mechanisms could explain the protection of LIP-1 by skimmed milk protein microcapsules.

Table 2: Survival rate during freeze drying (%), of LIP-1 as free cells or encapsulated using either $CaCO_3$ or $CaCl_2$

	$CaCO_3$	$CaCl_2$	Free cell
Survival rate (%)	64.733±0.210[a]	63.867±0.560[a]	7.933±0.470[b]

ab means in the same row followed by different lower case letters are significantly different from each other ($P < 0.05$).

Morphology of microcapsules

The optical and SEM morphology of two different microencapsulations were presented in Fig. 1, 3 and 4. As seen in Fig. 1, microcapsules prepared using $CaCO_3$ were smaller, more homogeneous and showed a narrower size distribution than microcapsules prepared using $CaCl_2$. Fig. 3 showed the morphology of two different microencapsulations dyed by methylene blue, in which we can see that many LIP-1 were involved in skimmed milk microcapsules. And Fig. 4 showed the morphology of two different microcapsules by SEM.

As seen in Fig. 4, microcapsules prepared using $CaCl_2$ and temperature-triggered gelation, were non-spherical and had an porous organization. (Fig. 4a and b). We believe these characteristics may due to the fast sublimation of frozen water from the skimmed milk matrix resulting in the formation of pores in areas where ice crystals had been present and had not enough time to shrink, as has been observed by others.[46] (Smrdel *et al.*, 2008) While microcapsules prepared by $CaCO_3$ with acid-triggered gelation were smoother (Fig. 4d). When compared at a higher resolution (8000×), there was a more compact substructure in microcapsules produced using $CaCO_3$ (Fig. 4e) than microcapsules produced by $CaCl_2$ (Fig. 4b). Moreover, some LIP-1 were discovered in the section of microencapsulations in Fig. 4f, which is a stronger evidence that LIP-1 being encapsulated in the microencapsulations.

In the $CaCl_2$ microcapsules produced by emulsification the crosslinking was

achieved instantly by raising the temperature during stirring, and this may be why the microcapsules are always agglomerated (Fig. 4c). By the time the calcium solution contacts the dispersed casein phase, gelling occurs. Thus, the gelation kinetic is heterogeneous, which has often been shown previously to lead to capsules of irregular shape and agglomerated.[47,48] (Muthukumarasamy *et al*., 2006; Sheu *et al*., 1993) In contrast to this, glacial acetic acid induced Ca^{2+} release into casein suspensions from $CaCO_3$ underwent a homogeneous internal gelation process, leading to dispersed and homogeneous small microcapsules, which are preferable because there are fewer negative sensorial impacts on the resulting food. So the use of $CaCO_3$ always induced a compact microcapsule structure, regular shape and a finer more dispersed substructure.

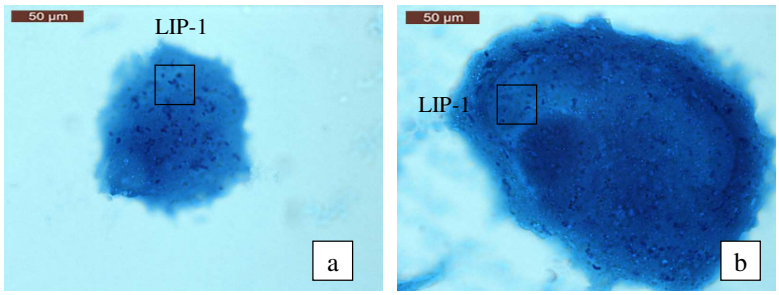

Figure 3: Morphology of two different microencapsulations dyed by methylene blue: (a) prepared by $CaCO_3$ (bar=50µm); (b) prepared by $CaCl_2$ (bar=50µm).

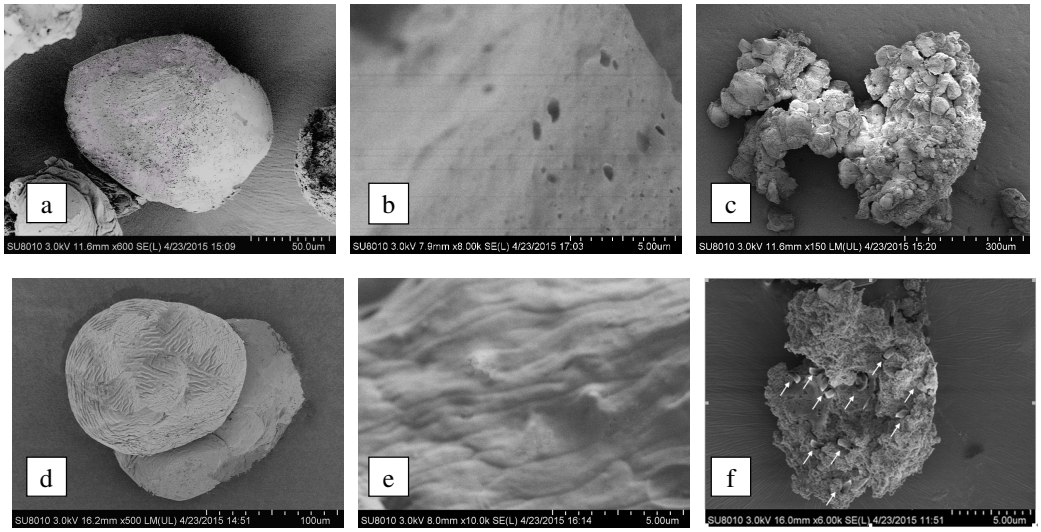

Figure 4: SEM images of two different microencapsulations: (a) prepared by $CaCl_2$

(bar=50μm); (b) prepared by $CaCl_2$ (bar=5μm); (c) prepared by $CaCl_2$ (bar=300μm); (d) prepared by $CaCO_3$ (bar=100μm); (e) prepared by $CaCO_3$ (bar= 5μm); (f) the section of microencapsulation prepared by $CaCO_3$ (bar=5μm).

Release of encapsulated probiotics under simulated intestinal condition

The release of cells from microcapsules in the colon is essential for growth and colonization of probiotics. If this does not happen then the probiotics in the microcapsules will be washed out of the body before they have exerting any beneficial effect.[49] (Sabikhi *et al*., 2010) In our study the encapsulated *L. plantarum* prepared using $CaCO_3$ was completely released within 90 min and, in fact, more than 70% was released within 30 min (Fig. 5). In contrast the encapsulated *L. plantarum* prepared by $CaCl_2$ took 120 min to be fully released, and by 30 min less than 40% had been released (Fig. 5). The release profile indicated that $CaCO_3$ microcapsules had significantly faster release properties in SIF than $CaCl_2$ microcapsules. Many studies have investigated the release of encapsulated probiotics in SIF. For example Sabikhi *et al*. (2010)[49] reported that microencapsulated *Lactobacillus acidophilus* were released after 2.5h in SIF (pH 7.4 ± 0.2), which was actually slower than in our study. We speculate that differences in speed of release amongst different types of microcapsules, is due to the difference in size and distribution status of those microcapsules.[50] (Chen and Subirade, 2006) In our study the microcapsules produced by $CaCO_3$ were dispersed better and the particle size was smaller and more homogeneous compared with microcapsules produced using $CaCl_2$, which were irregular and agglomerated (Fig. 1, and 4). This resulted in a larger specific surface area for enzyme activity, increasing the speed of disintegration.

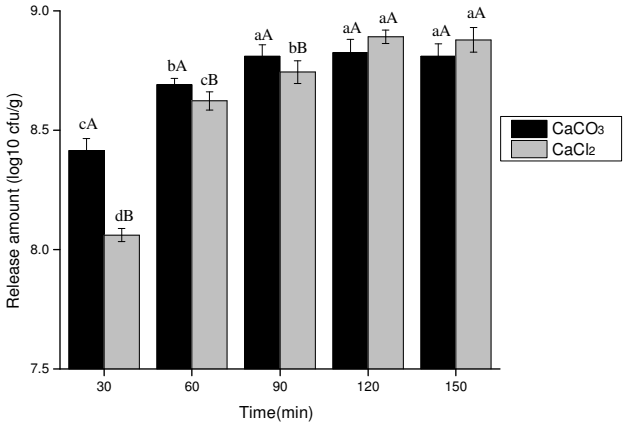

Figure 5: Release characteristics of encapsulated cells in SIF

Bars labelled with a different lower case letter (e.g. a, b or c) means in the same microencapsulation methods with different incubation time are significantly different from each other ($P < 0.05$)

Bars labelled with a different upper case letter (e.g. A, B or C) means at the same incubation time with different microencapsulation methods are significantly different from each other ($P < 0.05$).

Survival of encapsulated and free cells of LIP-1 in simulated gastric juice

Probiotic bacteria are, in general, sensitive to acidic environments, which presents a challenge for the industrial use of these bacteria as well as for consumer effectiveness. In order to exert positive health effects, probiotics should resist the harsh conditions of the stomach to remain active when they reach the colon. One main purpose of encapsulation is to improve the tolerance of probiotics to low pH in the stomach.

In our study free cells of LIP-1 were sensitive to low pH and they lost 2 log cfu/g when exposed to acidic conditions for 120 min (Fig. 6). This is in line with many articles reporting that unprotected cells of probiotics are easily damaged by stomach acid. For example, Heidebach *et al.* (2009)[14] reported that *Lactobacillus paracasei* isolate F19 lost viability almost entirely within 60 min at a SGF pH of 2.5. In contrast, encapsulation of LIP-1 in skimmed milk microspheres significantly improved survival in SGF (pH 2.0) compared with free cell ($p<0.05$) (Fig. 6). Furthermore, microspheres prepared by $CaCO_3$ with acid-triggered gelation survived significantly longer than microcapsules produced by $CaCl_2$ ($p<0.05$) (Fig. 6). In SGF, the viable counts of LIP-1 that had been encapsulated by either method were greater

than 8 log cfu/g after 150 min exposure (Fig. 6). Excellent pH tolerance of encapsulated LIP-1 was probably due to the buffering ability of skimmed milk and low porosity on the surface of the microspheres. Our findings agree with those of other studies. Guerin *et al*. (2003)[51] reported that the buffering ability of whey protein contributed to high survival rates for *Bididobacterium* spp. encapsulated in alginate–pectin–whey protein microspheres when exposed to SGF at pH 2.5. Heidebach *et al*. (2009b)[14] reported that viability of *L.paracasei* and *Bifidobacterium lactis* exposed to SGF at pH 2.5 was improved when they were encapsulated in casein microcapsule. Gbassi *et al*. (2009)[52] reported that viability of *L.plantarum* isolates was improved when encapsulated in whey protein microspheres.

After 150 min of exposure to SGF the viabiity of cells encapsulated using $CaCO_3$ had dropped to 8.591 log cfu/g compared with cells encapsulated using $CaCl_2$ where the viability had dropped to 8.190 log cfu/g under the same conditions, demonstrating that the microencapsulation process using $CaCO_3$ provided much better protection for *L. plantarum* under acidic conditions. This might be explained by the different surface structure of the $CaCO_3$ microcapsules compared with the $CaCl_2$ microcapsules. As the surface of the $CaCO_3$ microcapsules is dense, it is likely to be more resistant to the penetration of SGF (Fig. 4e). In contrast, the surface of the $CaCl_2$ microcapsules was porous allowing the SGF to enter easily (Fig. 4b).

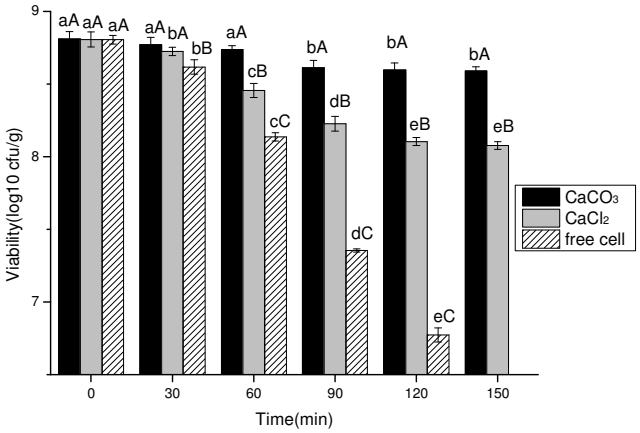

Figure 6: Survival of encapsulated *L. plantarum* in SGF

Bars labelled with a different lower case letter (e.g. a, b or c) means in the same microencapsulation methods with different incubation time are significantly different to each other ($P < 0.05$)

Bars labelled with a different upper case letter (e.g. A, B or C) means at the same incubation time with different microencapsulation methods are significantly different to each other ($P < 0.05$)

Survival of microencapsulated LIP-1 during storage

One of the key properties for any microorganism that is being considered as a probiotic is its capacity to survive storage as a formulated product.[53] (Sathyabama & Vijayabharathi,2014) Many studies have reported that encapsulation could improve the viability of probiotics during storage. For example, Su *et al*. (2011)[54] reported that alginate-human-like-collagen microspheres improved the stability of *Bifidobacterium longum* isolate BIOMA 5920 during storage for 3 weeks compared to free cells. Brinques & Ayub (2011)[55] concluded that an alginate-chitosan system had good potential to improve storage stability of *L. plantarum* isolate BL011, which lost little viability during the storage period evaluated.

In our study the encapsulating material, temperature, and storage time all significantly affected the viability of LIP-1(Fig. 7). Viability of LIP-1 encapsulated using $CaCl_2$ with temperature-triggered gelation was significantly lower than when encapsulated by $CaCO_3$ with acid-triggered gelation ($P<0.05$). For $CaCO_3$-produced microcapsules the average number of viable cells was 7.724±0.328 log cfu/g at 4°C after 28 days, which was significantly higher ($p<0.05$) than that of microcapsules stored at 20°C (7.176±0.414 log cfu/g) and 37°C (6.275±0.496 log cfu/g). For $CaCl_2$-produced microcapsules, there was also a smaller decrease in viability of *L. plantarum* (6.362±0.325 log cfu/g) at 4°C compared with storage at 20°C (5.987±0.286 log cfu/g) and 37□(4.886±0.375 log cfu/g). Regardless of storage temperature, the pattern of reduction in viability was similar for all treatments and, even after 28 d storage, the viability of *L. planttarum* encapsulated by $CaCO_3$ remained higher than 6 log cfu/g (Fig. 7), the threshold value recommended for a food to exert its probiotic benefits.[56,57] (Hamilton-Miller *et al*., 1999; Reid *et al*., 2003)

The maintenance of high viable cell counts after 28 d of storage, as presented in this study, may be attributed to several factors, such as the intrinsic resistance

characteristics of the isolate we used, the encapsulating material, the low moisture content of the particles, the type of packaging and storage temperature.[58,59] (Morgan *et al*., 2006;Abe *et al*., 2009) The barrier characteristics of the packaging material and the structure of microcapsules may have contributed to the microbiological stability of LIP-1 during storage. Milk protein is a good barrier against water vapor, gas and light, thereby preventing photo-oxidation and minimizing the oxygen levels in the interior of the package.[60] (Alves *et al*., 2008) Furthermore, the milk protein present in the packing may have contributed to minimizing oxygen diffusion to the cell membrane, favouring survival during storage.[61,62] (Teixeira *et al*., 1996; Chávez and Ledeboer, 2007) Rodrigues *et al*. (2011)[63] embedded *Lactobacilus acidophilus* isolate Ki, *L. paracasei* isolate L26 and *Bifidobacterium animalis* isolate BB-12 in whey protein, and the viability was higher than 6 log cfu/g after storage for up to 6 months at 5°C.

From the morphology visible using SEM we know that the microcapsules prepared using $CaCl_2$ are porous, while $CaCO_3$-produced microcapsules are compact (Fig. 4). Thus, microcapsules prepared using $CaCO_3$ provided better protection of LIP-1 than $CaCl_2$-produced microcapsules, and a higher viability after storage for 28d. Shi *et al*. (2013)[28] studied storage stability of *L. bulgaricus* in alginate–milk microspheres, and found that full viability of encapsulated *L. bulgaricus* could be preserved during storage for 1 month at 4 °C. This was probably due to the dense alginate–milk membrane formed around the microcapsules, which may have provided effective protection. In our study, it is possible that denser skimmed milk membranes were produced in $CaCO_3$-produced microcapsules than $CaCl_2$-produced microcapsules and that this provided better protection for LIP-1during storage.

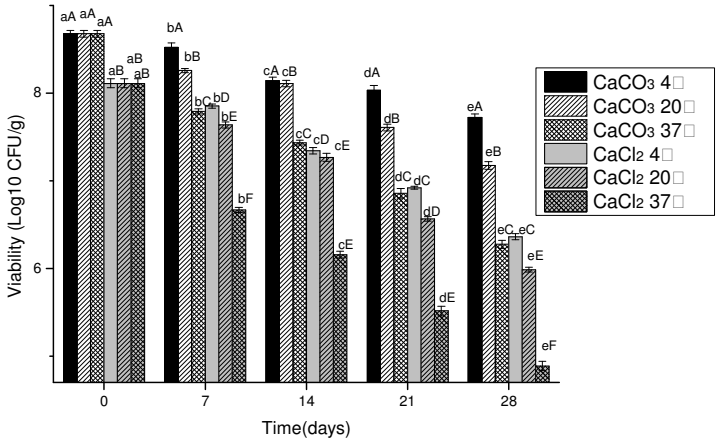

Figure 7: Survival of encapsulated LIP-1 during storage at different temperatures

Bars labelled with a different lower case letter (e.g. a, b or c) means in the same microencapsulation methods with different incubation time are significantly different to each other ($P < 0.05$)

Bars labelled with a different upper case letter (e.g. A, B or C) means at the same incubation time with different microencapsulation methods are significantly different to each other ($P < 0.05$).

Conclusions

This investigation compared the effect of $CaCl_2$ and temperature-triggered gelation with $CaCO_3$ and acid-triggered gelation on the biological characteristics and stability of microencapsulated LIP-1. The type of calcium salt and the method for triggering gelation significantly affected the surface and microstructure of the resulting microcapsules. The use of $CaCO_3$ with acid-triggered gelation provided better protection for LIP-1 against freeze-drying and adverse gastrointestinal conditions than $CaCl_2$ and temperature-triggered gelation. The $CaCO_3$ encapsulation processing method is not only easy to scale up, but also provides the encapsulated probiotics with better long-term preservation at different temperatures. These results demonstrate that $CaCO_3$ with acid-triggered gelation might be a better method for the preparation of microencapsulated LIP-1, than the $CaCl_2$ and temperature-triggered gelation method in order to increase protection against deleterious environmental factors. This study could also be useful for other probiotics used in the production of functional food or other related products.

Acknowledgments

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