Survivability of Freeze-Dried Probiotics Encapsulated in Cross-Linked Alginate: Influence of Divalent Cations

Dr. Ivana Kovačević[1]*

[1]University of Zagreb, Faculty of Food Technology and Biotechnology, Zagreb, Croatia

Key words

Delivery Systems; Probiotics; Cross-linkers; Cryoprotectant; Dessication; Encapsulation

Abstract

Alginate encapsulation is amongst the most studied probiotic encapsulation formulations in the literature, preferred for its low toxicity, low cost and ability to protect entrapped probiotics against acidic pH. Despite extensive academic studies, there has been little commercial translation of alginate-encapsulated probiotic formulations. In this study, we hypothesized that factors affecting freeze-drying survivability of alginate-encapsulated probiotics could have limited its translation potential. Factors including: 1) encapsulant material, 2) cryoprotectant, 3) cross-linker, 4) Concentration of crosslinker, and 5) other Group II divalent cations cross-linkers were investigated, using two model probiotics – *Lactobacillus rhamnosus GG* (LGG) and *Escherichia coli* Nissle 1917 (ECN). Divalent cations, which are used to cross-link alginates, were found to have antagonistic effects on the freeze-drying survivability of LGG and ECN. To mitigate the deleterious effects of divalent cations, the addition of sucrose was found to be useful, possibly due to its membrane stabilization mechanism.

1. Introduction

In recent years, probiotics have garnered increasing attention for its therapeutic and prophylactic effects in human hosts. Probiotics are defined as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). An important criterion for probiotics is that they should retain viability throughout the manufacturing process, storage and passage through the upper gastrointestinal tract (GIT). Encapsulation or immobilisation technologies have been devised to enhance the viability counts of probiotics, and alginate has emerged as the most commonly utilised material. Alginate is a negatively charged linear polysaccharide consisting of 1→4 linked ß-(D)-guluronic and α-(L)-mannuronic acids derived from brown algae or bacterial sources. Due to its biocompatibility, generally recognized as safe (GRAS) status, low cost, and mild gelation in the presence of divalent cation crosslinkers, alginate is particularly well suited for probiotics encapsulation (Lee & Mooney, 2012). Additionally, crosslinked alginate hydrogels are insoluble in acid, and numerous studies have demonstrated the success of an alginate-encapsulation technique in protecting probiotics against simulated gastric acid insults (Cheow & Hadinoto, 2013; Guimarães et al., 2013; Kim et al., 2008; Ramos et al., 2018).

Despite the myriad of alginate-based probiotic formulations published in literature, there has been little commercial translation (Burgain et al., 2011). One possible reason is the poor survivability of alginate-encapsulated probiotics upon drying (Chavarri et al., 2012; Meng et al., 2008; Ross et al., 2005). Drying is an important process that involves removal of water from probiotic formulations to enhance its shelf-life. Among numerous methods of drying, freeze-drying (i.e. lyophilisation) is considered a milder technique, and is used traditionally for the preservation of starter cultures and probiotic bacteria (Marques da Silva et al., 2018; Raddatz et al., 2020; Terpou et al., 2019; Ying et al., 2010). Still, several studies have shown significant losses (1 to 4 $\log_{10}$(CFU/ml)) of alginate-encapsulated probiotics upon freeze-drying (Brachkova et al., 2010; Cheow & Hadinoto, 2013; Donthidi et al., 2010; Etchepare et al., 2016; Halim et al., 2017; Martin-Dejardin et al., 2013). Despite such studies, there remains a poor understanding of the factors that affect probiotics' survivability after freeze-drying.

In pursuit of this question, we hypothesized that there are concomitant factors (i.e. encapsulation materials, cryoprotectants, crosslinking agents, etc.) that may be antagonistic to the viability of encapsulated probiotics, especially after freeze-drying. Two model probiotics were selected for this study: Gram-positive *Lactobacillus rhamnosus* GG (LGG) and Gram-negative *Escherichia coli* Nissle 1917 (ECN). LGG and ECN were individually exposed to various components of the calcium alginate matrix such as the alginate material and various divalent cation cross-linkers ($CaCl_2$/$SrCl_2$/$BaCl_2$) to identify the causation factor behind freeze-drying antagonism. The freeze-drying survivabilities of LGG and ECN encapsulated in Ca/Sr/Ba-crosslinked alginates were also studied in the presence/absence of a sucrose cryoprotectant. We envisage that a systematic investigation of antagonistic factors would provide key information to overcome the shortcomings of the much-studied alginate delivery system for probiotics encapsulation.

2. Experimental section

2.1. Materials

Lactobacillus rhamnosus GG – LGG in short – was isolated from a purchased Culturelle® LGG probiotic pill (i-Health, Inc., Crownwell, USA). *Escherichia coli* Nissle 1917 – ECN in short – was isolated from a purchased Mutaflor® pill (Pharma-Zentrale GmbH, Herdecke, Germany). Protanal® LFR5/60 sodium alginate – ALG for short – was purchased from FMC BioPolymer, Philadelphia, USA. Sucrose, sodium chloride (NaCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), strontium chloride ($SrCl_2$), barium chloride ($BaCl_2$) and sodium citrate ($Na_3C_6H_5O_7$) were purchased from Sigma-Aldrich, St. Louis, USA. Luria-Bertani (Lennox) broth and Bacto-agar were purchased from BD Biosciences, USA. de Man-Rogosa-Sharpe (MRS) broth and Live/Dead™ BacLight™ Bacterial Viability Kit were purchased from Thermo Fisher Scientific, USA.

2.2. Preparation of probiotics

To activate the probiotic organisms, a single colony of LGG/ECN was inoculated in sterile MRS/LB broth and incubated aerobically at 37 °C for 24 hours. Both bacteria grew to ~10^8 colony forming units/ml (CFU/ml). The cells were then washed once with 0.9% (w/v) NaCl and twice with sterile deionised water, with centrifugation at 10,000 xg for 5 min between each wash. Washed cells were then resuspended them in water at one-tenth of the original volume, for a ten-times concentrated probiotic mixture.

2.3. Addition of polymer, salts and cryoprotectant to probiotics

To evaluate the effect of the alginate material on LGG and ECN survivability, 900 µL of 2.22% (w/v) sodium alginate (ALG) was added to 100 µL of ten-times concentrated LGG/ECN mixture, yielding a final mixture of 2% (w/v) sodium alginate with ~10^8 CFU/ml cells. Salts, namely NaCl, $MgCl_2$,

$CaCl_2$, $SrCl_2$ and $BaCl_2$, were added similarly by mixing 900 µL of salt solution with 100 µL of ten-times concentrated LGG/ECN mixture, to yield a final salt concentration of 0.1M unless otherwise specified. For samples containing sucrose (Suc), sucrose was dissolved in the alginate/salt solutions, then added to the probiotic suspension at 10% (w/v) concentration. The control set indicates samples without any added salt or sucrose, i.e. probiotic cells suspended in sterile deionised water.

2.4. Encapsulation of probiotics in alginate

2.22% (w/v) sodium alginate with or without 11.11% (w/v) sucrose was mixed with ten-times concentrated LGG/ECN mixture at a 9:1 (v/v) ratio, yielding a mixture of 2% (w/v) sodium alginate and 10% (w/v) sucrose (or without sucrose) with ~10^8 CFU/ml cells. The cell suspension was extruded via a syringe pump (KD Scientific, USA) through a 26G needle into 40 ml of sterile 0.1M divalent cation (e.g. $CaCl_2$/$SrCl_2$/$BaCl_2$) crosslinking bath. $MgCl_2$ was not used as it was unable to crosslink the sodium alginate to form alginate beads. The crosslinking bath was kept stirring at 250 rpm using a magnetic stirrer during the extrusion process, and particles were left to crosslink for 5 min. Crosslinked alginate beads were then harvested and washed thrice in sterile water to remove any unencapsulated bacteria. Needle-to-crosslinking bath distance used was 1 cm and flow rate used was 1 ml/min.

2.5. Freeze-drying procedures

LGG/ECN with polymer, cryoprotectant and salt mixtures were frozen in liquid nitrogen (L. N_2) for 5 min or at -20 °C for 4 hours, before being transferred to the freeze-dryer equipment.

Crosslinked probiotic-containing alginate beads were suspended in water or 10% (w/v) sucrose in a 1:1 (v/v) ratio, then flash-frozen in L. N_2 and freeze-dried. The FreeZone 4.5 Plus freeze-dryer (Labconco, USA) was used in this experiment, operated with a -84 °C collector at 0.027 bar. All samples were dried for 72 hours.

2.6. Bacteria enumeration

Dried LGG/ECN with polymer, cryoprotectant and salt mixtures were rehydrated in 0.9% (w/v) NaCl and dissolved thoroughly. 0.9% (w/v) NaCl was deliberately chosen over phosphate buffered salts (PBS) solution as NaCl does not cause any precipitation of insoluble metal salts. A standard drop plating method was used to enumerate viable bacteria, where LGG was plated on MRS agar plates, while ECN was plated on LB (Lennox) agar plates. The plates were incubated in aerobic conditions at 37 °C for 48 hours (LGG) and 24 hours (ECN), then visible colonies were counted. To evaluate the CFU losses from the freezing step of selected samples, frozen samples were thawed at 37 °C for 20 min until fully liquid, before drop plating. For alginate-encapsulated probiotics, 0.2M sodium citrate was added to the beads, followed by 10 seconds of homogenisation to completely release entrapped cells. This method of homogenisation to release entrapped cells has been verified prior to this experiment as being non-deleterious towards LGG and ECN viability. The homogeniser used was Bio-Gen PRO200 Homogenizer (PRO Scientific, USA).

2.7. Flow cytometry

Flow cytometry was used to analyse the live/dead ratio of SYTO 9 and Propidium Iodide (PI) stained cells. Selected freeze-dried samples were rehydrated and washed thrice in 0.9% (w/v) NaCl to remove unbound sucrose and salts. Each sample was then diluted 1:100 times and stained with 1000-times diluted SYTO 9 and PI in 0.9% (w/v) NaCl. Samples were stained for 15 min in dark conditions.

Flow cytometric measurements were performed on BD Accuri™ C6 Plus equipped with 488 nm air-cooled argon laser (BD Biosciences, USA). For each measurement, forward scatter (FSC), side scatter (SSC), FL1 533/30 nm (SYTO 9) and FL3 > 670 nm (PI) fluorescence were recorded, amplified, and converted into digital signals for further analysis. Cells with intact cell membranes are stained with SYTO 9 and fluoresce more in the green channel, while cells with injured membranes exhibit significantly less green fluorescence and more red fluorescence(Thermo Fisher, 2004). A threshold of 1000 on FL1 was used to exclude unstained cell events.

All data were analysed using FlowJo version 10.6.1 (BD, USA). Gating of live and dead cells was done based on FL1 against FL3 channel readouts of 24 hours incubated live LGG/ECN or heat-killed LGG/ECN. The “injured” cells gate was considered to be the region between the live and dead cells population. % live/dead/injured cells were calculated based on the number of events that lie within the gated regions respectively, as a percentage of the total number of events.

3. Results

3.1. Effect of Ca-alginate hydrogel components on survivability of probiotics

The formation of freeze-dried alginate encapsulated LGG/ECN beads involved an initial gelation of alginate-probiotic mixtures by extrusion into a crosslinking bath. Ca^{2+} is the most common crosslinker used to form alginate hydrogels, and we are interested to evaluate if key components of the Ca-alginate hydrogel – the alginate material and Ca^{2+} ions – affect freeze-drying survivability of encapsulated LGG and ECN probiotics. The presence of the cryoprotectant sucrose was subsequently also investigated, and the results are presented in **Figure 1**.

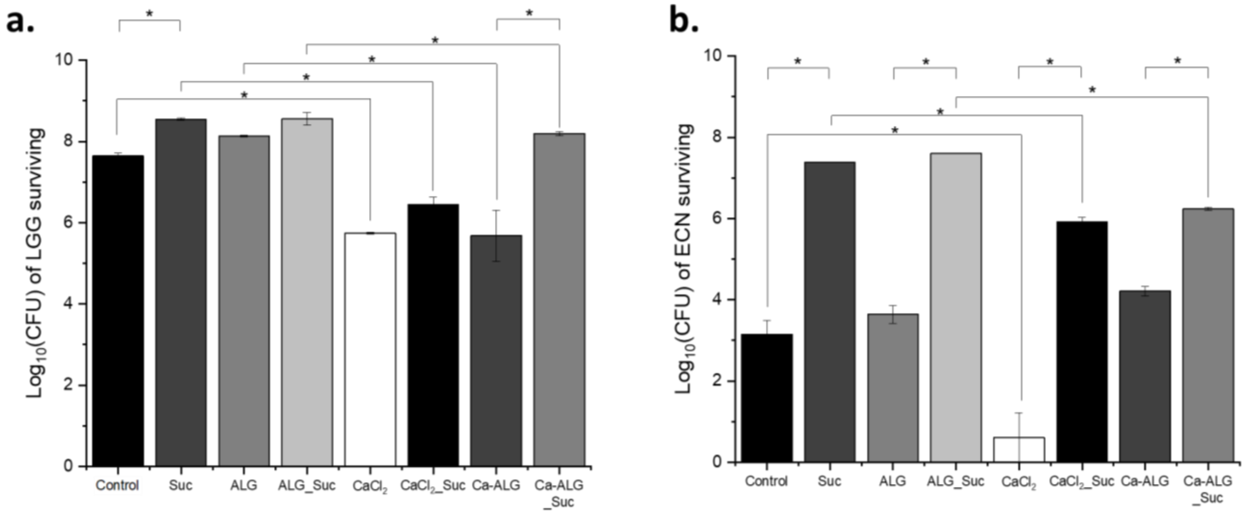

Figure 1. Log_{10}(CFU) of (a) LGG and (b) ECN surviving after freeze-drying in various Ca-alginate hydrogel components. Alginate was used at 2% (w/v), $CaCl_2$ at 0.1M and sucrose at 10% (w/v). Ca-ALG samples were alginate beads crosslinked in 0.1M $CaCl_2$ by the extrusion method. Initial average $log_{10}(CFU_0)$ of LGG and ECN were 8.60 and 8.30 respectively. Stars based on $p < 0.01$ (one-way analysis of variance (ANOVA) and post-hoc Tukey test, n = 3). Data are expressed as mean with standard error bars.

From **Figure 1**, It can be observed that LGG exhibited higher freeze-drying survivability than ECN. Lower freeze-drying survivability has been generally observed for Gram-negative bacteria than Gram-positive ones. This can be explained by the thinner peptidoglycan layer and the presence of lipopolysaccharides on the cell wall of the Gram-negative ECN bacteria (Miyamoto-Shinohara et al., 2008). Alginate appeared to be non-deleterious towards freeze-drying survivability of both LGG and ECN. However, the addition of 0.1M $CaCl_2$ significantly decreased both LGG and ECN survivability ($p < 0.01$). This suggests an antagonistic effect of the divalent Ca^{2+} ion during lyophilisation of both tested strains. LGG and ECN encapsulated in Ca-crosslinked alginate gels also showed lower survival than in un-crosslinked liquid alginates, substantiating the likely cause to be the crosslinking agent, i.e. $CaCl_2$.

Notably, the addition of 10% (w/v) sucrose enhanced survivability of both LGG and ECN for all samples. The addition of sucrose had a greater cryoprotective effect for ECN than LGG. Sucrose is a commonly used cryoprotectant for preservation of microbes and its mechanism of action is known to be its ability to lower the temperature of the membrane phase transition and to maintain protein structure in the dry state (Leslie et al., 1995; Saarela et al., 2005).

3.2. Effect of CaCl2 concentration and freezing rate

Since $CaCl_2$ showed significant antagonism towards freeze-drying survivability of LGG and ECN, the effect of this salt was further studied in different concentrations (0.5M, 0.1M, 0.05M, 0M) in **Figure 2**. The presence of the mono-cationic salt, NaCl, was also tested at 0.1M to determine if the antagonistic effect could be attributed to the divalent Ca^{2+} cation. As an additional step, LGG and ECN survival were determined after the freezing process (in which the frozen samples were thawed and enumerated) as well as after the freeze-drying process, to evaluate whether most of the CFU losses occurred in the freezing or drying step. The effect of freezing rate of cell suspensions was also studied using L. N_2 and at -20 °C as the freezing methods.

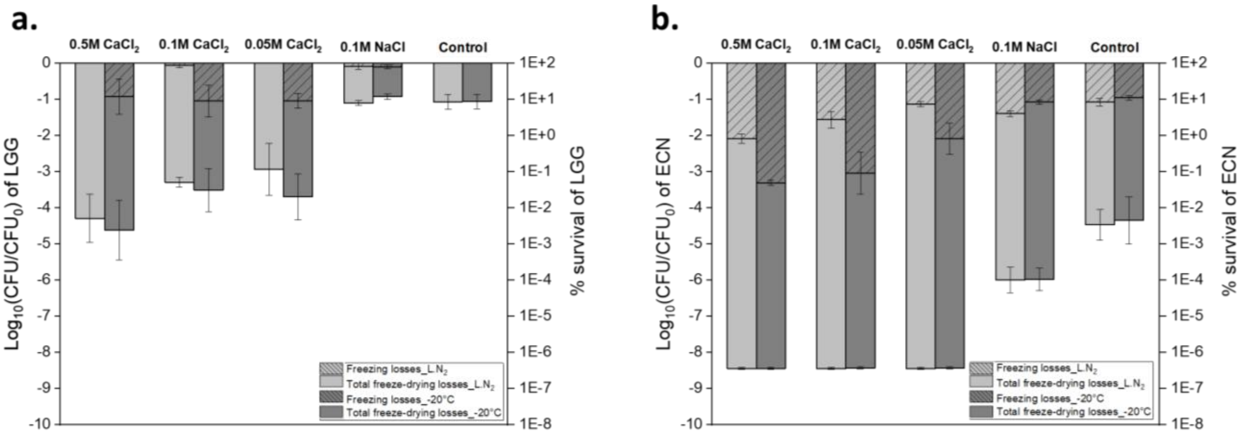

Figure 2. $Log_{10}(CFU/CFU_0)$ of (a) LGG and (b) ECN after freezing and after freeze-drying in presence of different salts. Initial average $log_{10}(CFU_0)$ of LGG and ECN were 8.60 and 8.30 respectively. Data are expressed as mean of triplicates with standard error bars.

LGG in the presence of $CaCl_2$ saw an average $log_{10}(CFU)$ reduction of between 3 to 5 for the various concentrations applied, while ECN in all concentrations of $CaCl_2$ (0.5M, 0.1M and 0.05M) did not survive the freeze-drying process (**Figure 2**). The effect of varying $CaCl_2$ concentration on LGG and ECN survivability was not pronounced, but a significant difference could be observed when comparing $CaCl_2$-containing samples versus NaCl-containing or control samples ($p < 0.05$). Higher viability losses were observed for both LGG and ECN in the presence of $CaCl_2$ as compared to the NaCl and control samples. In addition, it can be observed that 0.1M NaCl resulted in lower viability losses than 0.05M $CaCl_2$ for both LGG and ECN. In the presence of $CaCl_2$ salts, a slower rate of freezing at -20 °C was more antagonistic than L. N_2 freezing, resulting in higher CFU losses from the freezing step for both LGG and ECN. However, in terms of total freeze-drying losses, freezing at -20 °C and L. N_2 were found to yield similar freeze-drying losses.

3.3. Effect of different divalent ions

Different divalent-cation salts, namely $MgCl_2$, $SrCl_2$ and $BaCl_2$, in addition to $CaCl_2$, were studied to determine if they displayed similar antagonistic effects on freeze-drying survivability of LGG and ECN. Results are shown in **Figure 3**.

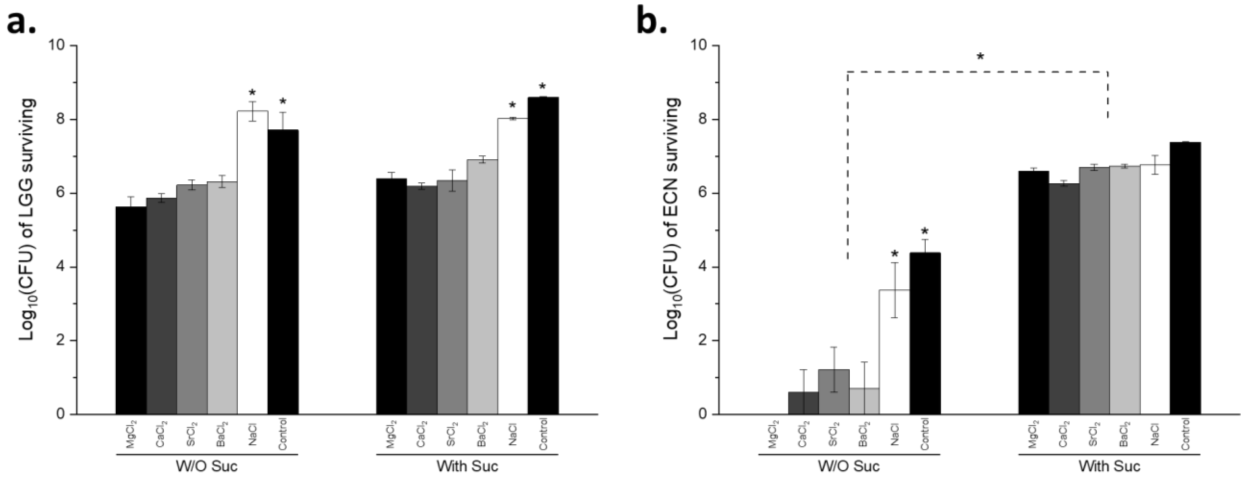

Figure 3. Log_{10}(CFU) of (a) LGG and (b) ECN surviving after freeze-drying in presence of various divalent ions. All salts were used at 0.1M concentration. Sucrose was used at 10% (w/v) concentration. Initial average $log_{10}(CFU_0)$ of LGG and ECN were 8.83 and 8.52 respectively. Stars based on $p < 0.05$ (one-way ANOVA and post-hoc Tukey test, n = 3). Star with dashed-line bracket

indicate significant difference between the two groups. Data are expressed as mean with standard error bars.

No significant difference in lyophilisation survivability was observed for LGG and ECN amongst the different divalent-cations, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}, used. However, a significant difference was observed for both LGG and ECN containing divalent cationic salts, versus samples with the monovalent salt NaCl or without any salts (control). This suggests a specific antagonism of divalent cations towards LGG and ECN freeze-drying survivability (see section 4 for a discussion of the literature on the antagonism of divalent cations). Nevertheless, the addition of 10% (w/v) sucrose greatly enhanced freeze-drying survivability of ECN for all the divalent-cations studied (3 to 6 $\log_{10}$(CFU) increase), whereas this effect was less pronounced for LGG (approximately 1 $\log_{10}$(CFU) increase).

3.4. Flow cytometry

The effect of divalent-cation salts on freeze-drying survivability of LGG and ECN was further studied using flow cytometry with live/dead staining (**Figures 4** and **5**, respectively). Different from CFU counts which register the number of culturable bacteria on nutrient-rich agar, live/dead staining probes the cell membrane integrity of bacteria cells, giving an insight to potential interactions between the salts and the bacteria membrane. In the live/dead stain used, PI penetrates only cells with disrupted membranes, and intercalates with DNA to emit a red fluorescence. SYTO 9 enters both live and dead cells, but when both PI and SYTO 9 are present, SYTO 9 is displaced by PI due to the latter's stronger affinity for nucleic acids (Stiefel et al., 2015).

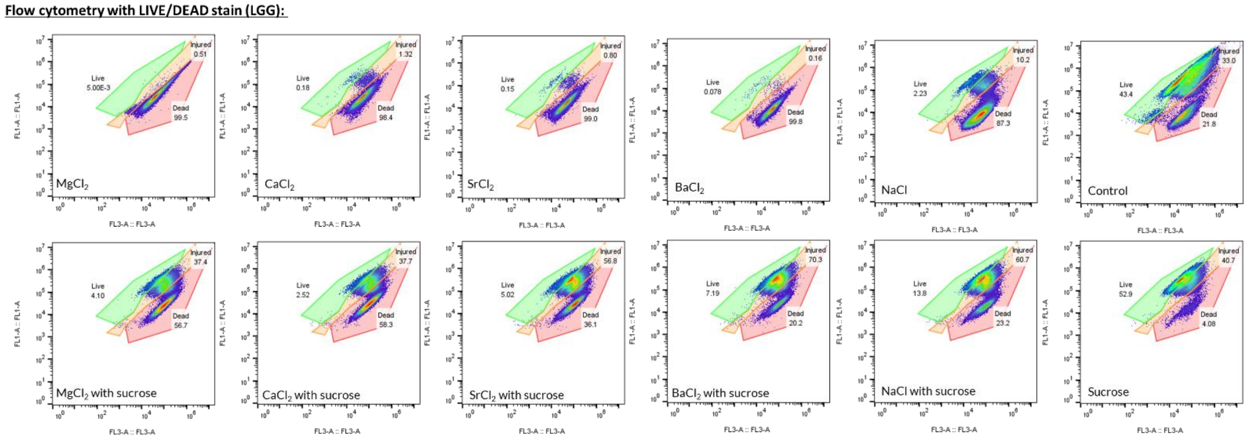

Figure 4. Flow cytometry dot plots for rehydrated freeze-dried LGG samples. FL1 (SYTO 9) vs. FL3 (PI) plots are depicted. Percentages of live, injured and dead populations are as indicated in each plot. All salts used were at 0.1M concentration, while sucrose was used at 10% (w/v) concentration. CFU of LGG added before freeze-drying was 6.8E8. (Figure to be coloured in print)

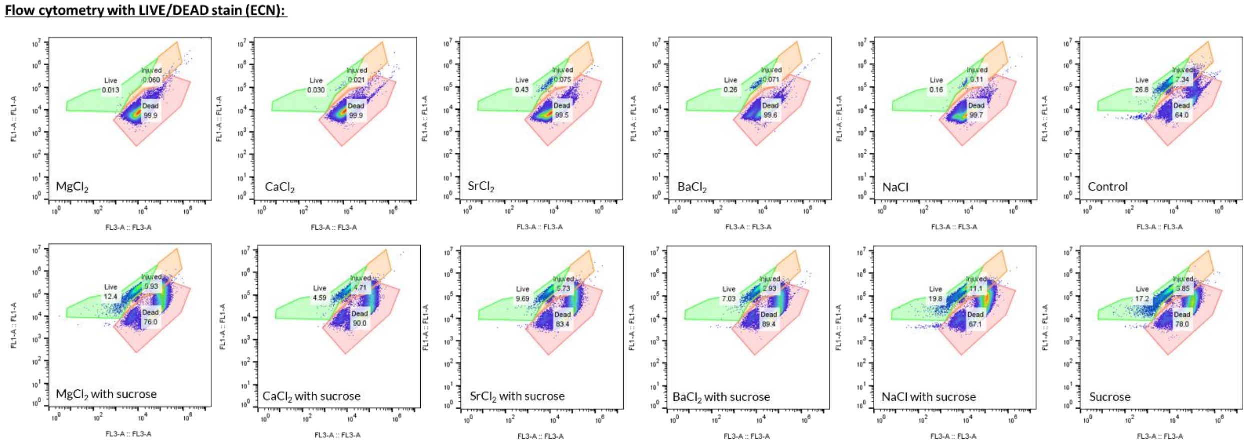

Figure 5. Flow cytometry dot plots for rehydrated freeze-dried ECN samples. FL1 (SYTO 9) vs. FL3 (PI) plots are depicted. Percentages of live, injured and dead populations are as indicated in each plot. All salts used were at 0.1M concentration, while sucrose was used at 10% (w/v) concentration. CFU of ECN added before freeze-drying was 3.3E8. (Figure to be coloured in print)

Bacterial populations as observed in the flow cytometry dot plots (**Figures 4** and **5**) were segregated into live, injured or dead segments, and their population percentages were determined. For LGG, NaCl and no-salt containing (control and sucrose) samples show higher preservation of membrane integrity as compared to divalent cationic salts-containing samples, which is consistent with CFU data reported in **Figure 3a**. A higher "live" population of LGG was observed for samples containing 10% (w/v) sucrose. Data for divalent salts with sucrose also show a decreasing "dead" cell population from Ca (58.3%) to Sr (36.1%) to Ba (20.2%), indicating that larger divalent ions may be less disruptive towards LGG cell membranes. In **Figure 3a**, a similar trend of increasing LGG survivability in terms of average CFU count has also been observed for Ca < Sr < Ba, although the increase was not found to be significant by statistical analysis.

For ECN, higher preservation of membrane integrity was similarly observed for samples with 10% (w/v) sucrose added than samples without sucrose. This observation corresponds well with the CFU data presented in **Figure 3b**. For samples without sucrose, a significantly larger "live" population was observed for control samples (26.8%) as compared to salt-containing samples (0.01-0.5%). No obvious difference in live/dead populations could be observed between samples with different salts used.

3.5. Encapsulating probiotics into alginate

Earlier data suggests that divalent ions have a significant antagonism towards freeze-drying survivability of LGG and ECN probiotics. The effects of different divalent ions were so far not significantly different towards LGG and ECN lyophilisation survivability. In order to study the effect of these ions, in the presence of alginate matrix, LGG and ECN were respectively encapsulated in Ca/Sr/Ba-crosslinked alginate beads and freeze-dried. $MgCl_2$ was not used as a crosslinker as it was unable to crosslink the sodium alginate to form alginate beads. For samples without sucrose, 2% (w/v) alginate was used and beads were suspended in water before freeze-drying. For samples containing sucrose, 2% (w/v) alginate and 10% (w/v) sucrose were used for encapsulation, and crosslinked beads were suspended in 10% (w/v) sucrose before freeze-drying. The results are presented in **Figure 6**.

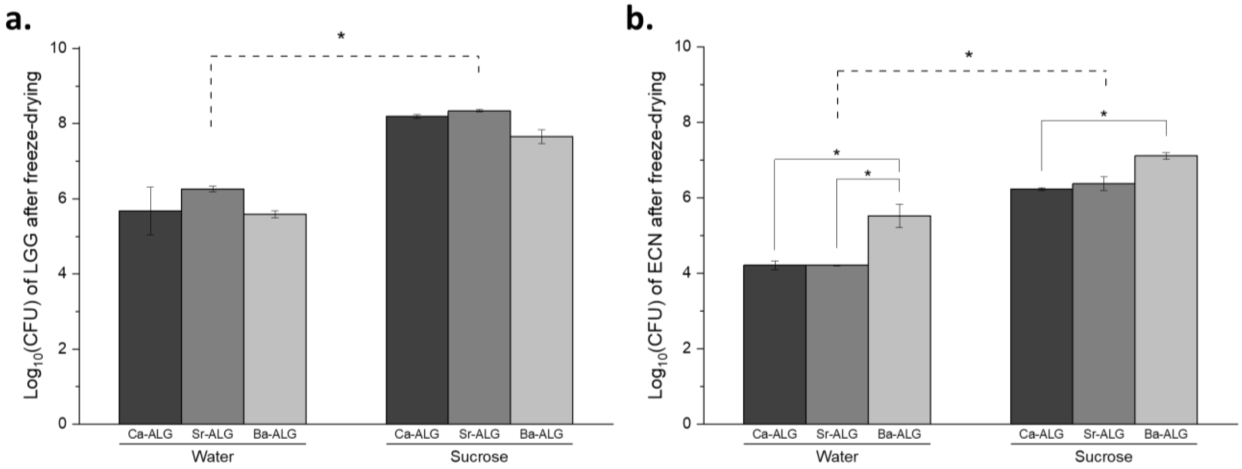

Figure 6. Log_{10}(CFU) of (a) LGG and (b) ECN encapsulated in crosslinked alginate beads after freeze-drying. Initial average log_{10}(CFU) of encapsulated LGG and ECN per sample were 8.78 and 8.34 respectively. Letters on bars based on $p < 0.05$ (one-way ANOVA and post-hoc Tukey test, n = 3). Data are expressed as mean with standard error bars.

As observed from **Figure 6**, similarly, significant difference in lyophilisation survivability could be observed for both LGG and ECN in the presence of 10% (w/v) sucrose as compared to without sucrose. This, concurrent with the observations made earlier, suggests that the presence of sucrose can reverse the deleterious effects of divalent cations on lyophilisation survivability. No significant difference in survivability was observed for LGG across Ca-/Sr-/Ba-crosslinked alginates.

For ECN, encapsulation in Ba-alginate beads showed enhanced survival upon freeze-drying. This trend is contrary to earlier data in **Figures 3** and **5**, where no significant differences in survivability of unencapsulated ECN were observed between Ba salt-containing samples versus Ca or Sr. This suggests that an additional effect, associated with the action of crosslinking, could have led to the enhanced freeze-drying survivability of ECN in the presence of Ba. One possibility is the increased protection conferred by Ba-crosslinked alginate due to the higher degree of crosslinking (refer to Supplementary **Figure S3**) by the Ba ion than Sr and Ca (Mørch et al., 2006).

4. Discussion

This study aimed to identify the antagonistic factors that result in poor freeze-drying survivability of probiotics encapsulated in alginate beads. Variables including: 1) alginate as encapsulant material, 2) sucrose as cryoprotectant, 3) $CaCl_2$ as cross-linker, 4) Concentration of $CaCl_2$, and 5) other Group II divalent cations as cross-linkers, were investigated. The results determined that the alginate as an encapsulant material was non-deleterious towards LGG and ECN survival (**Figure 1**), which is consistent with general knowledge that alginate is a non-toxic and bio-compatible material (U.S. Food & Drug Administration, n.d.). Strong evidence in the data points to the antagonistic role of divalent cations in reducing lyophilisation survival of probiotics LGG and ECN, whereby divalent cations Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} were found to be more antagonistic than monovalent cation Na^+ and un-salted samples. For the gram-positive bacteria LGG, flow cytometry data showed a decreasing "dead" population for Ca > Sr > Ba samples, indicating decreasing cell membrane disruption of LGG cells in the order Ca > Sr > Ba. However, such a trend was not observed for ECN. Overall, LGG exhibited higher survival than ECN after freeze-drying, consistent with other trends observed in which Gram-negative bacteria tends to have lower desiccation resistance than Gram-positive (Janning & in't Veld, 1994; Miyamoto-Shinohara et al., 2008).

The effect of divalent cations on bacteria survivability has been studied in literature(Choi et al., 2018; Clifton et al., 2015; Fontana et al., 1979; Xie & Yang, 2016; Zheng et al., 2015), albeit not in the context of freeze-drying. Freeze-drying consists of two main steps. In the initial freezing process, water within and surrounding the cells gradually converts into ice, and solutes accumulate in residual free water. This results in a localised increase in salt concentration, much higher than the original salt concentration added (supersaturation). In the second drying step, the ice formed after freezing is removed by conversion from solid to vapor, through sublimation (De Paoli, 2005). In this study, a slower freezing rate at -20 °C was found to be more antagonistic than L. N_2 freezing, resulting in higher CFU losses from the freezing step for both LGG and ECN (**Figure 2**). A similar observation has also been made by (Zhao & Zhang, 2005), where *Lactobacillus brevis* and *Oenococcus oeni* strains showed increased viability when frozen quickly at -65 °C as compared to -20 °C. The higher viability at a faster freezing rate is likely due to a less solute concentration effects. However, in terms of total freeze-drying losses, freezing at -20 °C and L. N_2 were found to yield similar freeze-drying losses for the tested conditions (**Figure 2**). Additionally, all three concentrations of $CaCl_2$ salt (0.5M, 0.1M, 0.05M) showed similar antagonism towards both LGG and ECN when frozen in liquid nitrogen conditions (**Figure 2**), indicating that 0.05M $CaCl_2$ was sufficient to induce significant freeze-drying viability losses. Also interesting is that 0.1M NaCl resulted in lower viability losses than 0.05M $CaCl_2$ for both LGG and ECN, although osmolarity of the former (0.2 Osmol/L NaCl) was higher than the latter (0.15 Osmol/L $CaCl_2$). This suggests that the divalent cation plays a bigger role than osmolarity in causing lyophilisation losses for both LGG and ECN.

Current literature of the effects of divalent cations on bacteria survivability presents two conflicting sides. Some suggest that divalent cations enhance bacteria survivability, such as in (Fontana *et al.*, 1979), which reported that when Mg^{2+} and Ca^{2+} were removed from Gram-negative *Klebsiella pneumoniae* Mir M7 cocci with EDTA treatment, the cocci rigid layer collapsed and lost any definite morphology. This led to the conclusion that "the accumulation of divalent cations appeared necessary for the peptidoglycan to acquire sufficient rigidity for shape determination and cell protection". The role of divalent ions Mg^{2+} and Ca^{2+} in outer membrane stabilisation of Gram-negative bacteria has been further studied in (Clifton *et al.*, 2015). (Choi *et al.*, 2018) reported a similar observation – removal of Mg^{2+} and Ca^{2+} from a cryoprotective soy powder reduced the survivability of lyophilised Gram-positive *Lactobacillus brevis* WiKim0069. Choi *et al* suggests that the influx of cations may have increased viability by the activation of calcineurin or by minimising the damage caused by osmotic pressure. (Zheng *et al.*, 2015) studied the mechanisms of the protective effects of reconstituted skim milk (RSM) during convective droplet drying of lactic acid bacteria. The authors suggested that the protective effect in RSM could be attributed to Ca^{2+}, as it stabilises cellular structures under heat stress, possibly the cytoplasmic membrane. These studies suggest that divalent cations enhance the survival of bacteria upon drying. In both studies by Fontana *et al* and Choi *et al* however, focus was on the removal of divalent cations, and the effect of adding excess divalent cations was not studied.

Divalent cations have been reported in other studies to have an antagonistic role towards bacteria cells. (Xie & Yang, 2016) reported a disruptive role of Ca^{2+} and Mg^{2+} on *Staphylococcus aureus* membranes through binding of the divalent cation with cardiolipin, a major lipid component of *S. aureus* membranes. The cardiolipin synthase gene has been identified in LGG (Kwon et al., 2018) and the *E. coli* species (Renner & Weibel, 2011), and it is possible that the divalent cations had interacted similarly with cardiolipin molecules in LGG and ECN membranes, resulting in loss of viability. Another possibility is the interaction of divalent ions with the anionic groups of other lipids, proteins or capsular polysaccharides present on the cell surface of bacteria. Differences in electronegativities of Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} could have resulted in these respective ions interacting to different extents with the polar groups on bacteria surfaces.

The strong evidence obtained from this work on the deleterious effect of divalent ions during freeze drying, aided to understand some of the survivability losses observed previously (Brachkova *et al*., 2010; Cheow & Hadinoto, 2013; Donthidi *et al*., 2010; Etchepare *et al.*, 2016; Halim *et al*., 2017; Martin-Dejardin *et al*., 2013) for alginate encapsulated probiotics. Being essential components in encapsulation formulations to crosslink alginate gels, the observed deleterious effects of divalent cations towards probiotics lyophilisation survivability has to be overcome, to enhance the usefulness of the encapsulation system. The inclusion of 10% (w/v) sucrose in the formulation of alginate beads showed enhanced freeze-drying survivability of encapsulated probiotics (**Figure 6**), indicating that a cryoprotectant like sucrose can reverse the deleterious effects of divalent cations on lyophilisation survivability. This may be due to interactions between the disaccharide sucrose and the bacteria membrane. Sucrose is known to have a stabilising effect on cell membranes upon drying, and one of the explanations for this effect is that sucrose molecules intercalate between lipid headgroups and form cross-linking hydrogen bonds with them (Stachura et al., 2019). This mechanism may have interfered with the binding of divalent cations with the lipid component of LGG and ECN cell membranes, hence mitigating the antagonism of divalent cations upon lyophilisation.

Comparing different divalent cations as crosslinkers in alginate encapsulation, Ba-crosslinked alginates exhibit higher survivability of ECN than Ca- and Sr-crosslinked alginates. For LGG, Ca-/Sr-/Ba-alginates were not found to be significantly different towards LGG lyophilisation survivability. It is possible that the higher degree of crosslinking of Ba-crosslinked alginates may have conferred a cryoprotective property on ECN, although this mechanism remains to be elucidated. However, considering the importance of non-toxicity in probiotic formulations, the use of cryoprotectant sucrose is still preferable to Ba^{2+} ions for ECN-alginate formulations. Barium compounds are known to pose human toxicity and are generally not recommended for ingestion (Moffet *et al*., 2007). In comparison, Ca and Sr have been utilised in commercial health supplements. Overall, Ca^{2+} and Sr^{2+} ions are recommended to be used for alginate-crosslinking in probiotic formulations, with the addition of sucrose to enhance freeze-drying survivability.

5. Conclusion

Divalent cations, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} were found to be antagonistic towards freeze-drying survival of probiotics LGG and ECN. Reduced freeze-drying survival of LGG and ECN was observed in presence of divalent cations, as compared to monovalent cations and without salts. Higher CFU losses from the freezing step was observed at a slower freezing rate in the presence of divalent cations for both LGG and ECN. The antagonism of divalent cations is likely the main reason behind low probiotics survivability in freeze-dried alginate-encapsulated formulations. The addition of the sucrose cryoprotectant in alginate formulations was found to reverse the deleterious effects of divalent cations and restore viability of freeze-dried encapsulated LGG and ECN formulations. Between Ca-/Sr-/Ba-crosslinked alginates, freeze-drying survivability of LGG was not found to be significantly different, whereas ECN showed enhanced survival in Ba-crosslinked alginates. Overall, the use of calcium or strontium ions for alginate-crosslinking with the addition of sucrose in probiotic formulations is recommended.

Acknowledgements

The first author is a recipient of the Nanyang President Graduate Scholarship from Nanyang Technological University, Singapore. This work was also supported by the Singapore Centre for Environmental Life Sciences Engineering (SCELSE) (MOE/RCE: M4330019.C70), Ministry of Education AcRF grant (RG19/18 & RT08/19), Agri-Food & Veterinary Authority of Singapore (APF

LCK102), Biomedical Research Council (BMRC) – Therapeutics Development Review (TDR-G-004-001), NTU-HSPH grant (NTU-HSPH 17002), and the Bill and Melinda Gates Foundation (OPP1199116).

References

Brachkova, M. I., Duarte, M. A., & Pinto, J. F. (2010). Preservation of viability and antibacterial activity of Lactobacillus spp. in calcium alginate beads. *European Journal of Pharmaceutical Sciences*, *41*(5), 589–596. https://doi.org/10.1016/j.ejps.2010.08.008

Burgain, J., Gaiani, C., Linder, M., & Scher, J. (2011). Encapsulation of probiotic living cells: From laboratory scale to industrial applications. In *Journal of Food Engineering*. https://doi.org/10.1016/j.jfoodeng.2010.12.031

Chavarri, M., Maranon, I., & Carmen, M. (2012). Encapsulation Technology to Protect Probiotic Bacteria. In *Probiotics*. https://doi.org/10.5772/50046

Cheow, W. S., & Hadinoto, K. (2013). Biofilm-like Lactobacillus rhamnosus probiotics encapsulated in alginate and carrageenan microcapsules exhibiting enhanced thermotolerance and freeze-drying resistance. *Biomacromolecules*, *14*(9), 3214–3222. https://doi.org/10.1021/bm400853d

Choi, I., Kim, H., Wi, S., Chun, H., Hwang, I., Choi, H.-J., & Park, H. (2018). Effect of Cation Influx on the Viability of Freeze-Dried Lactobacillus brevis WiKim0069. *Applied Sciences*, *8*(11), 2189. https://doi.org/10.3390/app8112189

Clifton, L. A., Skoda, M. W. A., Le Brun, A. P., Ciesielski, F., Kuzmenko, I., Holt, S. A., & Lakey, J. H. (2015). Effect of divalent cation removal on the structure of gram-negative bacterial outer membrane models. *Langmuir*, *31*(1), 404–412. https://doi.org/10.1021/la504407v

De Paoli, P. (2005). Biobanking in microbiology: From sample collection to epidemiology, diagnosis and research. In *FEMS Microbiology Reviews* (Vol. 29, Issue 5, pp. 897–910). John Wiley & Sons, Ltd. https://doi.org/10.1016/j.femsre.2005.01.005

Donthidi, A. R., Tester, R. F., & Aidoo, K. E. (2010). Effect of lecithin and starch on alginate-encapsulated probiotic bacteria. *Journal of Microencapsulation*, *27*(1), 67–77. https://doi.org/10.3109/02652040902982183

Etchepare, M. de A., Raddatz, G. C., Cichoski, A. J., Flores, É. M. M., Barin, J. S., Queiroz Zepka, L., Jacob-Lopes, E., Grosso, C. R. F., & de Menezes, C. R. (2016). Effect of resistant starch (Hi-maize) on the survival of Lactobacillus acidophilus microencapsulated with sodium alginate. *Journal of Functional Foods*, *21*, 321–329. https://doi.org/10.1016/j.jff.2015.12.025

FAO/WHO. (2001). Probiotics in food: Health and nutritional properties and guidelines for evaluation. *Food and Nutrition Paper*. https://doi.org/10.1201/9781420009613.ch16

Fontana, R., Canepari, P., & Satta, G. (1979). Alterations in peptidoglycan chemical composition associated with rod-to-sphere transition in a conditional mutant of Klebsiella pneumoniae. *Journal of Bacteriology*, *139*(3), 1028–1038. http://www.ncbi.nlm.nih.gov/pubmed/113382

Guimarães, R. R., Vendramini, A. L. do A., Santos, A. C. dos, Leite, S. G. F., & Miguel, M. A. L. (2013). Development of probiotic beads similar to fish eggs. *Journal of Functional Foods*, *5*(2), 968–973. https://doi.org/10.1016/j.jff.2013.01.002

Halim, M., Mohd Mustafa, N. A., Othman, M., Wasoh, H., Kapri, M. R., & Ariff, A. B. (2017). Effect of encapsulant and cryoprotectant on the viability of probiotic Pediococcus acidilactici ATCC 8042 during freeze-drying and exposure to high acidity, bile salts and heat. *LWT - Food*

Science and Technology, *81*, 210–216. https://doi.org/10.1016/j.lwt.2017.04.009

Janning, B., & in't Veld, P. H. (1994). Susceptibility of bacterial strains to desiccation: a simple method to test their stability in microbiological reference materials. *Analytica Chimica Acta*. https://doi.org/10.1016/0003-2670(94)85092-5

Kim, S. J., Cho, S. Y., Kim, S. H., Song, O. J., Shin, I. S., Cha, D. S., & Park, H. J. (2008). Effect of microencapsulation on viability and other characteristics in Lactobacillus acidophilus ATCC 43121. *LWT - Food Science and Technology*, *41*(3), 493–500. https://doi.org/10.1016/j.lwt.2007.03.025

Kwon, Y. W., Bae, J.-H., Kim, S.-A., & Han, N. S. (2018). Development of Freeze-Thaw Tolerant Lactobacillus rhamnosus GG by Adaptive Laboratory Evolution. *Frontiers in Microbiology*, *9*, 2781. https://doi.org/10.3389/fmicb.2018.02781

Lee, K. Y., & Mooney, D. J. (2012). Alginate: properties and biomedical applications. *Progress in Polymer Science*, *37*(1), 106–126. https://doi.org/10.1016/j.progpolymsci.2011.06.003

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., & Crowe, L. M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology*. https://doi.org/10.1128/aem.61.10.3592-3597.1995

Marques da Silva, T., Jacob Lopes, E., Codevilla, C. F., Cichoski, A. J., Flores, É. M. de M., Motta, M. H., da Silva, C. de B., Grosso, C. R. F., & de Menezes, C. R. (2018). Development and characterization of microcapsules containing Bifidobacterium Bb-12 produced by complex coacervation followed by freeze drying. *LWT - Food Science and Technology*. https://doi.org/10.1016/j.lwt.2017.12.057

Martin-Dejardin, F., Ebel, B., Lemetais, G., Nguyen Thi Minh, H., Gervais, P., Cachon, R., &

Chambin, O. (2013). A way to follow the viability of encapsulated Bifidobacterium bifidum subjected to a freeze-drying process in order to target the colon: Interest of flow cytometry. *European Journal of Pharmaceutical Sciences*, *49*(2), 166–174. https://doi.org/10.1016/j.ejps.2013.02.015

Meng, X. C., Stanton, C., Fitzgerald, G. F., Daly, C., & Ross, R. P. (2008). Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2007.04.076

Miyamoto-Shinohara, Y., Sukenoba, J., Imaizumi, T., & Nakahara, T. (2008). Survival of freeze-dried bacteria. *Journal of General and Applied Microbiology*, *54*(1), 9–24. https://doi.org/10.2323/jgam.54.9

Moffet, D., Smith, C., Stevens, Y., Ingerman, L., Swarts, S., & Chappell, L. (2007). Toxicological profile for barium and barium compounds. In *Agency for toxic substances and disease registry*.

Mørch, Ý. A., Donati, I., Strand, B. L., & Skjåk-Bræk, G. (2006). Effect of Ca2+, Ba2+, and Sr2+ on alginate microbeads. *Biomacromolecules*, *7*(5), 1471–1480. https://doi.org/10.1021/bm060010d

Raddatz, G. C., Poletto, G., Deus, C. de, Codevilla, C. F., Cichoski, A. J., Jacob-Lopes, E., Muller, E. I., Flores, E. M. M., Esmerino, E. A., & de Menezes, C. R. (2020). Use of prebiotic sources to increase probiotic viability in pectin microparticles obtained by emulsification/internal gelation followed by freeze-drying. *Food Research International*, *130*, 108902. https://doi.org/10.1016/j.foodres.2019.108902

Ramos, P. E., Silva, P., Alario, M. M., Pastrana, L. M., Teixeira, J. A., Cerqueira, M. A., & Vicente,

A. A. (2018). Effect of alginate molecular weight and M/G ratio in beads properties foreseeing the protection of probiotics. *Food Hydrocolloids*, *77*, 8–16. https://doi.org/10.1016/J.FOODHYD.2017.08.031

Renner, L. D., & Weibel, D. B. (2011). Cardiolipin microdomains localize to negatively curved regions of Escherichia coli membranes. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(15), 6264–6269. https://doi.org/10.1073/pnas.1015757108

Ross, R. P., Desmond, C., Fitzgerald, G. F., & Stanton, C. (2005). Overcoming the technological hurdles in the development of probiotic foods. *Journal of Applied Microbiology*. https://doi.org/10.1111/j.1365-2672.2005.02654.x

Saarela, M., Virkajärvi, I., Alakomi, H. L., Mattila-Sandholm, T., Vaari, A., Suomalainen, T., & Mättö, J. (2005). Influence of fermentation time, cryoprotectant and neutralization of cell concentrate on freeze-drying survival, storage stability, and acid and bile exposure of Bifidobacterium animalis ssp. lactis cells produced without milk-based ingredients. *Journal of Applied Microbiology*. https://doi.org/10.1111/j.1365-2672.2005.02742.x

Stachura, S. S., Malajczuk, C. J., & Mancera, R. L. (2019). Does Sucrose Change Its Mechanism of Stabilization of Lipid Bilayers during Desiccation? Influences of Hydration and Concentration. *Langmuir*. https://doi.org/10.1021/acs.langmuir.9b03086

Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K., & Ren, Q. (2015). Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. *BMC Microbiology*, *15*(1), 36. https://doi.org/10.1186/s12866-015-0376-x

Terpou, A., Papadaki, A., Lappa, I. K., Kachrimanidou, V., Bosnea, L. A., & Kopsahelis, N. (2019).

Probiotics in food systems: significance and emerging strategies towards improved viability and delivery of enhanced beneficial value. In *Nutrients* (Vol. 11, Issue 7). MDPI AG. https://doi.org/10.3390/nu11071591

Thermo Fisher, S. (2004). *LIVE/DEAD BacLight Bacterial Viability and Counting Kit*. https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fmp34856.pdf&title=TElWRS9ERUFEICZsdDtpJmd0O0JhYyZsdDsvaSZndDtMaWdodCBCYWN0ZXJpYWwgVmlhYmlsaXR5IGFuZCBDb3VudGluZyBL

U.S. Food & Drug Administration. (n.d.). *CFR - Code of Federal Regulations Title 21 Part 184 -- Direct Food Substances Affirmed as Generally Recognized as Safe*. Retrieved March 10, 2020, from http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=50.25

Xie, Y., & Yang, L. (2016). Calcium and Magnesium Ions Are Membrane-Active against Stationary-Phase Staphylococcus aureus with High Specificity. *Scientific Reports*, *6*(1), 1–8. https://doi.org/10.1038/srep20628

Ying, D. Y., Phoon, M. C., Sanguansri, L., Weerakkody, R., Burgar, I., & Augustin, M. A. (2010). Microencapsulated Lactobacillus rhamnosus GG Powders: Relationship of Powder Physical Properties to Probiotic Survival during Storage. *Journal of Food Science*, *75*(9), E588–E595. https://doi.org/10.1111/j.1750-3841.2010.01838.x

Zhao, G., & Zhang, G. (2005). Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying. *Journal of Applied Microbiology*. https://doi.org/10.1111/j.1365-2672.2005.02587.x

Zheng, X., Fu, N., Duan, M., Woo, M. W., Selomulya, C., & Chen, X. D. (2015). The mechanisms of the protective effects of reconstituted skim milk during convective droplet drying of lactic acid bacteria. *Food Research International*, *76*, 478–488. https://doi.org/10.1016/j.foodres.2015.07.045