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Encapsulation of *Lactobacillus rhamnosus* GG in chymosin treated milk protein- alginate microgel

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Abstract

Encapsulation of probiotic bacteria helps to protect its viability in food and enhances bioavailability in the human body. Alginate, a widely used gellant, singly cannot offer adequate protection to the encapsulated probiotics because the porosity of its micro-particles limits its stability in acidic conditions. Milk protein concentrate (MPC) is known to enhance gel strength. This study attempts to use chymosin treated MPC (1.0% solids w/w) as a co-gelling agent with sodium alginate (1.0%, 1.5% and 2.0% solids w/w) to enhance encapsulation of *Lactobacillus rhamnosus* GG (LGG) by adopting a continuous impinging aerosol technique using $CaCl_2$.

The moisture content of microgel paste of test formulations ranged from 88.1% to 90.4% (w/w) ($P>0.05$). Amongst the alginate MPC composite formulations, microparticles comprising of 1.0% alginate and 1.0% MPC solids exhibited highest ($P<0.05$) probiotic count (7.27 log CFU/g solids) and lowest viability reduction ($P<0.05$). Confocal image of its microparticle illustrate the presence of live bacteria, which appear as green, rod-shaped entities, entrapped within dark gel matrix. Under simulated gastric condition of pH 2 at 37^{o}C, its microgel particle exhibited detectable viability upto 15 minutes. In case of 1.0% alginate control microgel, comparatively higher viability was noted in the 5th minute, which was undetectable by the 10th minute.

With a progressive increase in alginate concentration among test formulations, cell count decreased, suggesting milk protein positively impacted viability. Microgel of 1.0% MPC control exhibited lowest loss of viable cells (0.93 log CFU/g solids). Optical image of its microparticles appeared as large flocculate rather than spherical microgel, as observed with alginate control microparticles, suggesting MPC alone is unable to produce microgels.

While this study infers better viability of microparticles comprising of 1.0 % alginate and 1.0 % MPC, it opens avenues for further research for strengthening co-gelation for probiotic survival in low pH.

Keywords: Chymosin-treated MPC, *Lactobacillus rhamnosus* GG, encapsulation, viability

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Introduction

The health promoting potential of functional bioactive components have opened up new avenues for food processing industries. Probiotics represent 70% of global functional food market and is expected to have a market value of $7.59 billion by 2026 [1]. A complex collection of 10^{14} microorganisms comprising of over 100 types of bacteria, live in a symbiotic relationship with the host in human intestine [2]**.** This intestinal microflora is altered by environmental factors like dietary habits, antibiotic therapy and stress conditions which makes the host susceptible to diseases [3]. Additionally, the viability of beneficial bacteria in the gastrointestinal tract is impacted by many factors like flushing effect of peristalsis, enzymatic action of pepsin and low pH in stomach [4]. To replenish natural gut microflora, either microflora has to be immobilized or grown at a faster rate than rate of its removal. Supplementation of probiotics as live microbial feeds can improve the balance of intestinal microflora of host and boost immunity [2]. Human probiotic species include *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus* GG (LGG), *Lactobacillus reuteri, Bifidobacterium longum* and *Bifidobacterium lactis* [5].

Probiotic bacteria adhere to the gastrointestinal tract and discourages the growth of pathogens by limiting nutrient availability [6, 7]. Probiotics provides relief from constipation, antibiotic associated diarrhea, allergic disorders, inflammatory bowel, ulcerative colitis, and tumor [2,8,9]. Bile salt hydrolase produced by probiotic bacteria facilitate reduction of serum cholesterol and assimilate cholesterol in intestinal conditions [10]. Probiotic organisms help in controlling low grade inflammation which is usually associated with obesity [11]. The bile tolerant B-galactosidase produced by probiotic organisms is beneficial for lactose intolerant people [12,13]. Dietary intervention with LGG and *Bifidobacterium lactis* lead to improvement in blood glucose level [14]. LGG supplemented diet improved weight control and reduced allergies in pregnant women [15]. Administration of LGG to children in daycare center

decreased risk of upper respiratory tract infections [16]. Probiotics maybe adopted as a safe means for reducing recurring respiratory issues in infants aged below 1 year [17].

The Food and Agriculture Organization and World Health Organization directive states that food claiming addition of probiotics should contain at least 10^6-10^7 CFU of viable probiotic organisms per gram [6,18]. The International Dairy Federation suggests minimum level of probiotics in any product to be at least 10^7 CFU/gm [19].

Encapsulation is a common technique which can be used to entrap material to protect and control the release of core material [20, 21]. Probiotic organism can be encapsulated in a hydrocolloid [22, 23] wherein such microgel particles offer cell protection in simulated gastric condition and allow its controlled release under simulated intestinal conditions [24, 25,26].

The usage of microcapsules of *Lactobacillus paracasei* prepared by gelation of sodium caseinate gellan gum in yoghurt demonstrated lower post acidification and higher viability during storage [27].

Alginate is a widely used natural biopolymer for encapsulation due to its biocompatibility, heat and acid resistance, better release properties, economical aspect, nontoxicity and easy to handle aspects [18]. It is a part of the family of unbranched polysaccharides that contains 1-4-linked β-D-mannuronic acid and α-L-guluronic acid residues [28]. In the presence of divalent cations like Ca^{2+}, instantaneous interfacial polymerization of alginate results in an egg case like structures that forms cross-linkages [29, 30, 31]. A dual aerosol method involving 2.0 % alginate solution containing microbial suspension and 0.1 M $CaCl_2$ was used to encapsulate probiotic LGG and *Lactobacillus acidophilus* in alginate hydrogel [23].

The porosity of alginate micro particles however, adversely impacts gel property hence additives like milk protein, gellan gum, carrageenan etc. are often used for structural modification [32]. Milk protein concentrate with its high solubility, good emulsifying and film forming properties and moderate viscosity facilitate proper dispersion of bacterial cells [33]. Furthermore, it forms gels with higher density giving better protection to entrapped bacteria [20]. Burgain et al. encapsulated LGG using varying formulations of milk proteins (micellar casein, native whey proteins and denatured whey protein) adopting emulsification method [22].

Casein micelle in milk is comprised of four main components namely αs1-, αs2, β- and κ-CN which are present in combination with colloidal calcium phosphate nanoclusters [34]. When κ-CN is cleaved by rennet at Phe105-Met106 bond to form para κ-CN, it results in reduction of net negative charge and release of hydrophilic fragments which causing it to aggregate [34, 35,36]. At a low temperature of 4°C, rennet cleaves κ casein bond but gel network is not initiated here. Coagulation occurs only at temperatures higher than 18◦C where gel is formed instantaneously [37, 38]. These phenomena is often utilized for entrapping bioactive component which is later securely contained within the gel matrix.

The present study aims to exploit this gel forming characteristics using a composite mixture of Chymosin treated MPC and sodium alginate to encapsulate LGG. An impinging aerosol technique will be used for this study as it is an easy, continuous, scalable and cost-effective method to produce microgel [39]. Microparticles of test formulation containing alginate (1.0%, 1.5% and 2.0%) and a constant concentration of 1.0% MPC will be assessed for viability of probiotics after encapsulation. Test formulations with the highest viability would be identified and acid resistance of microparticles will be tested by incubating it in an invitro gastric environment followed by assessment of its viability. Optical and confocal images will be used to infer about the microparticles structure. No such work has been reported previously. It can be anticipated that composite mixture of these gelling agents will enhance entrapment of probiotics and will provide protection in simulated gastric environments.

The scope of the present study has been drawn under limitations of academic timeline for project completion. The research results could pave way for further studies in the area of strengthening probiotic survival during encapsulation and its viability in low pH environment.

Material and methods

Materials

Commercial powder of LGG, 'Eczema shield' (Ethical nutrient) was obtained from Priceline Pharma, Brisbane, Australia. Sodium alginate (GRINSTED® Alginate FD 155) was purchased from Danisco, Australia. Calcium chloride dihydrate (99%) was obtained from Thermo Fisher Scientific Pty. Ltd., Australia. Milk protein concentrate (MPC) powder (80% Casein: 20% Whey protein) was obtained from Total FoodTec Pty Ltd, Australia. Vegetarian rennet, Chy-Max™ Plus was obtained from CHR Hansen Pty Ltd, Australia. de Man, Rogosa and Sharpe (MRS) broth, anaerogen packs (BD

GasPak™), tri-sodium citrate and sodium carbonate were purchased from Chem-supply Pty Ltd, South Australia. Bacterial viability kit, L7012 LIVE/*DEAD*®*BacLight*™ was obtained from Thermo Fisher Scientific Pty Ltd, Australia.

Encapsulation of LGG in chymosin treated MPC-alginate composite microgels

Solutions with 1.0%, 1.5% and 2.0% (w/w) alginate were prepared by dissolving measured amounts of sodium alginate powder in 1.5 kg of deionized sterile chilled water. Each of these test solutions was mixed for 60 minutes at 810 rpm using overhead stirrer (IKA® RW 20 D, Germany) at <4◦C. Similarly, 500 mL of 4% (w/w) MPC solution was prepared by mixing for 60 minutes at 625 rpm using the same mixer. This was followed by addition of 100 µL of vegetable rennet (200 IMCU/mL) to the MPC solution which was mixed using overhead stirrer (IKA® RW 20 D, Germany) for 45 minutes. Addition of 2 grams of LGG powder (1 gm =10^9 CFU) ensued and further mixing was carried out for 30 minutes at 4◦C. The chymosin treated MPC solution containing LGG culture was then added to alginate solution (<4^{0}C) and mixed for 15 minutes at 625 rpm.

0.1M of $CaCl_2$ prepared in sterilized deionized water was maintained at 42^{0}C for atomization. Encapsulation was carried out by patented dual aerosol method which involved atomizing alginate and chymosin treated MPC solution from the top nozzle (450 kPa) and $CaCl_2$ solution from the bottom nozzle (350 kPa) of a sanitized microencapsulator [40]. Micro gel particles were collected from the bottom outlet and vacuum filtered after settling for about 30 minutes before being stored under refrigerated conditions [41].

Microgels of two control formulations, 1.0 % (w/w) MPC solution (without alginate) and 1.0 % (w/w) alginate solution (without chymosin treated MPC) were also prepared. An outline of the encapsulation process adopted is illustrated in **Figure**.1. Microencapsulation was carried out as illustrated in **Figure**. 2 for three replicates of each test formulation and controls. The temperature of alginate MPC solution, volume of spraying solution consumed, time taken for spraying/atomization were monitored and documented.

Characterization of micro gel paste

Moisture content

Moisture content of microgel paste was analyzed by drying samples at 70◦C for 12 hours at 700 mbar in a vacuum oven (Thermoline Scientific, Australia) according to AOAC official method 925.09 [42]. Triplicate analysis was conducted for samples of each formulation.

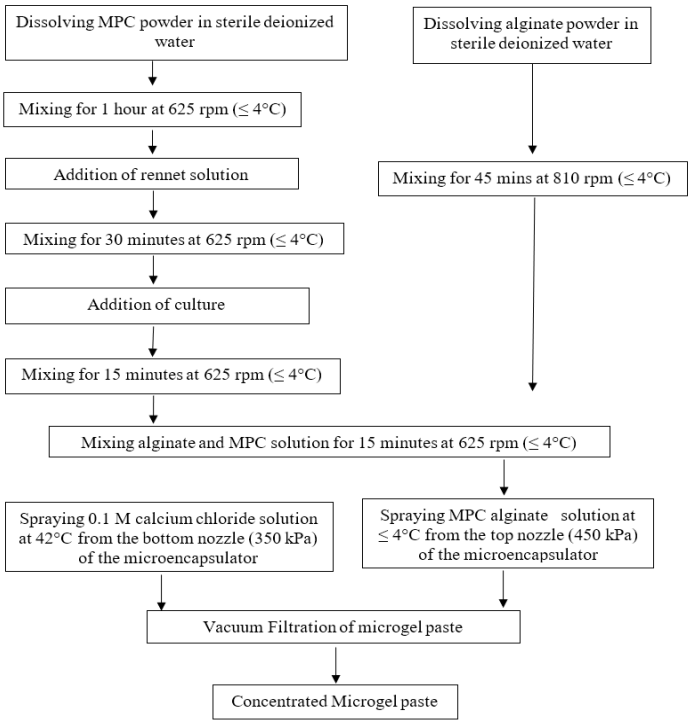

Figure 1. Flow chart of microencapsulation process adopted in this research.

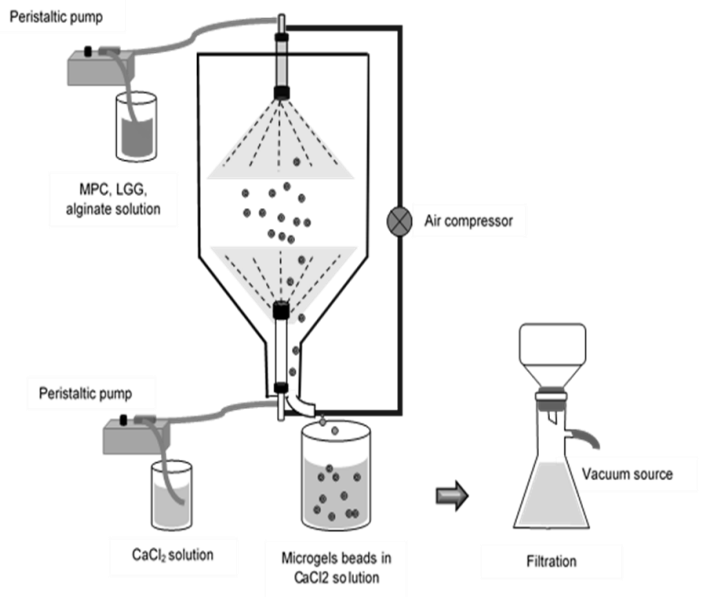

Figure 2. Illustration of impinging dual aerosol micro-encapsulation technique for production of microgels [40].

Enumeration of probiotic bacteria

Protocol for enumeration of viable probiotic bacteria in fresh microgel paste was developed by studying enumeration methods used by Abd El-Salam et. al [18]. Procedure adopted by Krasaekoopt et.al, 2004 was also referred during the study [24].

Rehydration of 1-gram micro gel paste was carried out by adding it to 9 mL of 0.5% (w/v) sterilized sodium citrate

solution followed by gentle shaking for 45 minutes at 500 rpm in an orbital shaker (IKA KS 260 Labtek). Sodium citrate, a chelating agent was used for breakdown of alginate microgels [23, 24]. Serial dilution was conducted using the same diluent and duplicate plating was done on MRS agar (Oxoid, Basingstoke, UK). The probiotic colonies were enumerated following incubation at 37^{0}C for 48 hours anaerobically using BD GasPak ™. Similar analytical method was adopted for enumeration of probiotic count in commercial sample of LGG using 0.1% peptone water as a diluent. Microgel filtrate was also analyzed for any presence of probiotic bacteria. Reduction in viability during encapsulation was calculated based on difference between probiotic cells incorporated per gram of solids in spray solution and gram solids of paste.

Acid tolerance test

Acid tolerance test was conducted on microgel paste of formulation that comprised of 1.0 % alginate and 1.0 % MPC and also on microgel paste of 1.0 % alginate control formulation by referring to method used by Ding and Shah [43]. Rehydration of 1-gram micro gel paste was carried out by adding it to 9 mL of sterilized MRS broth in labelled tubes. The tubes were incubated at 37^{0}C in a water bath. At intervals of 5, 10, 30 and 60 minutes of incubation, 1 mL sample was withdrawn from the tube and diluted in 9 mL of 0.5% sodium citrate. The solution was gently shaken in an orbital shaker for 45 minutes to breakdown the microgel particles. Further serial dilution was continued in 0.5% sodium citrate before being plated on a MRS agar plate for each time point. Serial dilution and plating of microgel sample without incubation was also carried out to estimate initial viability. Duplicate plating was carried out for relevant dilutions and media plates were incubated anaerobically at 37^{0}C for 48 hours using BD GasPak ™.

Optical images

Optical image of microgel paste was obtained by analyzing under a light microscope with resolution 40X, Prism Optical PRO 2300T (Scientific Instrument and Optical Sales, Australia) and images were recorded using software TSView7. Size of microspheres were calculated using a standardized scale bar.

Confocal Images

Confocal image of micro gel paste was obtained by analyzing under Zeiss LSM700 confocal microscope using a bacterial viability kit, L7012 LIVE/*DEAD*® *BacLight*™). Equal volumes of 1.67 mM SYTO 9 dye with excitation maxima of 480/500 nm and 1.67mM propidium iodide with excitation maxima of 490/635 nm were combined in a microfuge tube. A mixture of 3 µL of dye and 1mL of microgel paste suspension was incubated at room temperature in dark for 15 minutes. Then, 5µL of stained bacterial suspension was trapped between a slide and 18mm square coverslip followed by observation under a confocal microscope.

Statistical Analysis

Data derived from three independent experiments of each test formulation were presented as mean ± standard deviation (SD) of duplicate test results, wherever applicable. The number of sample data used for analysis is indicated by n. The significance of differences between the values (where applicable) were statistically analyzed by Minitab® R16 (Minitab Inc, Chicago) using Analysis of Variance (ANOVA) with Tukey's pair wise comparison at 95% confidence level.

Results and Discussion

Flow rate of spraying solutions during encapsulation

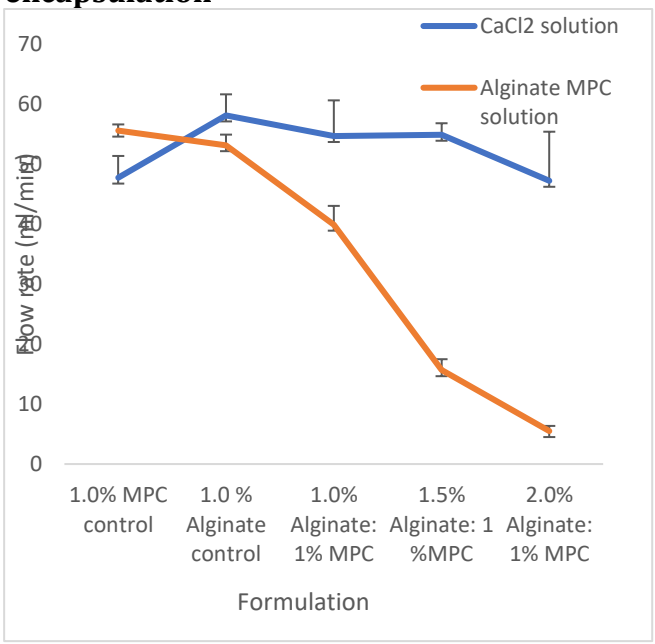

Figure 3. Flow rate of spraying solutions during encapsulation process.

With increasing alginate concentration among test formulations comprising of 1.0%, 1.5% and 2.0% alginate, the average flow rate of alginate-MPC spray solution progressively decreased from 39.89 mL/min to 15.05 and 5.5 mL/min. respectively. It is evident that formulation with 1.0 % alginate and 1.0% MPC had the highest flow rate among test formulations. The flow rate of each test formulation was significantly different from each other

(P<0.05) as illustrated in **Figure. 3**. The viscosity of an aqueous alginate sharply rises with its increasing concentration with a demonstrated increase in viscosity of upto 100-fold on a 10% increase in alginate [44].

The average flow rate of 1.0 % MPC control solution (55 mL/min) and 1.0 % alginate control solution (53.13 mL/min) were not significantly different (P>0.05) but were however higher than the flow rate of test formulations (P<0.05).

There was no significant difference in flow rate of calcium chloride solution among the formulations (P>0.05) which ranged from 45.04 mL/min to 58 mL//min.

Moisture content of microgel paste

The moisture content of paste of test formulations ranged from 88.1% to 90.4% (w/w). The results illustrate an increase in moisture content with a progressive increase in alginate concentration among test formulations. However, no significant difference in moisture content of microgel paste among test formulations (P>0.05) was observed, as has been illustrated in **Figure**. 4. The average moisture content of 1.0 % alginate control was 94.4%, which is significantly higher than the rest of the formulations (P<0.05). Alginate has the highest water holding capacity among hydrocolloids due to its extensive hydroxyl group [45].

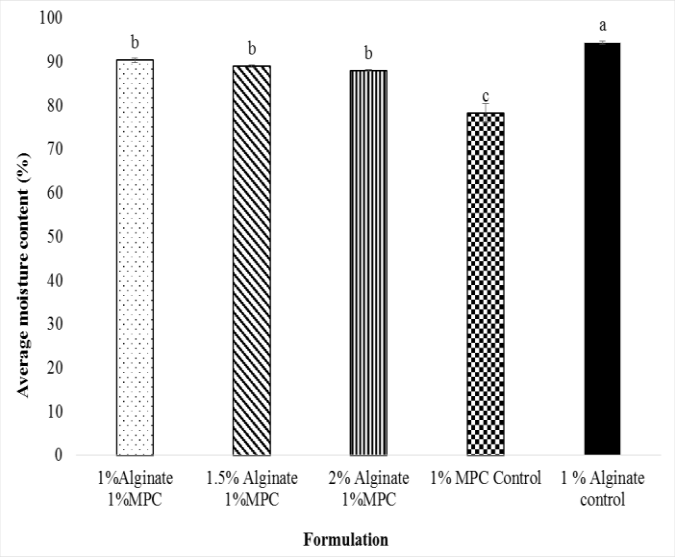

Figure 4. Moisture content of paste obtained after filtration of microgels for five different formulations after encapsulation. Results are expressed as mean ± SD. Values on the bars that do not share a letter is significantly different at p<0.05 (n=6).

The average moisture content of 1.0 % MPC control was 78.3% (w/w), which is significantly lower than the rest of the formulations (P<0.05). It can be inferred that presence of milk component in alginate reduces its moisture content. Decrease in the protein content in a composite alginate formulation result in absorption of excess moisture leading to lesser compact gel matrix [46]. The intactness of gel is known to enhance cell viability, hence any increase in moisture content within a microgel could impact the containment of bio actives.

Probiotic count of microgel paste

Amongst the test formulations, highest viability in microbial paste after encapsulation was observed in the formulation comprising of 1.0 % alginate and 1.0 % MPC, which exhibited an average probiotic count of 7.27 log CFU/g solids, as has been illustrated in **Figure**.5.

The viability of this formulation was however, not significantly different to the viability of 1.0 % alginate control formulation (P-Value of 0.700), to 1.0 % MPC control formulation (P-Value of 0.549) and with the rest of the test formulations (P>0.05). We can thus infer that there was no significant difference in viability among formulations.

The formulation containing 1.5% alginate and 1.0 % MPC and formulation containing 2.0 % alginate and 1.0 % MPC however, share much lower statistical difference (P-Value of 1.00) and have significantly lower viability than that of 1.0 % MPC control formulation (P<0.05).

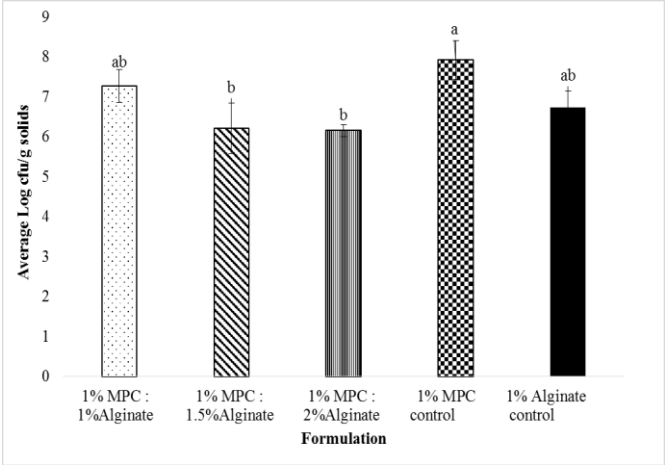

Figure 5. Cell count of LGG encapsulated in an alginate-milk protein microgel paste of different formulations. Results are expressed as mean ± SD. Values on the bars that do not share a letter is significantly different at p<0.05 (n=6).

This study result shows that with a progressive increase in alginate concentration among the three test formulations, a decrease in LGG viability was observed as has been illustrated in Figure 5. An increase in the overall concentration of solutions used for encapsulating bacteria decreases cell viability due to high shear force required to mix cells [43].

Shi et.al in their study inferred that larger microsphere provided better protection to probiotics [19]. Sera et.al. measured particle size of alginate MPC microgel as a part of this study and reported highest particle size in

microgel from formulation containing 1.0 % alginate and 1.0 % MPC [47]. This study results are in agreement with Shi et al [19].

We can infer that increasing alginate concentration resulted in reduced particle size and a marked decrease in viability among test formulations.

The impact of MPC on enhancing viability during encapsulation is evident in Figure 5. 1.0 % MPC control paste had highest probiotic count (7.92 log CFU/g solids) amongst all formulations. This was significantly higher than the formulation containing 1.5% alginate and 1.0 % MPC ($P<0.05$) and the formulation containing 2.0% alginate and 1.0 % MPC ($P<0.05$). This wasn't however, significantly different to 1.0 % alginate control and the formulation containing 1% alginate and 1.0 % MPC ($P>0.05$).

Shi et al. encapsulated *Lactobacillus bulgaricus* in 1.0 % alginate with varying concentration of milk protein (1.0 %, 2.0 %, 3.0 %, 4.0 %) and reported better protection of encapsulated bacteria at higher protein concentrations [19]. Skim milk help to stabilize cell membrane and protects cells during encapsulation [48]. Whey protein gel has been used to immobilize bio actives [30].

It can be observed from the present study that increasing the alginate concentration for encapsulating LGG in an alginate MPC complex led to decrease in microbial viability and reduction in size of microparticles. While the impact of MPC for reducing porosity of alginate gel has not been assessed here, a progressive reduction in MPC availability due to increasing alginate concentration among test formulation, may also have contributed to the decrease in viability. Hence, it can be inferred that presence of milk solids enhance viability in alginate MPC microgel.

Reduction in viability during encapsulation

Amongst the test formulations, lowest reduction in viability during encapsulation was noted in the formulation containing 1.0 % alginate and 1.0 % MPC mixture which exhibited a reduction in viability of 1.29 (Log CFU/g solids). This formulation comprised of proportionately higher amount of milk solids as compared to other two test formulations. There was no significant difference in the reduction in viability among formulations during encapsulation ($P>0.05$) as illustrated in **Figure**. 6. Lowest viability reduction of 0.93 log CFU/g solids was observed in 1.0 % MPC control as illustrated in **Figure** 6.

Alginate microgels are porous and can protect encapsulated bacteria better, if combined with polymers like milk protein which help form high density gel [49]. The buffering potential of milk solids, its film forming properties and high solubility aid in the encapsulation of probiotic bacteria [33]. The result of the present study reiterates this encapsulation potential of milk solids and its role in enhancing viability during encapsulation. Higher milk concentration provided better protection of Lactobacillus bulgaricus encapsulated in an alginate milk-microsphere [19].

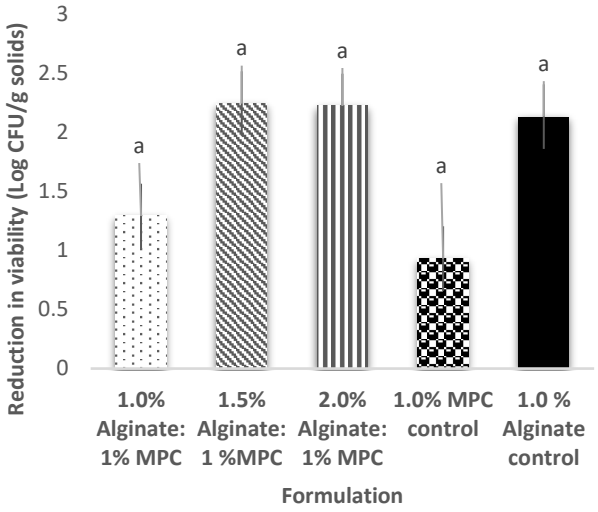

Figure 6. Reduction in viability during encapsulation (calculated based on difference in probiotic count in Log CFU/g solids in the spraying solution with culture and microgel paste of all formulations. CFU count per g solids in alginate MPC spray solution is calculated based on $7.15x10^9$ cfu/g of culture used at the rate of 2g/L spray solution in each experiment (n=3), SD is assumed to be 0. CFU count in microgel paste are expressed as mean ± SD (n=6). Values on the bars that do not share a letter is significantly different at $p<0.05$.

Apart from the choice of gelling agents, many other underlying factors could impact preservation of microbial viability during encapsulation. This includes selection of most appropriate encapsulation technique [50], presence of carrier materials [23], form of microbial culture, initial count in the culture and strain resistance to encapsulating conditions [51].

Enrichment of culture is also known to enhance cell viability during encapsulation. An alginate encapsulation study incorporated the enrichment of a frozen culture of LGG by inoculation it in MRS broth followed by harvesting of cells that involved centrifugation and washing [23].

In this study, a commercial powder of LGG, 'Eczyma shield', a probiotic nutritional supplement was used as the culture. There remains a possibility of interference of food grade coating of the culture with the alginate-milk protein gel during encapsulation. Presence of appropriate coating materials ensures higher viability in

encapsulated particles. A viability of 9.0 to 9.3 (log CFU/g) was reported in chitosan coated alginate micro beads containing LGG and *Lactobacillus acidophilus* encapsulated by extrusion method using an initial culture suspension of 9.2 to 9.4 (log CFU/ml) [23].

No protective carrier material or culture enrichment was involved in the present study. Any loss of culture through filtrate during vacuum filtration was ruled out, as probiotic count was not detected in filtrate during microgel filtration for any formulation.

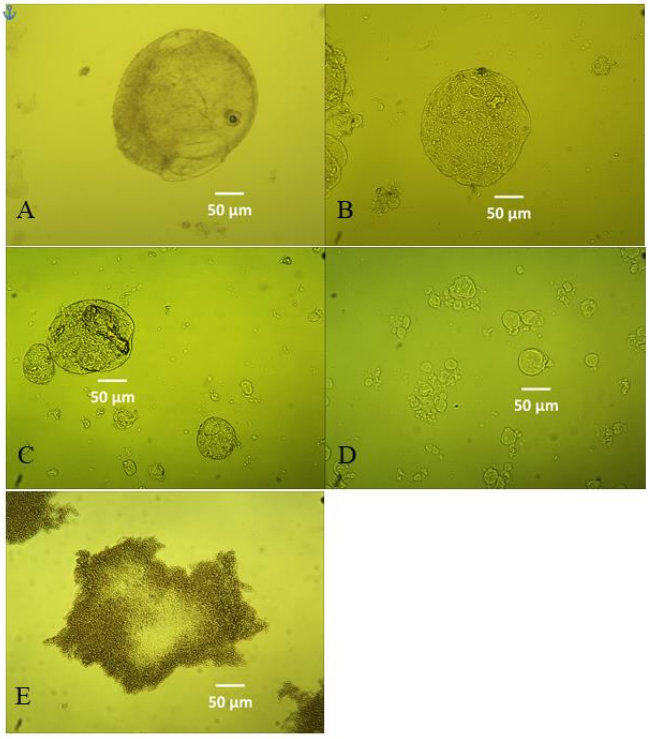

Figure 7. Optical microscopic image of alginate-milk protein composite microgel particles containing encapsulated LGG of five different formulations: (A) 1% alginate and 1% MPC, (B) 1.5 % alginate and 1% MPC (C) 2% alginate and 1% MPC (D) 1% alginate (E) 1% MPC.

Optical image of microgel particles

The optical images of microgel particles containing encapsulated LGG of all formulations have been presented in **Figure**. 7. Spherical microgels was observed in the test formulation and in the 1.0 % Alginate control formulation. Optical image of microgel particle of 1.0 % Alginate and 1.0 % MPC formulation was distinctly translucent and spherical in shape (Section A of Figure 7). With progressive increase in alginate concentration amongst test formulations, a decrease in translucency was observed. Optical images (Section B and C) of Figure 7 shows spherical microgels with increasing compactness of microgels embedded within particles, with progressive increase in alginate concentration. Alginate gels quickly on atomization. This explains the sphericity of formulations containing alginate as was seen in microgel particle containing 1.0 % Alginate control (Section D of figure 7).

Optical image of 1.0 % MPC control microgel appeared as aggregated particle with numerous microgels flocculated. Enzyme treated MPC do not gel quickly. During encapsulation using MPC, particles coalesce and form large flocculates even before the surface gelation of atomized particle is complete.

It is also evident that alginate microgel particle were smaller in size than the composite microgel of the test formulations containing alginate and MPC, which had higher solids concentrations. Particle size of microgels of the test formulations and alginate control formulation under this present study, ranged between 30-200 µm [47]. Alginate MPC microgel were reported to be bigger in size than alginate control microgel which has been illustrated in Section E of Figure 7. Furthermore, with decrease in proportion of MPC among the three test formulations, particle size significantly reduced. This is also in agreement with study of Shi et al. which finds a direct proportionate relation between microgel size and polymer concentration [19].

Confocal laser scanning microscopic image of microgel particles

The confocal images of microgel particles of formulation containing 1.0 % alginate and 1.0 % MPC and formulation containing 1.0 % alginate control have been illustrated in **Figure**. 8. Here live bacteria can be identified as bright green rod-shaped entities and dead bacteria appear as red rod-shaped entities. Live bacteria can be seen entrapped mostly in the greenish coagulated mass. Identifying live organisms by color difference in confocal microscopy is based on nucleic acid staining potential of dye mixture. Green fluorescing SYTO9 dyes penetrates live cells whereas red fluorescing propidium iodide enters only cells with damaged cytoplasmic membranes [52].

By comparing sections, A and B in Figure 8, it can be noted that alginate control microgel which does not contain milk protein, appears dark whereas areas within an alginate MPC microgel are comparatively light and stained slightly green. Higher concentration of live bacteria is seen in microgel particles containing 1.0 % alginate and 1.0 % MPC than the microgel matrix made of alginate only. The average probiotic count in the microgel particle made of 1.0 % alginate and 1.0 % MPC was log 7.27±0.41 CFU/gm solids. The microgel particle of 1.0 % alginate control exhibited an average count of log

Table 1. Comparison of the acid tolerance and % survival during incubation of 1% alginate and 1% MPC paste and 1 % alginate control paste which were exposed to pH 2 at 37◦ C for various incubation times.

Incubation time (min)	1% alginate and 1% MPC microgel		1% alginate control	
	Average Count± SD (log CFU/g)	% Survival during incubation	Count (log CFU/g)	% Survival during incubation
0	6.280±0.247	100.00	4.656± 0.09	100
5	4.599±0.489	73.23	3.917± 0.062	84.13
10	3.634±0.306	57.86	< 3.00	< 64.43
30	< 3.0	< 47.77	< 3.0	< 64.43
60	< 3.0	< 47.77	< 3.0	<64.43

6.73±0.41 CFU/gm solids. This presence of higher concentration of live probiotic bacteria entrapped within the composite microgel correlates with **Figure 8**.

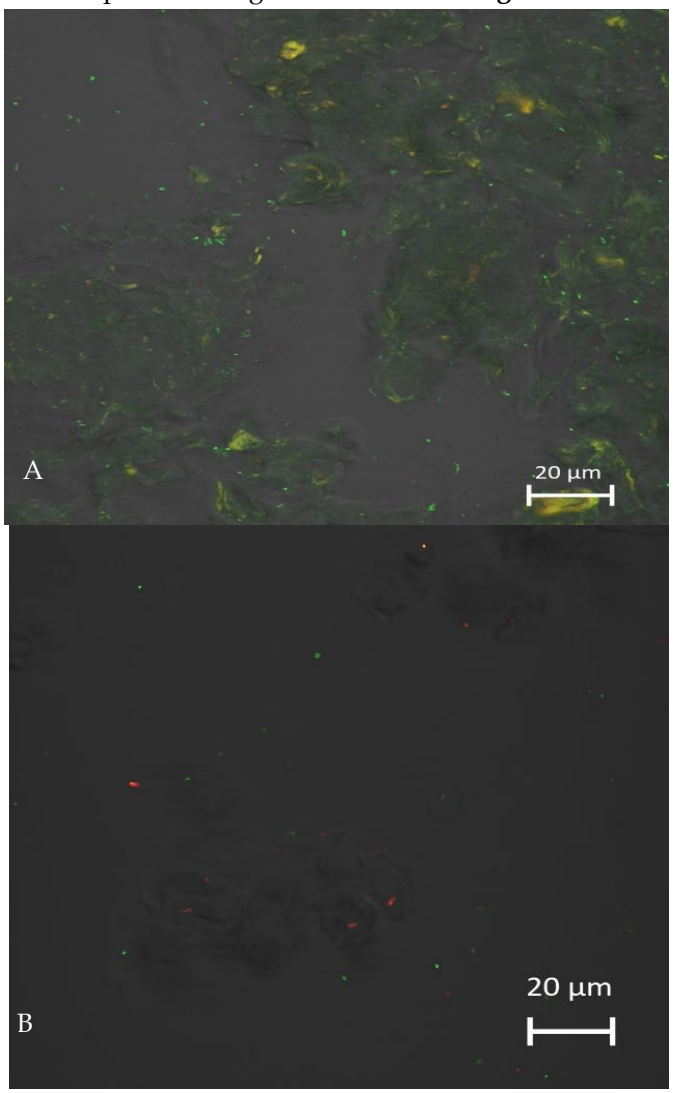

Figure 8. Confocal image (confocal) of alginate-milk protein composite microgel particles containing encapsulated LGG labelled with (A) Bright green rod-shaped bacteria representing live LGG in 1% alginate and 1% MPC composite gel particle (B) Bright green rod-shaped bacteria representing live LGG and red rod-shaped bacteria representing dead bacteria in 1% alginate gel control.

Acid tolerance of LGG microgel particle

The probiotic count of microgel particle in test formulation comprising of 1.0 % alginate and 1.0% MPC is higher (Log 6.280 CFU/g) than that of 1% alginate control formulation (Log 4.656 CFU/g). Probiotic count in food is known to be reduced by the gastric environment within the human body. A simple invitro model was used in this study to assess the acid tolerance of both of these formulations. The simulated gastric environment which comprised of an acidic medium (pH 2) maintained at 37◦C saw a progressive decline in microgel viability in both these formulations. Higher viability was observed in the composite formulation than in the alginate control formulation as outlined in **Table 1** On computing the acid tolerance of formulation comprising of 1% alginate and 1% MPC microgel, 73.23% survival was noted in the 5th minute and 57.86% in the 10th minute, after which the counts were undetectable in the 15th minute of observation (survival below 47.77%).

A higher survival of 84.13% was noted in 1.0 % alginate control microgels in the 5th minute of incubation however in the 10th minute, counts were undetectable (survival below 64.43%). The viability was detected for slightly longer duration in the composite microgel than in the alginate control. As the minimum dilution plated for both formulations was 10^{-2} (using 0.1 mL in spread plate), the undetectable count has been reported as less than 3 log CFU/g. This corresponds to a survival of less than 47.77% in the test formulation and survival less than 64.43%. in the control formulation.

Composite formulation of alginate MPC have dense microgels that aids entrapment of probiotic organisms. The buffering ability of milk along with its dense hydrogel network reduces the rate of diffusion of acid into the microsphere, thereby reducing loss of viability [4, 19].

Lower survival rate, was however, noted in Alginate MPC microgel as compared to alginate control at the observed test intervals. While the composite particle entrapped more bacteria than alginate control particle, its gel resistance to acidic conditions initially seemed lower than that of alginate microgel. Anal and Singh refer to vulnerability of probiotic bacteria to acidic conditions [6]. A 10^6-fold reduction in colony forming unit was observed

in commercially available probiotics within 5 minutes of its incubation in gastric fluids [1].

This necessitates the need to design a robust gel resistant to a simulated gastric environment. In this study, there remains a possibility that presence of an appropriate and robust carrier material for coating microgels could have helped preserve and maintain viability for extended time. Use of antacids like calcium carbonate and magnesium hydroxide helps protect encapsulated probiotics especially under stressed conditions [18]. Probiotic survivability improved after spray drying encapsulated microgel of *Lactobacillus acidophillus* NCFM and LGG using maltodextrin as carrier material [23]. LGG encapsulated in alginate microbeads demonstrated a survival for 40 minutes (4.38 log CFU/ml) at pH 2, after which colonies could not be detected [23]. However, chitosan coating of alginate microbeads resulted in extension of survival time of encapsulated LGG to 120 minutes (8.65 log CFU/mL). High loss of viability (reduction of 4.5 log CFU/g) of LGG and *Lactobacillus plantarum* was observed after 2 hours of incubation at pH 2.0. By using xanthan and gellan gum (1.0 %: 0.75%) as encapsulating materials, a significant increase in survival in gastric conditions up to 6 hours was noted [53].

It can thus be inferred that encapsulating gel matrix optimization is imperative for improving viability. Additionally, initial bacterial count in paste, the strength of bacterial strain and method used for assessing acid tolerance study also impacts acid tolerance [51].

Conclusion

Microencapsulation offers great potential to entrap bio-active entities and deliver them in targeted areas for enhancing functional properties. In this study, chymosin treated milk protein-alginate microgel encapsulated with probiotic bacteria, *Lactobacillus rhamnosus* GG (LGG) were produced by an impinging aerosol technique using $CaCl_2$ as a crosslinking solution. Test formulations containing enzyme treated 1.0 % MPC incorporated in 1.0 %, 1.5% and 2.0 % sodium alginate respectively were prepared for encapsulation. Similarly control formulation of both gelling agents were also encapsulated. Enumeration of *Lactobacillus rhamnosus* GG cells in all formulation were conducted after encapsulation and reduction in viability during encapsulation was also assessed.

Amongst the test formulations, microgel made from 1.0 % alginate and 1.0 % MPC showed highest viability ($P>0.05$) and lowest viability reduction during encapsulation ($P>0.05$). With a progressive increase in alginate concentration and proportionately lower availability of MPC amongst test formulations, the flow rate of spraying solutions decreased and microgels obtained were characterized by smaller particle size and reduced viability. While alginate alone is known to form porous gels, MPC singly forms large flocculates during encapsulation which was evident in the optical images.

When compared to other test formulations, the composite formulation comprising of 1% alginate and 1% MPC exhibited highest flowrate during encapsulation, yielded microparticles with highest particle size and lowest moisture content. Furthermore, it demonstrated lowest reduction in viability during encapsulation and had the highest viability in encapsulated microparticles. In an invitro study to assess acid tolerance, this formulation exhibited a viability of up till 10 minutes after which it was undetectable in the 15th minute in a simulated gastric environment.

This study has attempted to optimize the polymer mix of alginate and chymosin treated MPC for ensuring higher viability during encapsulation using a novel impinging aerosol technique. The results validate the role of MPC in enhancing film forming, flocculation and entrapment of bacteria. Many factors in the study including form of culture used for encapsulation, amount of culture inoculated for encapsulation and stress created during encapsulation may have affected study results especially the viability. The low survival in simulated gastric environment conditions observed reiterates the need for looking into prospects for strengthening gel matrix of the composite formulation. Incorporation of a robust carrier material and antacids to protect microgels against the harsh acidic environment of acidic environment in products or gastrointestinal tract could be a way forward. Overall, this study contributes to a better understanding of *Lactobacillus rhamnosus* GG encapsulation process using chymosin treated MPC with alginate and opens avenues for further research towards strengthening its viability.

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Declaration of Interest:

No conflict of interest to declare.

Reference

1 Sun Q, Yin S, He Y, Cao Y, Jiang C. Biomaterials and Encapsulation Techniques for Probiotics: Current Status and Future Prospects in Biomedical Applications. Nanomaterials. 2023 Jul 27;13(15):2185.

2 Floch MH, Kim AS. Probiotics: a clinical guide. Slack Incorporated; 2010.

3 Rao AV, Shiwnarain N, Maharaj I. Survival of microencapsulated *Bifidobacterium pseudolongum* in simulated gastric and intestinal juices. Canadian Institute of Food Science and Technology Journal. 1989 Oct 1;22(4):345-9.

4 Abd El-Salam MH, El-Shibiny S. Preparation and properties of milk proteins-based encapsulated probiotics: a review. Dairy science & technology. 2015 Jul; 95:393-412.

5 Doyle MP, BuchananR. Food microbiology: fundamentals and frontiers. 4th ed. Washington DC: ASM Press; 2013.

6 Anal AK, Singh H. Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. Trends in food science & technology. 2007 May 1;18(5):240-51.

7 Basu S, Chatterjee M, Ganguly S, Chandra PK. Efficacy of *Lactobacillus rhamnosus* GG in acute watery diarrhoea of Indian children: a randomised controlled trial. Journal of paediatrics and child health. 2007 Dec;43(12):837-42.

8 Fuller R. Probiotics in man and animals. The Journal of applied bacteriology. 1989 May 1;66(5):365-78.

9 Horvath A, Dziechciarz P, Szajewska H. Meta-analysis: *Lactobacillus rhamnosus* GG for abdominal pain-related functional gastrointestinal disorders in childhood. Alimentary pharmacology & therapeutics. 2011 Jun;33(12):1302-10.

10 Tomaro-Duchesneau C, Jones ML, Shah D, Jain P, Saha S, Prakash S. Cholesterol assimilation by *Lactobacillus* probiotic bacteria: an in vitro investigation. BioMed research international. 2014 Oct;2014.

11 Ilmonen J, Isolauri E, Poussa T, Laitinen K. Impact of dietary counselling and probiotic intervention on maternal anthropometric measurements during and after pregnancy: a randomized placebo-controlled trial. Clinical nutrition. 2011 Apr 1;30(2):156-64.

12 Krasaekoopt W, Bhandari B, Deeth H. Evaluation of encapsulation techniques of probiotics for yoghurt. International dairy journal. 2003 Jan 1;13(1):3-13.

13 Shetty K. Food biotechnology. Taylor & Francis Group; 2006.

14 Laitinen K, Poussa T, Isolauri E. Probiotics and dietary counselling contribute to glucose regulation during and after pregnancy: a randomised controlled trial. British Journal of Nutrition. 2008 Nov;101(11):1679-87.

15 Dotterud CK, Storrø O, Johnsen R, Øien T. Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. British Journal of Dermatology. 2010 Sep 1;163(3):616-23.

16 Hojsak I, Snovak N, Abdović S, Szajewska H, Mišak Z, Kolaček S. *Lactobacillus* GG in the prevention of gastrointestinal and respiratory tract infections in children who attend day care centers: a randomized, double-blind, placebo-controlled trial. Clinical Nutrition. 2010 Jun 1;29(3):312-6.

17 Rautava S, Salminen S, Isolauri E. Specific probiotics in reducing the risk of acute infections in infancy–a randomised, double-blind, placebo-controlled study. British Journal of Nutrition. 2008 Nov;101(11):1722-6.

18 Afzaal M, Saeed F, Saeed M, Azam M, Hussain S, Mohamed AA, Alamri MS, Anjum FM. Survival and stability of free and encapsulated probiotic bacteria under simulated gastrointestinal and thermal conditions. International Journal of Food Properties. 2020 Jan 1;23(1):1899-912.

19 Shi LE, Li ZH, Li DT, Xu M, Chen HY, Zhang ZL, Tang ZX. Encapsulation of probiotic *Lactobacillus bulgaricus* in alginate–milk microspheres and evaluation of the survival in simulated gastrointestinal conditions. Journal of food engineering. 2013 Jul 1;117(1):99-104.

20 Bhandari B, Roos YH. Food Materials Science and Engineering. 2012 Jul 30.

21 Sliwka W. Microencapsulation. Angewandte Chemie International Edition in English. 1975 Aug;14(8):539-50.

22 Burgain J, Gaiani C, Cailliez-Grimal C, Jeandel C, Scher J. Encapsulation of *Lactobacillus rhamnosus* GG in microparticles: Influence of casein to whey protein ratio on bacterial survival during digestion. Innovative Food Science & Emerging Technologies. 2013 Jul 1;19:233-42.

23 Sohail A, Turner MS, Coombes A, Bhandari B. The viability of *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* NCFM following double encapsulation in alginate and maltodextrin. Food and Bioprocess Technology. 2013 Oct;6:2763-9.

24 Krasaekoopt W, Bhandari B, Deeth H. The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. International dairy journal. 2004 Aug 1;14(8):737-43.

25 Corona-Hernandez RI, Álvarez-Parrilla E, Lizardi-Mendoza J, Islas-Rubio AR, de la Rosa LA, Wall-Medrano A. Structural stability and viability of microencapsulated probiotic bacteria: a review. Comprehensive Reviews in Food Science and Food Safety. 2013 Nov;12(6):614-28.

26 Rao AV, Shiwnarain N, Maharaj I. Survival of microencapsulated *Bifidobacterium pseudolongum* in simulated gastric and intestinal juices. Canadian Institute of Food Science and Technology Journal. 1989 Oct 1;22(4):345-9.

27 Moghaddas Kia E, Ghasempour Z, Ghanbari S, Pirmohammadi R, Ehsani A. Development of probiotic yogurt by incorporation of milk protein concentrate (MPC) and microencapsulated *Lactobacillus paracasei* in gellan-caseinate mixture. British Food Journal. 2018 Aug 7;120(7):1516-28.

28 de Vos P, Faas MM, Spasojevic M, Sikkema J. Encapsulation for preservation of functionality and targeted delivery of bioactive food components. International dairy journal. 2010 Apr 1;20(4):292-302.

29 Gbassi GK, Vandamme T. Probiotic encapsulation technology: from microencapsulation to release into the gut. Pharmaceutics. 2012 Feb 6;4(1):149-63.

30 Leon AM, Medina WT, Park DJ, Aguilera JM. Mechanical properties of whey protein/Na alginate gel microparticles. Journal of Food Engineering. 2016 Nov 1;188:1-7.

31 Gombotz WR, Wee SF. Protein release from alginate matrices. Advanced drug delivery reviews. 1998;31(3) :267-285.

32 Burgain J, Gaiani C, Linder M, Scher J. Encapsulation of probiotic living cells: From laboratory scale to industrial applications. Journal of food engineering. 2011 Jun 1;104(4):467-83.

33 Dong QY, Chen MY, Xin Y, Qin XY, Cheng Z, Shi LE, Tang ZX. Alginate-based and protein-based materials for probiotics encapsulation: a review. International journal of food science & technology. 2013 Jul;48(7):1339-51.

34 Lucey JA. Formation and physical properties of milk protein gels. Journal of dairy science. 2002 Feb 1;85(2):281-94.

35 Fox PF, McSweeney, PLH. Advanced dairy chemistry. 3rd ed. NewYork: Springer; 2003.

36 Wong, NP, Fundamentals of dairy chemistry. 3rd ed. New York; Van Nostrand Reihold Co, 1988.

37 Heidebach T, Först P, Kulozik U. Microencapsulation of probiotic cells by means of rennet-gelation of milk proteins. Food hydrocolloids. 2009 Oct 1;23(7):1670-7.

38 Ramdhan T, Ching SH, Prakash S, Bhandari B. Evaluation of alginate-biopolymers (protein, hydrocolloid, starch) composite microgels prepared by the spray aerosol technique as a carrier for green tea polyphenols. Food Chemistry. 2022 Mar 1;371:131382.

39 Ching SH. Alginate microgel particles: stability, rheology and application as bioactive carrier. [dissertation] St Lucia (AU): University of Queensland; 2015 [cited 2023 Nov 27]. Available from: *https://core.ac.uk/download/pdf/43374938.pdf*

40 Bhandari B, inventor; The University of Queensland, assignee. Australia patent PCT/AU2008/001695 . 2009 May 22 [cited 2023 Nov 28]. Available from: *https://espace.library.uq.edu.au/view/UQ:328393*.

41 Bokkhim H, Bansal N, Grøndahl L, Bhandari B. Characterization of alginate–lactoferrin beads prepared by extrusion gelation method. Food Hydrocolloids. 2016 Feb 1;53:270-6.

42 Official methods of analysis. Washington, DC: Association of Official Analytical Chemists; 2008.
43 Ding WK, Shah NP. Acid, bile, and heat tolerance of free and microencapsulated probiotic bacteria. Journal of food science. 2007 Nov;72(9):M446-50.
44 Pestovsky YS, Martínez-Antonio A. The synthesis of alginate microparticles and nanoparticles. Drug Des. Intellect. Prop. Int. J. 2019;3:293-327.
45 Venugopal V. Marine polysaccharides: Food applications. CRC press; 2016 Apr 19.
46 HJ, Smith MK, Mooney DJ. Designing alginate hydrogels to maintain viability of immobilized cells. Biomaterials. 2003 Oct 1;24(22):4023-9.
47 Sera CA, Pradhan M, Prakash S, Gaini C, Bhandari, B. Morphological, rheological and tribological properties of *Lactobacillus rhamnosus* GG (LGG) encapsulated protein-alginate composite microgels [master's thesis]. St Lucia (AU): University of Queensland; 2016.
48 Pirarat N, Pinpimai K, Rodkhum C, Chansue N, Ooi EL, Katagiri T, Maita M. Viability and morphological evaluation of alginate-encapsulated *Lactobacillus rhamnosus* GG under simulated tilapia gastrointestinal conditions and its effect on growth performance, intestinal morphology and protection against *Streptococcus agalactiae*. Animal Feed Science and Technology. 2015 Sep 1;207:93-103.
49 Mandal S, Puniya AK, Singh K. Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298. International Dairy Journal. 2006 Oct 1;16(10):1190-5.
50 Rokka S, Rantamäki P. Protecting probiotic bacteria by microencapsulation: challenges for industrial applications. European Food Research and Technology. 2010 May;231:1-2.
51 Ying DY, Phoon MC, Sanguansri L, Weerakkody R, Burgar I, Augustin MA. Microencapsulated *Lactobacillus rhamnosus* GG powders: relationship of powder physical properties to probiotic survival during storage. Journal of Food Science. 2010 Nov;75(9):E588-95.
52 Berney M, Hammes F, Bosshard F, Weilenmann HU, Egli T. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. Applied and environmental microbiology. 2007 May 15;73(10):3283-90.
53 Jiménez-Pranteda ML, Poncelet D, Náder-Macías ME, Arcos A, Aguilera M, Monteoliva-Sánchez M, Ramos-Cormenzana A. Stability of *Lactobacilli* encapsulated in various microbial polymers. Journal of bioscience and bioengineering. 2012 Feb 1;113(2):179-84.