In situ alginate crosslinking during spray-drying of lactobacilli probiotics promotes gastrointestinal-targeted delivery

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A B S T R A C T

Alginate-based formulations have shown desirable functional characteristics for probiotic encapsulation. However, current technologies used to produce these formulations are inefficient, detrimental to probiotics viability or do not produce dry, shelf-stable products. Herein, we developed a novel spray-drying technique that combines particle formation, alginate crosslinking and drying into a single step, thereby streamlining the production of encapsulated probiotics powder. *Lacticaseibacillus rhamnosus* GG (LGG) encapsulated in six encapsulation formulations were characterized and compared. Among the six formulations investigated, the crosslinked alginate with sucrose formulation (Ca-Alg-Suc) was found to be most promising, achieving $\sim 10^9$ CFU/g of surviving LGG after spray-drying and exposure to simulated gastric fluid (SGF). The Ca-Alg-Suc formulation was further evaluated with *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei*, and similar results of high post-spray-drying and post-SGF viabilities were obtained. Successful encapsulation of different lactobacilli probiotics via the proposed spray-drying technique highlights potential of this procedure to be scaled up for commercial applications.

1. Introduction

Probiotics, defined as "live microorganisms which when administered in sufficient amounts, confer a health benefit on the host" (World Health Organization (WHO) & Food and Agriculture Organization (FAO), 2001), have received escalating interest in recent years. Various therapeutic and prophylactic benefits on human hosts in aspects of digestive, immunity and neurological health have been attributed to probiotic use (Shreiner et al., 2015). Consequently, global consumer demand for probiotic products has seen a significant surge, and numerous innovations in probiotic formulation techniques have sprouted to improve on functional attributes of probiotic products. Lactobacilli are among the most used probiotics due to their beneficial properties and generally safe to consume nature, and lactobacilli probiotics for oral administration have been incorporated in various product formats including dietary supplements, such as in capsules, tablets or sachets, and functional food products, such as dairy, confectionary or beverages.

In the design of suitable probiotic formulations, encapsulation has emerged as a useful method to imbue functional characteristics such as enhance probiotics viability, improve shelf life, or confer gastro-protective effects. Spray-drying is a commercially viable technique which has been used in encapsulation of probiotics (Assadpour & Jafari, 2019; Huang et al., 2017). Via the combined action of atomization, heat and vacuum drying (Broeckx et al., 2016), spray-drying generates dried powder particles rapidly in a single-step, making spray-drying a highly efficient and economical technique. The resultant dry powder product is additionally advantageous in ways such as having prolonged shelf life, able to be versatilely incorporated into different product types, and able to be transported more efficiently.

In this paper, the use of alginate for probiotics encapsulation via spray-drying is explored. Alginate, a polysaccharide comprising (1,4)-linked α-(L)-guluronic and β-(D)-mannuronic acids, is a well-known material widely used for probiotics encapsulation. Advantages of

alginate include that it is natural, biocompatible, generally recognized as safe and low cost (Lee & Mooney, 2012). Crosslinking of alginate is achieved by exposure to multivalent cation crosslinkers, typically Ca^{2+}, and crosslinked alginate hydrogels are known to confer gastric acid-protective effects on entrapped microorganisms (Cheow & Hadinoto, 2013; Guimarães et al., 2013; Kim et al., 2008; Ramos et al., 2018). Crosslinked alginates also facilitate intestine-targeted release of encapsulated microorganisms, as higher concentration of phosphate ions in the small intestine sequesters cation crosslinkers (Zhou et al., 2001).

Prior alginate-based probiotic formulations mostly focused on utilizing fabrication techniques such as extrusion (Cook et al., 2011; Gu et al., 2019), emulsification (Song et al., 2013; Yus et al., 2019), layer-by-layer encapsulation (Anselmo et al., 2016) and impinging aerosols (Sohail et al., 2013). Resultant particles formed via these techniques are in the "wet" state, and an additional drying step, such as by air drying, freeze-drying, fluidized bed drying or vacuum foam drying, is required to yield a final dry product. A milling or micronization step is also often performed after drying to yield a homogenous, fine powder product. Comparing these techniques to spray-drying, the major advantage which spray-drying offers is the ability to produce dry fine powder particles directly in a single step, which greatly streamlines and simplifies the formulation process. Having a single-step process can also minimize contamination risks and transfer losses, thereby ensuring higher quality and better yield of the resultant probiotic product.

Despite the advantages of using the spray drying technique for live microorganism encapsulation, there are various challenges associated with it. Firstly, the high temperature setting and rapid dehydration during the spray-drying process can greatly reduce probiotics survivability (Broeckx et al., 2016; Huang et al., 2017). To overcome this issue, various protective agents have been proposed in prior studies, including sucrose, inulin, trehalose, maltodextrin, skim milk and whey protein (Assadpour & Jafari, 2019; Huang et al., 2017). These materials showed varying extents of improvement on probiotics survivability; however, few investigations were done with regards to the gastroprotective effect of these materials. Secondly, the method of crosslinking alginate in a spray dryer setup can be quite tricky. Since spray-drying directly produces dry powder, it is counter-productive to spray-dry an alginate-probiotic slurry followed by crosslinking in an aqueous solution, as an extra step would still be required to dry the formulation. Feeding a crosslinked alginate formulation directly into the spray-dryer inlet would also be infeasible as high viscosity of the crosslinked gel may result in nozzle clogging. A method of spray-drying crosslinked alginate particles has been described by (Kawakita et al., 2021a, 2021b; Santa-Maria et al., 2012; Strobel et al., 2016; Strobel et al., 2018; Wong et al., 2020), however this method involves the use of a volatile base to facilitate the crosslinking process, which is potentially deleterious to many pH-sensitive probiotic microorganisms. By far, no methods of spray-drying with in situ alginate crosslinking for encapsulating live microorganisms for oral administration purposes has been described.

Herein, we examine a novel spray-drying technique of probiotics encapsulation which utilizes coaxial spraying for alginate crosslinking. Sucrose is used as a protective agent, and six formulations, namely 1) Sucrose only [Suc], 2) Alginate only [Alg], 3) Alginate with sucrose [Alg-Suc], 4) Crosslinked calcium-alginate [Ca-Alg], 5) Crosslinked calcium-alginate with sucrose [Ca-Alg-Suc] and 6) Crosslinked calcium-alginate with sucrose, using inverted feed channels [Ca-Alg-Suc-IFC] were studied with use of *Lacticaseibacillus rhamnosus* GG (LGG) probiotics. Survivability of probiotics after spray-drying, simulated gastric fluid (SGF) exposure and storage, were assessed. The resulting best performing formulation was additionally adapted for encapsulating other lactobacilli probiotics, namely *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei*. Findings of this study could provide relevant industries in food or pharmaceutical sectors with an improved method to produce encapsulated lactobacilli probiotic powder with gastroprotective properties, and thereby enhancing the survivability of the probiotic through the gastrointestinal tract.

2. Materials and methods

2.1. Materials

LGG was isolated from a purchased Culturelle® LGG probiotic pill (i-Health, Inc., USA). *L. plantarum* and *L. paracasei* were in-house strains isolated from fermented food sources. Protanal® LFR5/60 sodium alginate (M/G = 30/70, M_w = 20–60 kDa) was procured from FMC Biopolymer, USA. De Man-Rogosa-Sharpe (MRS) broth and Live/Dead™ BacLight™ Bacterial Viability Kit were purchased from Thermo Fisher Scientific, USA. All other chemicals used in this experiment were purchased from Sigma Aldrich, USA.

2.2. Methods

2.2.1. Growth and preparation of probiotics

Single colonies of LGG, *L. plantarum* and *L. paracasei* were inoculated in sterile MRS broths and incubated aerobically at 37 °C for 24 h (for LGG and *L. plantarum*) and at 30 °C for 72 h (for *L. paracasei*), wherein a stationary phase cell concentration of approximately 10^8–10^9 colony forming units/ml (CFU/ml) was attained for each bacterium. The probiotic cells were then washed thrice with 0.9% (w/v) NaCl, with centrifugation at 10,000 ×*g* for 5 min between each wash. Next, the probiotic cell pellets were resuspended in 0.9% NaCl at one-tenth of the initial inoculated volume to obtain a 10× concentrated probiotic sample. Drop plating on MRS agar was performed to determine the initial CFU concentration.

2.2.2. Preparation of alginate-probiotic slurry and crosslinking agent

2.22% (w/v) sodium alginate with or without 11.11% (w/v) sucrose was dissolved in deionized (DI) water and autoclaved (121 °C, 15 min) in advance. The 10× concentrated probiotics suspension was then added in a 1:9 (v:v) ratio with the alginate-sucrose polymeric slurry and thoroughly mixed, thereby yielding a final 2% (w/v) alginate with or without 10% (w/v) sucrose with 10^8–10^9 CFU/ml of probiotics. 10 mM calcium chloride was used as the crosslinking agent, and similarly prepared by dissolution in DI water and sterilized by autoclaving. 10 mM calcium chloride was chosen as higher concentrations of calcium chloride were observed to cause frequent spray-drier nozzle blockage.

2.2.3. Encapsulation of probiotics via spray-drying

The Buchi-290 spray-dryer (Buchi AG, Switzerland) operating in co-current mode was set up with assembly of the Buchi three-fluid coaxial nozzle (inner nozzle diameter: 1.4 mm, outer diameter: 2.5 mm), similar to that described in (Kharel et al., 2021). Nitrogen gas flow was turned on to 0.742 m^3/h for nozzle flow and 35 m^3/h for aspirator flow. Prior to spraying probiotics, the spray-dryer equipment was pre-heated at 140 °C to sterilize and dry-out any residual moisture in the apparatus. Next, the inlet temperature of the spray-dryer was set to 120 °C, and the alginate-probiotics slurry and calcium chloride crosslinking agent were respectively fed into the inner and outer feed channels. 1.5 ml/min inner channel flow rate and flow rate ratio (inner:outer) of 1:2 was used. For samples Suc, Alg and Alg-Suc, which did not require crosslinking, no solutions were fed through the outer channel. For the Ca-Alg-Suc-IFC formulation, the feeds of the inner and outer channels were switched, such that the alginate-probiotics slurry was fed to the outer channel while the crosslinking agent was fed to the inner channel, and flow rate ratio (inner:outer) was accordingly 2:1. Batch volumes of 25–50 ml of alginate-probiotics slurry were prepared at one go. Dried probiotics powders were generated in the collection chamber, and were maintained at outlet temperatures ranging from 62 to 79 °C for 20–35 min before collection.

2.2.4. Characterization of spray-dried powder

2.2.4.1. Extent of alginate crosslinking. The extent of alginate cross-linking was determined using the Periodic Acid Schiff's (PAS) assay adapted from (Houghton et al., 2014; Strobel et al., 2018). The aim of this assay was to determine the ratio of soluble alginate (un-crosslinked)

to total alginate. 10 mg of Ca-Alg and 60 mg of Ca-Alg-Suc particles, not containing any probiotics, were added into 5 ml of water and 5 ml of 0.05 M of sodium citrate separately. Particles were rotated at 30 rpm for 1 h at room temperature to dissolve the alginate. Samples were then centrifuged at 13,300 x*g* for 10 min, and the supernatants were diluted with dilution factors of 2× and 10× for water-dissolved and citrate-dissolved samples respectively. Alginate standards with concentrations of 0.3 to 0.025 mg/ml were also prepared. 200 μl of samples and standards were transferred to a 96-well plate, and 20 μl of 50% (*w/v*) periodic acid in 7% (*v*/v) acetic acid was added. Schiff's reagent was prepared by dissolving 16.7 mg of sodium metabisulfite per ml of Schiff's fuchsin-sulfite reagent. Both the plate and Schiff's reagent were then incubated at 37 °C for 1 h. Next, 20 μl of Schiff's reagent was added to each well and the plate was incubated for another 1 h at room temperature to allow color development. Absorbance of the samples was measured at 550 nm using Infinite M200 microplate reader (Tecan Group Ltd., Switzerland). The extent of crosslinking was computed as follows:

$$Extent\ of\ crosslinking\ (\%) = \left(1 - \frac{Concentration\ of\ soluble\ alginate\ in\ water}{Concentration\ of\ soluble\ alginate\ in\ citrate}\right) \times 100 \tag{1}$$

2.2.4.2. Powder yield measurement. Powder yields were determined based on the mass of the spray-dried powder collected relative to the total mass of solids within the formulation, as according to the equation shown below:

$$Powder\ yield\ (\%) = \frac{Mass\ of\ spray - dried\ powder\ collected}{Original\ mass\ of\ solids\ in\ sample} \times 100 \tag{2}$$

2.2.4.3. Scanning electron microscopy. Morphologies of the spray-dried powders were observed using a JEOL JSM-6360 Scanning Electron Microscope (SEM) (JEOL Ltd., Japan), with an accelerating voltage of 5 kV in the secondary electron mode. Spray-dried powders were mounted to a stub with carbon tape and coated with 10 nm of conductive gold prior to imaging.

2.2.4.4. Epifluorescence microscopy. Viability staining of spray-dried probiotic powder was performed using Live/Dead™ BacLight™ Bacterial Viability Kit. Selected spray-dried samples were rehydrated and washed thrice in 0.9% NaCl to remove any un-crosslinked polymer or probiotics. Each sample was then stained with

500-times diluted SYTO 9 and propidium iodide (PI) in 0.9% NaCl. Samples were stained for 15 min in dark conditions then fluorescently imaged using the Axio Observer Z1 Inverted Microscope (Carl Zeiss AG, Germany).

2.2.4.5. Residual moisture test. Spray-dried powders were further dehydrated in a VO400 vacuum oven (Memmert GmbH + Co. KG, Germany) for 3 days. Residual moisture content of powders was determined by gravimetric analysis based on the formula below:

$$Residual\ moisture\ content\ (\%) = \frac{Initial\ weight\ of\ powder - Dried\ weight\ of\ powder}{Initial\ weight\ of\ powder} \times 100 \tag{3}$$

2.2.4.6. Enumeration of probiotics after spray-drying. Viable probiotic counts following spray-drying were evaluated by dissolving the powder in 0.05 M sodium citrate followed by drop-plating on MRS agars. LGG and *L. plantarum* were incubated at 37 °C, aerobic conditions for 48 h, and *L. paracasei* was incubated at 30 °C, aerobic conditions for 72 h. Bacterial colonies at specific dilution factors were counted and surviving probiotics were calculated in CFU/g.

2.2.4.7. Exposure to SGF. The encapsulated probiotic powders were exposed to SGF to determine their ability to persist in the human gastric environment. SGF was prepared as 0.2 M NaCl, 2000 units/ml porcine pepsin, pH 2, and 50 ± 2 mg of spray-dried powders were added to 5 ml of SGF. Other tested powder: SGF ratios include 25 ± 1 mg: 5 ml and 15 ± 0.6 mg: 7.5 ml. SGF adjusted to pH 2.5 and pH 3, were also tested for the Suc samples, to determine the intrinsic acid resistance of various probiotics. Samples were incubated at 37 °C, 200 rpm shaking conditions for 2 h. Viable probiotic counts after SGF exposure were determined by dissolution in 0.05 M sodium citrate and drop-plating.

2.2.4.8. Storage viability testing. Storage viabilities of spray-dried powders were determined over a 4- and 8-week period. To maintain a dry environment, MiniPax absorbent packets (Sigma-Aldrich, USA) were placed as desiccants together with the spray-dried powders. Samples were stored at 4 °C refrigerated or 25 °C (room temperature) conditions. Following storage, viable probiotic counts were determined by dissolution in 0.05 M sodium citrate and drop-plating. Samples were also exposed to SGF pH 2 after storage, following the method described in Section 2.2.4.7, to determine if gastroprotective effects of the formulations were retained after storage.

2.2.5. Statistical analysis

Results were expressed as mean of triplicates with standard deviation bars. For the statistical evaluation of numerical data, one way analysis of variance and post-hoc Tukey test was used. Letters on bars were based on * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns – no significant difference.

3. Results and discussion

3.1. Extent of alginate crosslinking in Ca-Alg and Ca-Alg-Suc

In this paper, a novel method of in situ alginate crosslinking via coaxial spray-drying was proposed. The efficacy of this alginate crosslinking process was determined using the PAS assay. Based on the
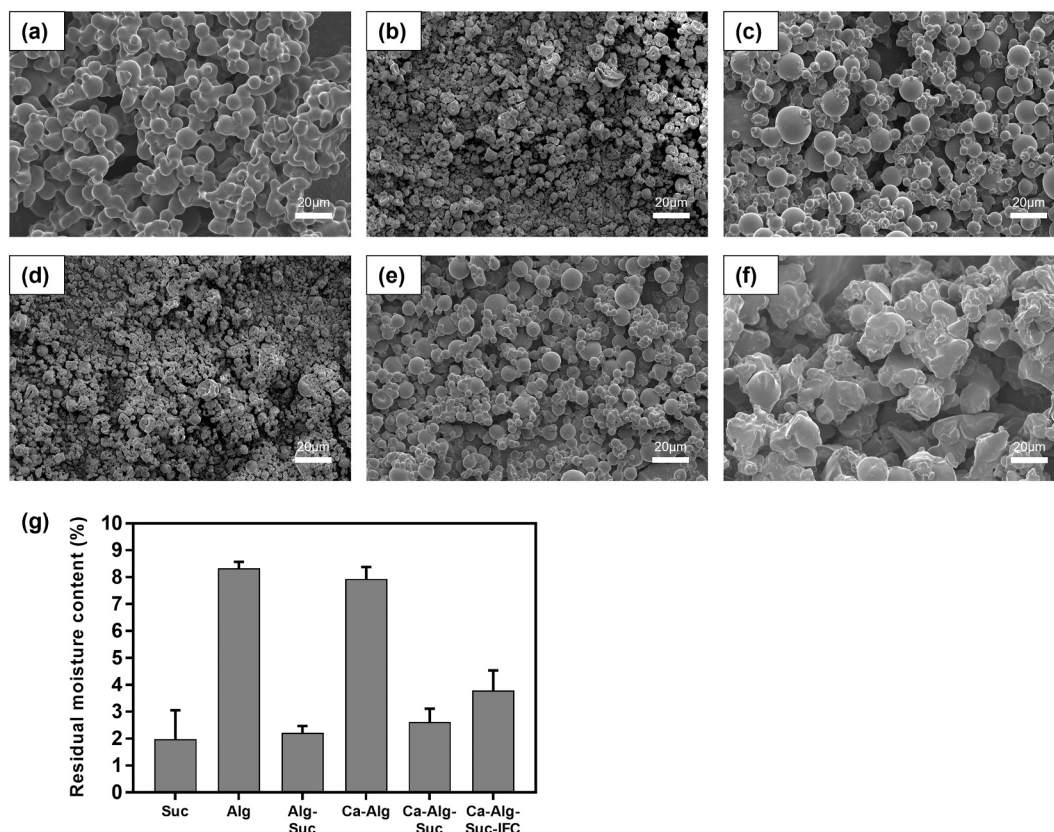
Fig. 1. Physical properties of spray-dried *L. rhamnosus* GG (LGG) in various formulations. SEM images of (a) Sucrose (Suc), (b) Alginate (Alg), (c) Alginate-sucrose (Alg-Suc), (d) Calcium-alginate (Ca-Alg), (e) Calcium-alginate-sucrose (Ca-Alg-Suc) and (f) Calcium-alginate-sucrose, using inverted feed channels (Ca-Alg-Suc-IFC) are included. Residual moisture content in various formulations are described in (g).

concentration of soluble alginate released from Ca-Alg and Ca-Alg-Suc powders, the extent of crosslinking was calculated and expressed as a percentage of insoluble crosslinked alginate that remained in the polymer matrix. Extent of crosslinking were 92.49% and 99.12% for Ca-Alg and Ca-Alg-Suc, respectively, indicating that the majority of alginate polymers were crosslinked via the described spray-drying technique. Addition of sucrose did not hamper the alginate crosslinking process. Instead, better crosslinking was achieved for the Ca-Alg-Suc formulation, possibly due to effects of sucrose in modifying the alginate crosslinking process. Sucrose has been reported in other studies to modify alginate crosslinking by acting as an intermolecular inert spacer (Al-Remawi, 2012). Overall, high degree of alginate crosslinking was achieved by the coaxial spray-drying method for both Ca-Alg and Ca-Alg-Suc formulations, and following sections describe characteristics of probiotics-loaded spray-dried formulations.

3.2. Physical characterization of spray-dried powders

Powder yields ranged from 58 to 75%, consistent with typical yields of B-290 spray-driers (Kemp et al., 2016). SEM morphologies of spray-dried LGG-loaded powders are shown in Figs. 1a-f. Particle sizes ranged between 2 and 34 μm, within the expected size range of powders produced from the B-290 spray-drier (Büchi, 2012). Alg (Fig. 1b) and Ca-Alg (Fig. 1d) powders appeared smaller with textured surfaces, while sucrose-containing formulations were larger and had smoother surfaces. This is likely due to the lower solids content in formulations without sucrose, hence individual atomized droplets of Alg and Ca-Alg had lower solid mass and yielded smaller particles. The Suc formulation (Fig. 1a) showed irregular powder morphologies, seemingly caused by agglomeration or coalescence of several atomized particles. Addition of 2% alginate, as in the Alg-Suc sample (Fig. 1c), yielded more spherical and

separate particles, possibly due to differences in viscosities and glass transition temperatures for alginate-containing samples (Verdurmen et al., 2006). Apparent differences were also observed between Ca-Alg-Suc (Fig. 1e) and Ca-Alg-Suc-IFC (Fig. 1f) particles. Ca-Alg-Suc-IFC particles were significantly larger granules with lumpy surface characteristics, and these particles do not appear well separated as compared to Ca-Alg-Suc. This suggests that the inversion of feed channels had a significant impact on crosslinking and particle formation. The effects of calcium crosslinking (compare Alg and Ca-Alg or Alg-Suc and Ca-Alg-Suc) did not show significant differences in powder morphology.

Residual moisture contents of spray-dried powders are presented in Fig. 1g. Alg and Ca-Alg powders showed high residual moisture content of ~8%, significantly higher than the sucrose-containing formulations, which showed residual moisture of <4%. Such is likely due to more water solvent present in formulations without sucrose, hence the Alg and Ca-Alg samples were not dried as thoroughly during the spray-drying process. Comparing Ca-Alg-Suc and Ca-Alg-Suc-IFC, Ca-Alg-Suc-IFC showed higher residual moisture. This may be due to some moisture from the calcium chloride crosslinking solution being trapped within the Ca-Alg-Suc-IFC powder, as the crosslinking solution was fed via the inner channel in the Ca-Alg-Suc-IFC sample. Overall, residual moisture content of <4% were considered good-quality for spray-dried powders (Ananta et al., 2005), and the Suc, Alg-Suc and Ca-Alg-Suc formulations fulfil this criterion.

3.3. Survivability of LGG after spray-drying

Viable CFU counts of LGG after spray-drying are reported in Fig. 2a. All six formulations yielded $>10^9$ CFU/g of surviving LGG after spray-drying. The Alg sample yielded highest CFU/g, due to the lower solids content in Alg as compared to sucrose-containing samples, which
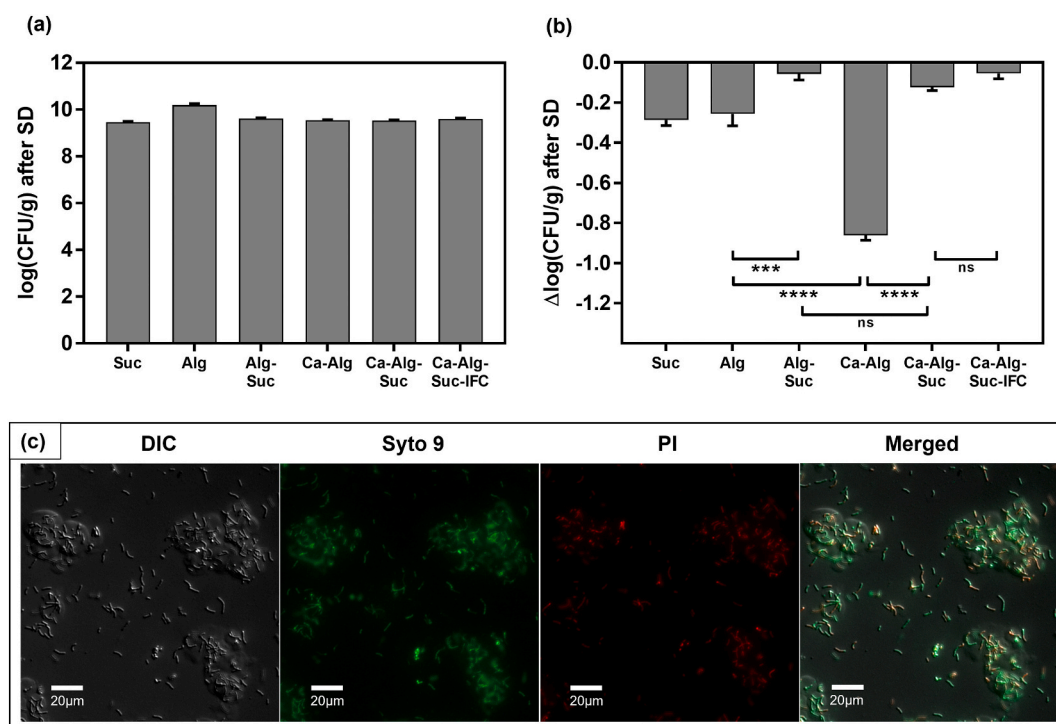
Fig. 2. Survivability of *L. rhamnosus* GG (LGG) in various formulations post-spray-drying. (a) $Log_{10}(CFU/g)$ data of LGG in various formulations following spray-drying. (b) Change in $log_{10}(CFU/g)$ counts of LGG in various formulations, as compared to before spray-drying. (c) Epifluorescence microscopy image of live/dead stained LGG bacteria in calcium-alginate-sucrose (Ca-Alg-Suc) matrix.
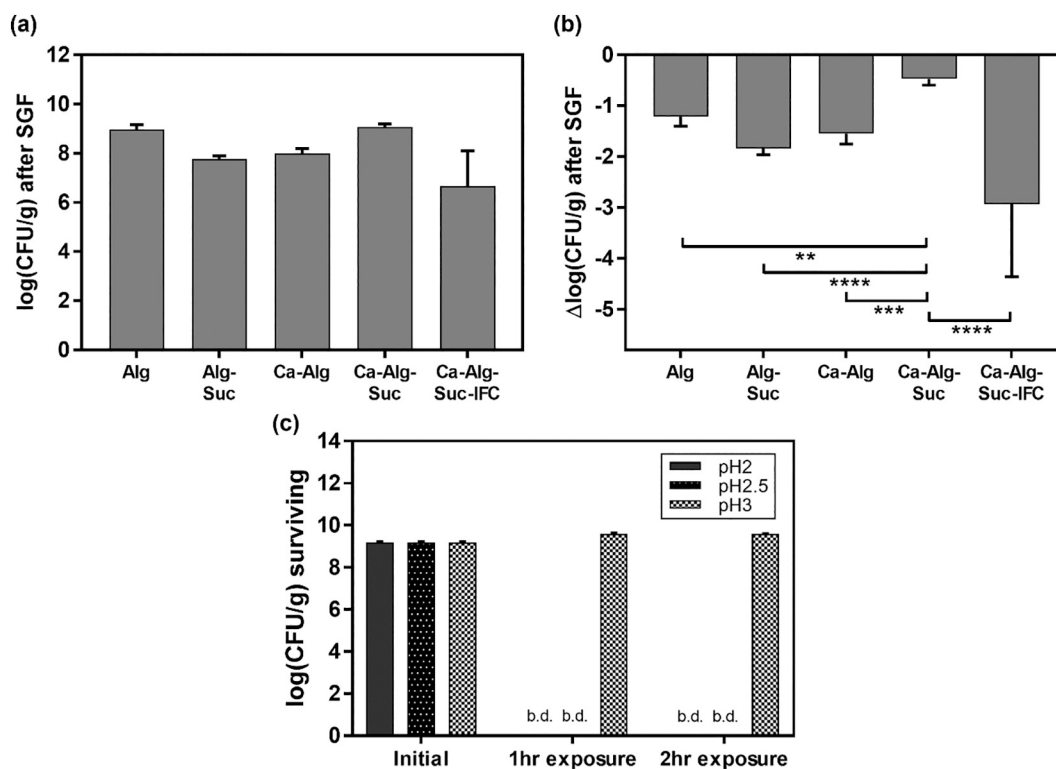

Fig. 3. Survivability data of *L. rhamnosus* GG (LGG) in various spray-dried formulations after exposure to simulated gastric fluid (SGF). (a) $Log_{10}(CFU/g)$ data of LGG in various spray-dried formulations following exposure to SGF pH 2. (b) Change in $log_{10}(CFU/g)$ counts of LGG in various formulations after SGF pH 2 exposure. (c) $Log_{10}(CFU/g)$ data of LGG in Suc formulation, exposed to SGF of various pH. B.d. indicates that the cell count is below the detection limit of ~1.5 $log_{10}(CFU/g)$.

contributed to higher initial CFU per dry weight in formulations without sucrose. The CFU losses graph (Fig. 2b) provides a better representation of the CFU reduction for various formulations associated with the spray-drying process. Comparing Alg versus Alg-Suc and Ca-Alg versus Ca-Alg-Suc, formulations with sucrose retained significantly higher viability counts of LGG, indicating that sucrose was effective as a protective agent in enhancing LGG survival during the spray-drying process. This is consistent with prior studies, as sucrose is known to enhance desiccation tolerance of microorganisms by stabilizing their cellular membrane and proteins (Goderska, 2012; Marcial-Coba et al., 2019). Comparing crosslinked and un-crosslinked samples (Alg vs Ca-Alg and Alg-Suc vs Ca-Alg-Suc), calcium crosslinking greatly reduced LGG survivability in Alg samples, but did not have a significant impact on LGG survivability in Alg-Suc. A similar phenomenon has been observed in freeze-dried crosslinked alginate, where divalent cations, including Ca^{2+}, demonstrated antagonistic properties in reducing desiccation survivability of probiotics (Tan et al., 2020). In the same study (Tan et al., 2020), sucrose was found to reverse the deleterious effects of divalent cations and restore viability of probiotics in freeze-dried calcium alginate particles. Possibly, a similar mechanism of Ca^{2+} antagonism towards LGG survivability occurred in this spray-drying process, and the addition of sucrose as a protective agent alleviated this effect. Between Ca-Alg-Suc and Ca-Alg-Suc-IFC samples, no significant differences in survivability of LGG were found.

Fig. 2c shows an epifluorescence image of rehydrated Ca-Alg-Suc with live/dead stained LGG. As seen, Ca-Alg-Suc particles formed insoluble gels upon rehydration in aqueous saline, indicating that crosslinking was effectively achieved during the coaxial spray-drying process. LGG bacteria were seen embedded within crosslinked Ca-Alg-Suc matrices with strong fluorescence in the SYTO 9 channel, indicating good preservation of LGG membrane integrity following spray-drying.

3.4. Survivability of LGG in SGF

Various spray-dried formulations were exposed to SGF, and the consequent viable LGG counts are presented in Fig. 3. Fig. 3c indicates survivability of LGG in the Suc formulation, i.e. without alginate added, when exposed to SGF pH 2, 2.5 and 3 for 2 h. As seen, LGG in the Suc formulation showed complete susceptibility to pH 2 and pH 2.5 within 1 h of SGF exposure, indicating that sucrose alone did not confer gastro-protective effects on spray-dried LGG. Formulations which incorporated alginate provided higher survivability of LGG. In Fig. 3a, the Alg and Ca-Alg-Suc formulations retained highest viability of LGG after SGF pH 2 exposure, with $\sim 10^9$ CFU/g LGG surviving. From Fig. 3b, the Ca-Alg-Suc matrix was identified as best performing in conferring gastroprotection to LGG, with lowest CFU/g losses of 0.47 $\log_{10}$(CFU/g) following SGF exposure. Significant LGG survivability differences were identified between the un-crosslinked Alg-Suc and crosslinked Ca-Alg-Suc samples, indicating that the effect of crosslinking was important in enhancing the gastric acid-protective ability of the alginate matrix. Comparing Ca-Alg-Suc and Ca-Alg-Suc-IFC, Ca-Alg-Suc-IFC performed significantly worse in protecting LGG against SGF, possibly due to improper alginate crosslinking. Coaxially spray-dried particles involving multiple feed solutions are known to confer particles with heterogenous core-shell-like inner structures (Kašpar et al., 2013). In the IFC formulation, the alginate-probiotics slurry was fed to the outer channel while the calcium chloride crosslinking agent was fed to the inner channel. This process
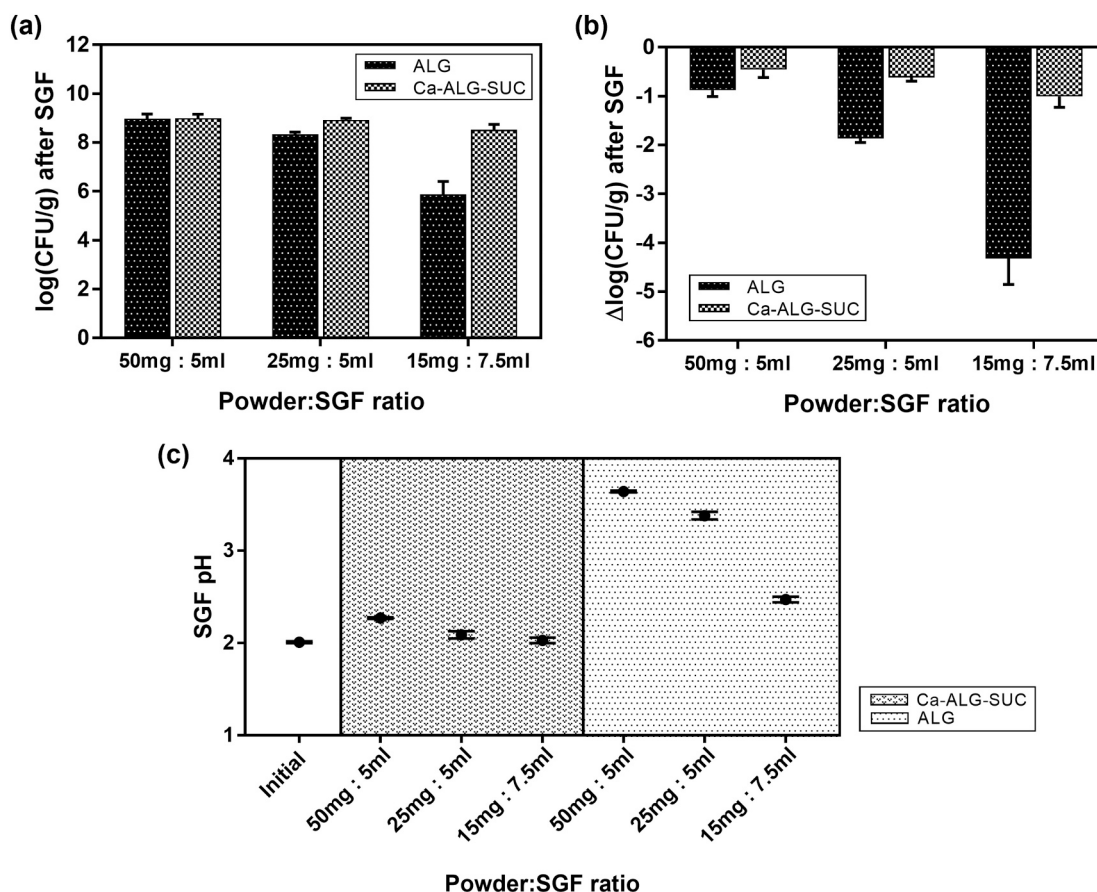

Fig. 4. Survivability data of *L. rhamnosus* GG (LGG) in various formulations after exposure to simulated gastric fluid (SGF) in varying powder:SGF ratios. (a) Log$_{10}$(CFU/g) data of LGG in un-crosslinked (Alg) and crosslinked (Ca-Alg-Suc) formulations exposed to SGF in varying powder:SGF ratios. (b) Change in log$_{10}$(CFU/g) counts of LGG, as compared to before SGF exposure. (c) pH of SGF media following addition of Ca-Alg-Suc and Alg powder.

likely yielded Ca-Alg-Suc-IFC particles which were crosslinked at the core, but un-crosslinked near the surface, resulting in significant viability losses of LGG which were present near the particle surface. In contrast, Ca-Alg-Suc particles were likely to be better crosslinked at its surface, hence forming a calcium alginate surface layer which better protects encapsulated LGG from gastric acid insults.

The effect of alginate crosslinking on gastroprotection of entrapped probiotics was further tested by exposing the un-crosslinked Alg and crosslinked Ca-Alg-Suc formulations to SGF in varying powder mass to SGF volume ratios. As seen in Fig. 4a, high $\log_{10}$(CFU/g) counts were obtained for the crosslinked Ca-Alg-Suc formulation, even with decreasing powder mass to SGF volume ratio. In contrast, there was a considerable decline in viable LGG counts for the un-crosslinked Alg formulation, from 9 $\log_{10}$(CFU/g) to 5.9 $\log_{10}$(CFU/g), with decreasing powder mass added relative to SGF volume. Likewise in Fig. 4b, changes in the CFU/g reduction of viable LGG in Ca-Alg-Suc powder were minor compared to the significant changes in the CFU/g reduction of viable LGG in Alg powder, for decreasing powder mass ratios. Final pH values of the SGF milieu after incubation with the spray-dried powders were also measured and documented in Fig. 4c. pH values of SGF containing Ca-Alg-Suc powder did not increase significantly from the original pH 2 and were relatively constant with different powder mass to SGF volume

ratios. However, for Alg, SGF pH was substantially increased to pH 3.7 upon addition at 50 mg: 5 ml ratio, and a considerable decline in SGF pH values was observed with decreasing powder mass to SGF volume ratio.

In acidic pH, alginate is known to sequester protons and convert to insoluble alginic acid, thereby achieving a pH buffering effect which promotes survivability of encapsulated probiotics (Lee & Mooney, 2012). Data from Fig. 4 suggest that the un-crosslinked Alg formulation conferred gastroprotection to encapsulated LGG primarily through buffering pH of the entire gastric fluid environment. Accordingly, as the powder mass to SGF volume decreased, the pH buffering capacity of un-crosslinked Alg was hampered, thereby exposing LGG to the detrimental acidic pH, causing significant viability losses. The pH buffering capacity of crosslinked Ca-Alg-Suc was however, independent of the SGF volume, likely due to a localized pH buffering effect achieved within each spray-dried particle, facilitated by the formation of crosslinked calcium alginate gels surrounding each particle. The crosslinked calcium alginate surface layer has potentially immobilized encapsulated probiotics within the matrix. By the conversion of alginate to alginic acid, excess protons in the vicinity of embedded LGG were sequestered, thereby maintaining a localized pH higher than the external SGF milieu, and maintaining high LGG viability.

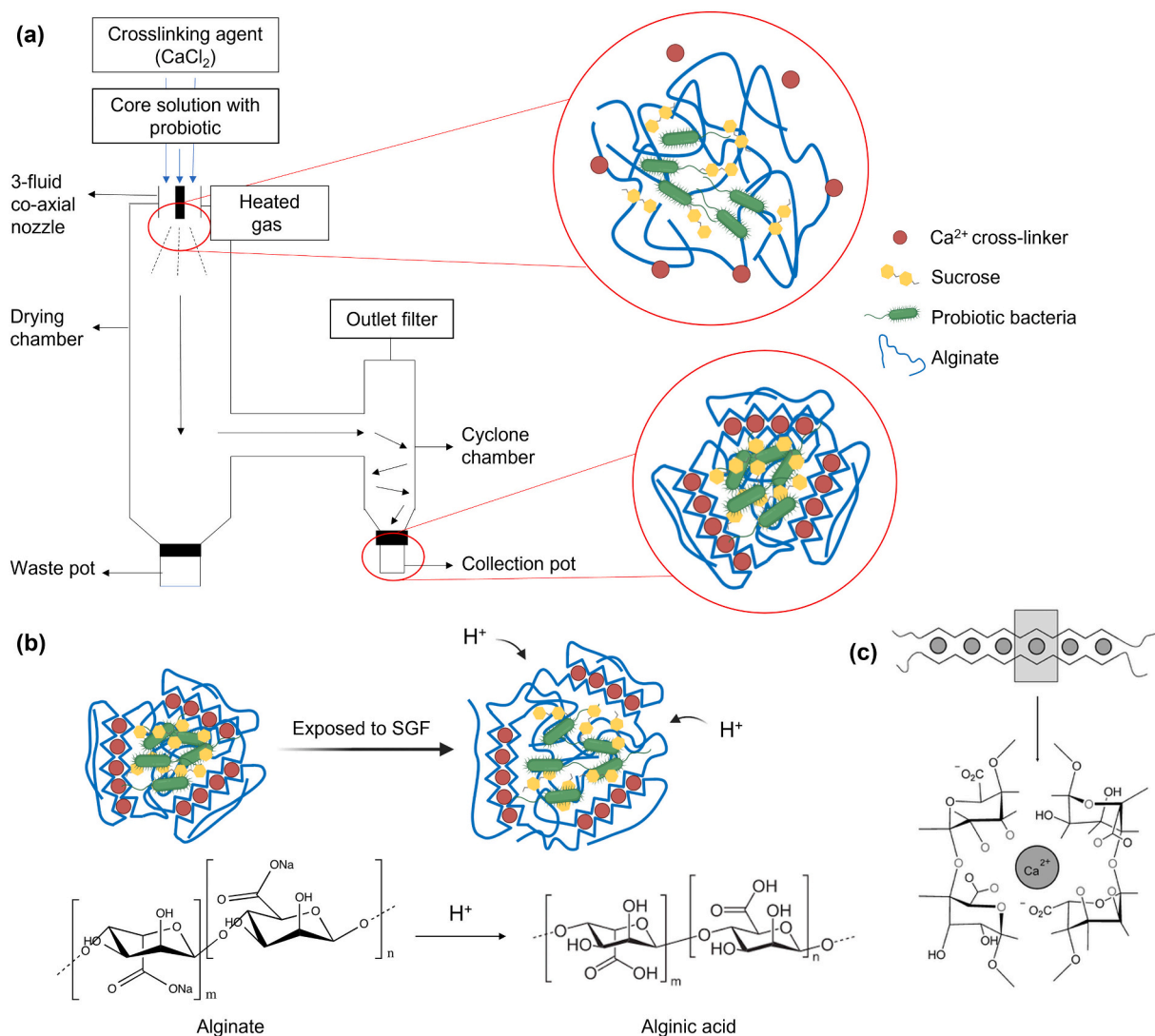A proposed mechanism of in situ alginate crosslinking by the

Fig. 5. Proposed mechanism of in situ alginate crosslinking during spray-drying. (a) Schematic diagram of calcium-alginate-sucrose (Ca-Alg-Suc) particle formation process during coaxial spray-drying. (b) Mechanism of gastroprotection conferred by Ca-Alg-Suc matrix. (c) "Egg-box" chemical structure for calcium alginate (Leick et al., 2010).
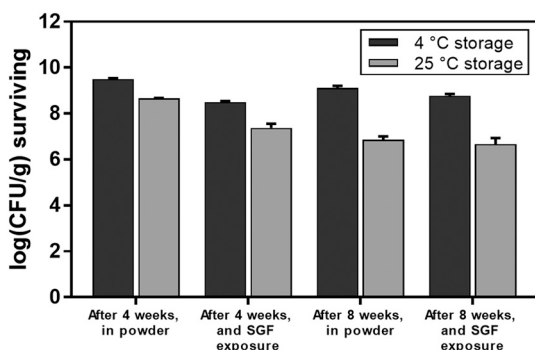
Fig. 6. Survivability of *L. rhamnosus* GG (LGG) in spray-dried calcium-alginate-sucrose (Ca-Alg-Suc) formulation after storage within the powder, and after storage followed by exposure to simulated gastric fluid (SGF).

described coaxial spray-drying technique is hence illustrated in Fig. 5a. The three-fluid coaxial nozzle enables simultaneous flow of the alginate-probiotics solution and the calcium crosslinking agent in the inner and outer feed channels respectively. Crosslinking likely occurred at the tip of the spray-dryer nozzle when both the alginate polymer and calcium chloride crosslinking agent were ejected and in contact. "Egg-box"-like crosslinked calcium alginate structures (Fig. 5c) were rapidly formed at the surface of each atomized alginate-probiotic particle (Leick et al., 2010), immobilizing entrapped probiotics within. This process of crosslinking was likely facilitated by the rapid dehydration of the outer feed channel, which was proximally closer to the heated atomizing gas. Calcium chloride in the outer feed channel hence preferentially interacted with alginate from the inner feed channel to rapidly form crosslinked calcium alginate structures, achieving effective crosslinking during the spray-drying process. Upon exposure to acidic SGF (Fig. 5b), individual Ca-Alg-Suc particles rehydrate and swell slightly, but the integrity of each particle was kept intact due to the crosslinked calcium-alginate layer surrounding each particle. Excess protons from the acidic milieu could hence be sequestered by alginate within each particle, providing a localized pH buffering effect which enhances survivability of entrapped probiotics in SGF.

3.5. Storage viability of LGG

The Ca-Alg-Suc formulation was found to be most effective in achieving high viable doses of LGG probiotics after spray-drying and SGF exposure. Hence, further studies on the storage stability of LGG encapsulated in Ca-Alg-Suc were conducted. From Fig. 6, Ca-Alg-Suc formulations stored at 4 °C refrigerated conditions maintained $>10^9$ CFU/g of LGG after 8 weeks. Gastroprotective function of Ca-Alg-Suc was also retained after 8 weeks storage, with $>10^8$ CFU/g LGG

surviving after refrigerated storage and SGF exposure. Powders that were stored at ambient temperatures performed poorer and saw significant LGG viability losses. Approximately 1 and 2 $\log_{10}$(CFU/g) reductions in LGG were observed after 4 and 8 weeks of room temperature storage respectively. Some degree of gastroprotection was retained in Ca-Alg-Suc stored at room temperature, with $>10^{6-7}$ CFU/g LGG surviving after SGF exposure. Lower temperatures generally led to higher microorganism survival due to reduced rates of chemical reactions, such as oxidation of lipids or degradation of proteins (Fu & Chen, 2011; Heidebach et al., 2010). Overall, spray-dried Ca-Alg-Suc was found to be stable under refrigerated storage conditions for the tested period of up to 8 weeks.

3.6. Spray-drying encapsulation of other lactobacilli probiotics

The Ca-Alg-Suc formulation was further adapted for encapsulation of other probiotics, specifically *L. plantarum* and *L. paracasei*. Viabilities of *L. plantarum* and L. *paracasei* after spray-drying in Suc and Ca-Alg-Suc are shown in Fig. 7. Both probiotic strains attained high post-spray-drying viabilities of $>10^9$ CFU/g in the Suc and Ca-Alg-Suc formulations, with <0.2 $\log_{10}$(CFU/g) losses. This suggests that sucrose was useful in protecting *L. plantarum* and *L. paracasei* probiotics against the harsh conditions involved in spray-drying.

Spray-dried L. *plantarum* and *L. paracasei* were also exposed to SGF, and results are presented in Fig. 8. From **Fig. 8c** and **d**, both *L. plantarum* and *L. paracasei* in Suc formulations showed complete susceptibility to pH 2 within 1 h of SGF exposure. *L. plantarum* and *L. paracasei* in Suc formulations also showed a degree of susceptibility to acidic pH 2.5 and pH 3, with >8 $\log_{10}$(CFU/g) reduction in pH 2.5 and ~ 1 $\log_{10}$(CFU/g) reduction in pH 3 after 2 h exposure. In **Fig. 8a** and **b**, the Ca-Alg-Suc matrix demonstrated significant improvement in gastroprotection of encapsulated *L. plantarum* and *L. paracasei*. $\sim 10^9$ CFU/g (0.7 $\log_{10}$(CFU/g) reduction) of *L. plantarum* in Ca-Alg-Suc remained viable following SGF pH 2 exposure, while $\sim 10^8$ CFU/g (1.9 $\log_{10}$(CFU/g) reduction) of *L. paracasei* remained viable. This finding highlights the suitability of the Ca-Alg-Suc formulation for high throughput spray-drying encapsulation and gastroprotection of other lactobacilli strains. Overall, the findings demonstrated the utility of the proposed spray-drying method and various functional benefits of the Ca-Alg-Suc formulation.

4. Conclusion

Encapsulation of LGG in various formulations was performed using a novel spray-drying technique which utilizes coaxial spraying for in situ alginate crosslinking. This technique could effectively achieve more than 90% crosslinked alginate in Ca-Alg and Ca-Alg-Suc polymer matrices. Among the six formulations investigated, the Ca-Alg-Suc formulation was most promising for probiotic encapsulation via the
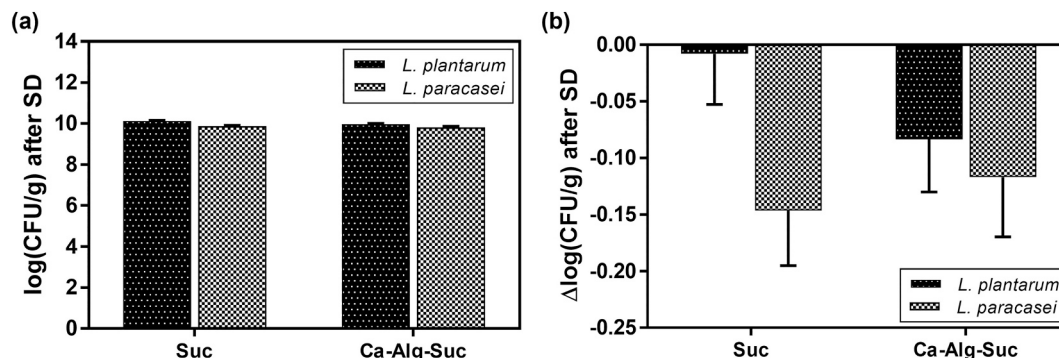

Fig. 7. Survivability data of *L. plantarum* and *L. paracasei* in the crosslinked spray-dried polymeric formulation post-spray-drying. (a) Log$_{10}$(CFU/g) data of *L. plantarum* and *L. paracasei* in sucrose (Suc) and calcium-alginate-sucrose (Ca-Alg-Suc) formulations following spray-drying. (b) Change in log$_{10}$(CFU/g) counts of *L. plantarum* and *L. paracasei*, as compared to before spray-drying.
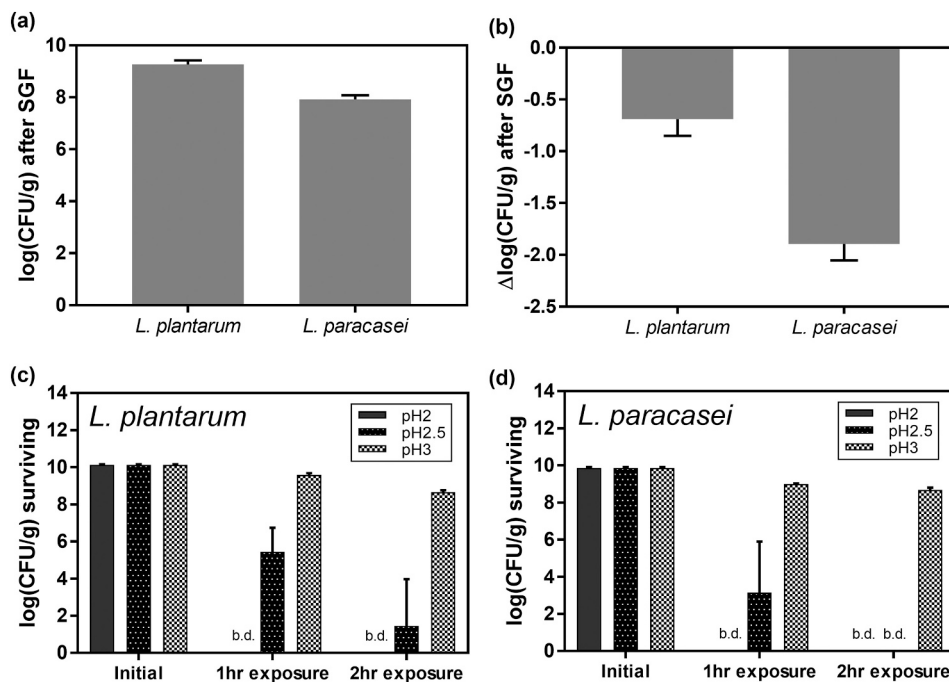
Fig. 8. Survivability data of spray-*dried L. plantarum* and *L. paracasei* in sucrose (Suc) and calcium-alginate-sucrose (Ca-Alg-Suc) formulations after exposure to simulated gastric fluid (SGF). (a) $Log_{10}(CFU/g)$ data of *L. plantarum* and *L. paracasei* in the Ca-Alg-Suc formulation following exposure to SGF pH 2. (b) Change in $log_{10}(CFU/g)$ counts of *L. plantarum* and *L. paracasei* in Ca-Alg-Suc after SGF pH 2 exposure. (c) $Log_{10}(CFU/g)$ data of *L. plantarum* in Suc formulation, exposed to SGF of various pH. (d) $Log_{10}(CFU/g)$ data *of L. paracasei* in Suc formulation, exposed to SGF of various pH. B.d. indicates that the cell count is below the detection limit of ~1.5 $log_{10}(CFU/g)$.

described spray-drying method. A high 10^9 CFU/g of surviving LGG, indicating less than 1 $log_{10}(CFU/g)$ viability losses, was retained after spray-drying and SGF exposure, highlighting the ability of the Ca-Alg-Suc formulation to protect entrapped probiotics against desiccation and thermal stresses during spray-drying, as well as confer gastro-protection. Enhanced survivability of probiotics was attributed to the formation of crosslinked calcium-alginate surrounding each particle, which immobilizes encapsulated probiotics within the matrix. Upon exposure to acidic SGF, excess protons could be sequestered by conversion of alginate to alginic acid, thereby providing a localized pH buffering effect which enhances survivability of entrapped probiotics in SGF. Similar trends in spray-drying and SGF survivals of *L. plantarum* and *L. paracasei* encapsulated in Ca-Alg-Suc were observed. LGG encapsulated in Ca-Alg-Suc formulation also demonstrated shelf-stability after 8 weeks of refrigerated storage. With further adaptation of spray-drying parameters, it will be possible to extend this approach for encapsulation of different probiotics species to produce shelf-stable probiotic powder for commercial applications such as in the agro-food and medical industries.

Author contributions

LL Tan planned and conducted all experiments and analyses, with assistance from SY Chan. M Mahotra conducted spray-drying experiments. LL Tan and SY Chan drafted the manuscript. SCJ Loo provided project guidance and revised the manuscript.

Data availability statement

The datasets generated for this study can be found in Tan (2021).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.119279.

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