

## RESEARCH ARTICLE

# Chitosan coated alginate beads as probiotic delivery system for New Zealand black footed abalone (*Haliotis iris*)

Seyedehsara Masoomi Dezfooli<sup>1</sup>  | Chloe Bonnot<sup>2</sup> | Noemi Gutierrez-Maddox<sup>3</sup> |  
Andrea C. Alfaro<sup>4</sup> | Ali Seyfoddin<sup>2</sup> 

<sup>1</sup>Aquaculture Biotechnology Research Group, Drug Delivery Research Group, School of Science, Faculty of Health and Environmental Sciences, Auckland University of Technology, Auckland, New Zealand

<sup>2</sup>Drug Delivery Research Group, School of Science, Faculty of Health and Environmental Sciences, Auckland University of Technology, Auckland, New Zealand

<sup>3</sup>School of Science, Faculty of Health and Environmental Sciences, Auckland University of Technology, Auckland, New Zealand

<sup>4</sup>Aquaculture Biotechnology Research Group, School of Science, Faculty of Health and Environmental Sciences, Auckland University of Technology, Auckland, New Zealand

## Correspondence

Ali Seyfoddin, Drug Delivery Research Group, School of Science, Faculty of Health and Environmental Sciences, Auckland University of Technology, Auckland 1010, New Zealand.  
Email: [ali.seyfoddin@aut.ac.nz](mailto:ali.seyfoddin@aut.ac.nz)

## Funding information

Auckland University of Technology (AUT), New Zealand; Ministry for Primary Industries; Auckland University of Technology (AUT)

## Abstract

The potential benefits of probiotics for growth of black-footed abalone (*Haliotis iris*) have been highlighted in previous studies. However, traditional methods of probiotic administration in aquaculture are inefficient due to environmental contamination and loss of bacterial viability. This study investigates a new delivery system for delivery of probiotics to *H. iris*. An extrusion technique coupled with ionotropic gelation was utilized to produce chitosan coated alginate beads (CCALG). Concentration of alginate, chitosan, and coating time were optimized to obtain beads with a desired morphology, stability, sinking time, and release profile. The results showed that increasing alginate concentration up to 1.5% w/w can improve the morphological characteristics of the beads while reducing the sinking time in seawater. Chitosan coating improved the stability of beads and reduced the release of the encapsulated probiotics in seawater. A higher probiotic load was found in the digestive tract of animals fed with CCALG beads ( $1.3 \times 10^8$  CFU) compared to the control group ( $6 \times 10^3$  CFU). High palatability and stability of CCALG beads in seawater combined with controlled release of viable probiotics show that encapsulation of probiotics in CCALG beads is an efficient method for delivery of probiotics in aquaculture.

## KEYWORDS

abalone, alginate, aquaculture, chitosan, encapsulation, probiotic delivery, probiotics

## 1 | INTRODUCTION

A great number of studies have shown the beneficial effects of probiotics to promote growth and control diseases in aquatic animals.<sup>1,2</sup> Traditionally, probiotics are applied in aquaculture settings through water treatment or as feed additives. However, the traditional methods of probiotic delivery in aquaculture raise several key concerns such as environmental contamination due to the release of live microorganisms into farm effluent water. Moreover, these techniques are not ideal since the initial dosages may be diluted in the culturing water before they reach the target animals. The loss in viability of probiotics during delivery is another major concern which limits the beneficial effects of the administered probiotics.<sup>3</sup> Therefore, the design of new delivery methods that can retain the viability of the probiotics and release them into a target site in the gastrointestinal tract (GIT) of the animal is essential for an efficient delivery of probiotics in aquaculture.

In recent years, controlled and targeted drug delivery strategies used in pharmaceuticals have been adopted by marine scientists to develop new delivery methods for probiotics in aquaculture.<sup>4–7</sup> Although the application of encapsulation techniques is quite established for pharmaceutical purposes, not many research has been carried out to develop controlled release systems of probiotics for aquaculture application. Different types of materials including alginate, chitosan, whey protein, skim milk, and polylactic-co-glycolic acid (PLGA) have been utilized to create confinement structures for delivery of probiotics, among which alginate has been one of the most commonly used natural polymer.<sup>5,8–11</sup> Alginate offers desired properties for encapsulating probiotics, such as biocompatibility, water solubility, biodegradability, and it is widely used as a food ingredient.<sup>12</sup> Probiotics can be immobilized in an alginate matrix simply by extrusion into a cross-linking solution while retaining a high survival rate of up to 95% for probiotics after encapsulation.<sup>13</sup> Alginate has also been used to enhance the shelf life of encapsulated probiotics.<sup>14</sup> In addition, the negatively charged polymer chains of alginate and its pH-dependent solubility has made alginate an excellent candidate for designing targeted delivery of probiotics to GIT of aquatic animals.<sup>11</sup>

Although alginate is a great candidate for delivery of probiotics in aquaculture, the properties of its hydrogels should be carefully optimized with regards to its application. Some properties such as size, morphology, floatability, sinking time and stability should be critically adjusted considering the physiology of the target animal (e.g., pH of GIT), feeding habit (e.g., bottom- or surface- feeder) and culturing conditions (e.g., pH of

culturing water). The morphology of alginate beads can influence the bead stability and release profile of the encapsulated probiotics.<sup>15</sup> The size and sinking time of particles can have significant impact on the accessibility of hydrogels to animals. For example, previously floating alginate microcapsules were successfully used for delivery of probiotics to fish.<sup>16</sup> However, these microcapsules would probably be less efficient in delivery of probiotics to bottom-feeders, such as abalone. The characteristics of alginate hydrogels can be adjusted by modifying the concentration of polymer, type and concentration of hardening solutions, incubation time and temperature.<sup>15</sup>

Most previous studies have focused on the effect of the encapsulated probiotics on the growth and health of the target species with minimum attention toward optimizing the probiotic carrier. However, optimizing the physiochemical properties of the probiotic carrier should be prioritized before performing a feeding trial. Therefore, the aim of this study is to use a stepwise optimization approach to develop a new delivery system of probiotics to New Zealand black-footed abalone. The experimental work utilizes an extrusion technique to encapsulate probiotics in chitosan-coated alginate hydrogels. The assessed physiochemical characterization of the beads is used to identify an optimized formulation with desired properties, such as spherical shape, high consumption rate and high stability in seawater. The viability of the encapsulated probiotics after encapsulation, storage, during incubation and transit within GIT of abalone are evaluated.

## 2 | MATERIALS AND METHODS

### 2.1 | Optimization process

A step-by-step approach was applied to optimize the properties of chitosan coated alginate beads (CCALG). The core and shell of the beads were characterized in two main experimental phases as listed in Table 1. In phase I, alginate was considered as the core material to produce plain polymeric alginate beads (ALG). Here, the percentage of alginate was optimized considering viscosity of obtained formulations, morphology and sinking time of ALG beads in seawater. The concentration of alginate which produced spherical beads with lowest settling time in seawater was selected as optimized and was further used in phase II of the optimization process. In the second phase, the optimized concentration of alginate was used to produce CCALG beads. Different concentrations of chitosan in the coating solution and three coating times were then assessed to determine a single

TABLE 1 Optimization process of CCALG beads: parameters and variables

| Phase | Parameters                                 | Formula code | Concentration of sodium alginate (% w/v) | Concentration of chitosan (% w/v) | Coating time (min) |
|-------|--------------------------------------------|--------------|------------------------------------------|-----------------------------------|--------------------|
| I     | Concentration of sodium alginate           | ALG-0.5      | 0.5                                      | -                                 | -                  |
|       |                                            | ALG-1.0      | 1.0                                      | -                                 | -                  |
|       |                                            | ALG-1.5      | 1.5                                      | -                                 | -                  |
|       |                                            | ALG-2.0      | 2.0                                      | -                                 | -                  |
| II    | Concentration of chitosan and coating time | CCALG-0.2-20 | 1.5                                      | 0.2                               | 20                 |
|       |                                            | CCALG-0.2-40 | 1.5                                      | 0.2                               | 40                 |
|       |                                            | CCALG-0.2-60 | 1.5                                      | 0.2                               | 60                 |
|       |                                            | CCALG-0.4-20 | 1.5                                      | 0.4                               | 20                 |
|       |                                            | CCALG-0.4-40 | 1.5                                      | 0.4                               | 40                 |
|       |                                            | CCALG-0.4-60 | 1.5                                      | 0.4                               | 60                 |
|       |                                            | CCALG-0.8-20 | 1.5                                      | 0.8                               | 20                 |
|       |                                            | CCALG-0.8-40 | 1.5                                      | 0.8                               | 40                 |
|       |                                            | CCALG-0.8-60 | 1.5                                      | 0.8                               | 60                 |

TABLE 2 Composition of ALG beads

| Formula code | SA (ml) | Media (ml) | dH <sub>2</sub> O (ml) | Final volume (ml) | Final SA concentration (w/v) |
|--------------|---------|------------|------------------------|-------------------|------------------------------|
| ALG-0.5      | 6       | 3          | 21                     | 30                | 0.5                          |
| ALG-1        | 12      | 3          | 15                     | 30                | 1                            |
| ALG-1.5      | 18      | 3          | 9                      | 30                | 1.5                          |
| ALG-2        | 24      | 3          | 3                      | 30                | 2                            |

formulation with minimum bacterial release and matrix erosion in seawater.

## 2.2 | Preparation of alginate beads

Sodium alginate (SA) (Acros Organics, China) stock solutions (2% w/v and 2.5% w/v) were prepared by dissolving sodium alginate powder in distilled water (dH<sub>2</sub>O), which was stirred overnight to obtain a fully dissolved alginate solution. The SA stock solutions were stored in a refrigerator (4°C) until use.

ALG beads were produced by mixing a solution of sodium alginate with marine broth. The mixture was extruded through a 20 ml syringe with an aperture size of 2.22 mm and allowing the mixture to free-fall from about 10 cm into a 100 ml CaCl<sub>2</sub> (Ajax Finechem, Australia) cross-linking bath (0.1 M). The produced particles were

collected using a sieve after a 30-min incubation period in the agitated cross-linking bath (200 rpm). Alginate beads were then washed with dH<sub>2</sub>O (100 ml) twice to remove unbound CaCl<sub>2</sub> from the surface of the beads. Several formulations were prepared to study the effect of concentration of sodium alginate on physical properties of ALG beads (Table 2). In order to facilitate the optimization process and reduce experimental cost, blank media (Marine broth) was used instead of probiotic cultures, unless otherwise stated.

## 2.3 | Preparation of chitosan-coated alginate beads

Chitosan coating solutions with different concentrations of 0.2, 0.4, and 0.8% w/v were prepared by dissolving appropriate amounts of medium molecular weight chitosan (Sigma,

USA) in lactic acid (1% v/v in dH<sub>2</sub>O) with pH adjusted to 5.70 by gradual addition of NaOH (1 M). The chitosan solution was stirred (200 rpm) until a clear solution was obtained. ALG beads obtained previously, were transferred into an agitated chitosan solution (100 rpm) and incubated over various times (20, 40, and 60 min). The CCALG beads were then separated using a plastic mesh and washed with dH<sub>2</sub>O (100 ml) twice for further characterization.

## 2.4 | pH and viscosity

The pH and viscosity were assessed using a pH meter (GOnDO, Taiwan) and rheometer (Brookfield AMETEK, USA). Each property was measured for three identical samples and the average value was reported for each test. Viscosity measurements were carried out using a rheometer fitted with cone NO. CCT-25 accepting 15 ml of sample. Measurements were made at room temperature (24°C) while the shear rate was increased from 50 to 400 s<sup>-1</sup>. The duration time was 120 s with a total of 100 measurement points for each sample.

## 2.5 | Microscopy

The mean diameter of 50 beads was measured using a light microscope equipped with a scaled eyepiece and a stage micrometer to calculate the average size (Leica, USA). The morphology and microstructure of the beads were examined using an optical light microscope (Olympus CX31, Philippine) and scanning electron microscope (SEM) (Hitachi SU-70, Japan). For SEM imaging, samples were dehydrated at 35°C and randomly selected for SEM imaging. The dried beads were placed on double sided adhesive carbon tapes on aluminum stubs and then coated with a thin layer of platinum under vacuum for 60 sec by an ion sputter coater (Hitachi E-1045, Japan). The elemental analysis was performed using an energy dispersive spectrometer (EDS). Dried beads were observed under SEM at 15 kV and EDS spectra were obtained to screen the elemental composition of the beads using Noran System 7 (NSS) microanalysis system software (ThermoScientific, USA).

## 2.6 | Sinking time in seawater

The sinking time of particles ( $n = 10$ ) was measured by placing individual beads in a measuring cylinder filled with seawater. The height of the seawater column was 16 cm. The time that a particle traveled the column

height was recorded as its sinking time. The measurements were repeated for at least 10 individual particles.

## 2.7 | Probiotic cultivation and encapsulation

A probiotic cocktail consisting of three species of bacteria was used in this study. Bacterial isolates were kindly provided by the Aquaculture and Biotechnology Research Group at Auckland University of Technology (Auckland, New Zealand). All the three probiotic bacteria namely *Exiguobacterium* sp., *Vibrio* sp., and *Enterococcus* sp. were revived from glycerol stocks. *Exiguobacterium* sp. and *Vibrio* sp. were cultured in marine broth. The cultures were incubated at room temperature (20–22°C) for 18 h under agitation at 100 rpm. *Enterococcus* sp. was cultured in the same media for 18 h under anaerobic condition at room temperature (20–22°C) using Gaspak™ EZ anaerobic container system (New Jersey, USA). A selective medium, oxide kanamycin esculin azide agar (KAA), was used to selectively culture *Enterococcus* sp. when required. After the incubation period, bacterial cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C. Pellets were resuspended in fresh marine broth before adding to a solution of sodium alginate for encapsulation. ALG beads were produced following the method described in Section 2.2. The ALG beads were subsequently transferred into an agitated chitosan solution (100 rpm). The CCALG beads were then collected and washed as described in Section 2.3 and utilized for characterization immediately after production.

## 2.8 | Matrix erosion

Beads containing *Exiguobacterium* sp., *Enterococcus* sp., and *Vibrio* sp. ( $7 - 9 \times 10^9$  CFU/g) were used to study the stability in seawater. The beads were then placed in beakers (0.20 g per beaker). Seawater was added to each beaker (40 ml), which was then incubated on a shaker (130 rpm) at room temperature. Particles were collected after 72 h and were dried in an oven (35°C). The dried weight of particles was measured and the percentage of matrix erosion in the particles was calculated using the following Equation (1):

$$\text{Matrix erosion (\%w/w)} = \frac{(W_0 - W)}{W_0} \times 100, \quad (1)$$

where  $W_0$  is the initial dry weight of the beads and  $W$  is the mass of dry beads after incubation in seawater.

## 2.9 | Bacterial staining with fluorescent dye

A single colony of *Exiguobacterium* sp. was inoculated in marine broth (100 ml). The inoculated medium was incubated at room temperature for 18 h while shaking (100 rpm) to ensure adequate aeration of the medium. The bacterial cells were subsequently collected by centrifugation at 4000 rpm for 15 min. A solution of 0.85% w/v NaCl was used to wash bacterial pellets twice. A LIVE/DEAD Bacterial Viability Kit (L7012, BacLight, USA) was used for the staining of bacteria. The kit contained SYTO9 and propidium iodide dyes with 485/498 and 535/617 nm excitation/emission wavelengths, respectively. Volumes equal to 30  $\mu$ l were combined in a dark tube from each prewarmed fluorescence dyes. The mixture was mixed with the recovered bacterial pellets and incubated in the dark for 15 min. The cells were then centrifuged at 4000 rpm for 15 min at 10°C. The supernatant was removed followed by two washing steps to remove free fluorophores.

## 2.10 | Bacterial release study

Fluorescent-labeled bacteria were used to encapsulate probiotics in CCALG and ALG beads. *Exiguobacterium* sp. was used in this test because it can be recognized easily under the microscope and has high survivability during the staining procedure. The encapsulated bacteria (1.00 g) were placed in a falcon tube with 5 ml of filtered seawater (0.22  $\mu$ m). The tubes were kept at 15°C to mimic the temperature at which abalone were maintained. Aliquots of 40  $\mu$ l were withdrawn from the release medium at various time intervals (1, 2, 3, 4, 5, 6, 24, 48, and 72 h). A volume of 10  $\mu$ l was immediately placed on a counting chamber to determine the number of released bacteria using a fluorescence microscope. The experiment was performed in triplicate for each formulation.

## 2.11 | Effect of encapsulation on the viability of probiotics

To measure the effect of encapsulation on the viability of probiotics, CCALG beads were dissolved using sodium citrate solution (10% w/v, pH: 6.0). Briefly, CCALG beads (4.0 g) containing probiotic bacteria were incubated for 20 min in the depolymerizing solution while shaking (300 rpm) at room temperature. After a complete degradation of the beads, the

obtained solution was serially diluted using sterile marine broth medium. The number of colony-forming units was quantified by culturing 100  $\mu$ l of each dilution on KAA and marine agar plates. Cultured KAA plates were incubated in anaerobic conditions for 48 h. Cultured marine agar plates were incubated at room temperature until the colony colors were fully developed. The percentage of bacterial survivability after encapsulation was calculated using Equation (2)<sup>17</sup>:

$$\text{Survivability (\%)} = \frac{N}{N_0} \times 100, \quad (2)$$

where  $N$  is the number of released viable bacteria from the beads and  $N_0$  is the number of viable bacteria added to the formulation before encapsulation.

## 2.12 | Observational evaluation of acceptability and palatability of the developed beads

Juvenile black-footed abalone (25–35 mm shell length) were provided by Moana New Zealand (Ruakaka, New Zealand). Seawater was collected from Okahu Bay, Auckland. The commercial feed manufactured by Marifeed (Hermanus, South Africa) was obtained from Moana™ New Zealand (Ruakaka, New Zealand). Four juvenile abalone were kept in a small tank with 10 L of filtered seawater at 15°C. The animals were kept in the tank for a week and fed daily with commercial feed (eight pellets) to acclimatize the animals. On the day of the experiment all uneaten feed was removed, and the tank water was replaced with 10 L of fresh seawater. Then, eight commercial pellets and eight encapsulated probiotic beads were placed in the tank simultaneously. Feeds were then distributed evenly within the tank. The number of uneaten feed pellets and CCALG beads were counted after 24 h of incubation. The experiment was performed in triplicate. The eating behavior of abalone was recorded using a digital single lens reflex camera. Animals were selected randomly and placed in a glass-bottom aquarium. The camera was set at the bottom of the aquarium to view the animals from underneath.

## 2.13 | Probiotic load in the digestive tract of abalone

The efficiency of beads in delivering probiotics to the abalone's gut was studied on CCALG beads containing *Enterococcus* sp. as a model strain. Six abalone were

transferred into two separate tanks and starved for 24 h before the beginning of the experiment. Each tank contained 10 L of filtered seawater and was aerated with two air stones. Water temperature was maintained at 15°C and the water was replaced with fresh seawater after removal of uneaten beads and before placement of fresh beads every 2 days. Three animals were fed with CCALG beads (4.0 g) containing probiotic bacteria and the other three abalone were fed empty beads (4.0 g) every 2 days. After 10 days, all the control and probiotic-fed animals were collected, shucked and placed in a stomacher bag. The digestive tract of each abalone was homogenized separately in sterile marine broth (10 ml) for 5 min using a stomacher. Several dilutions from  $10^1$  to  $10^7$  were prepared from each homogenized solution. A volume of 100  $\mu$ l from each dilution was cultured on KAA plate and incubated for 72 h at room temperature until the colors of colonies were fully developed.

## 2.14 | Effect of storage condition on the viability of encapsulated bacteria

The viability of encapsulated bacteria in optimized formulation of CCALG beads was investigated at two different storage conditions including refrigeration at 4°C and freeze drying. All three species of probiotic bacteria (*Exiguobacterium* sp., *Vibrio* sp., and *Enterococcus* sp.) were encapsulated in CCALG beads. The produced beads were then stored at 4°C for 96 h. The number of bacteria was enumerated every 2 days by dissolving the CCALG beads (4.0 g) in sodium citrate solution (10% w/v, pH: 6.0). The obtained solution was serially diluted and cultured (100  $\mu$ l) on KAA and marine agar plates. The number of bacteria was recorded after 72 h of incubation at room temperature when the color of the colonies was fully developed. The experiment was performed in triplicate.

The viability of encapsulated probiotics was assessed after freeze drying (lyophilization). Overnight cultures of the three probiotic bacteria were prepared as explained in Section 2.7. The bacterial suspensions were centrifuged at 4000 rpm for 15 min at 4°C. The pellets of the three bacterial species were resuspended in 2 ml of fresh marine broth medium. A volume equal to 1 ml of the bacterial suspension was added to the bead formulation. CCALG beads were prepared as described in Sections 2.2 and 2.3. The produced beads (4.0 g) were incubated in a freezer (−20°C) for 12 h and subsequently lyophilized for 24 h (−75°C with 0.0014 mbar pressure). Freeze dried CCALG beads were collected and dissolved in 10 ml of sodium citrate

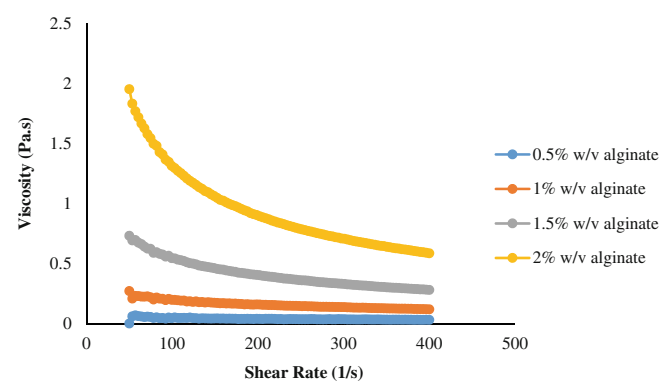
solution (10% w/v, pH: 6.0) for 30 min with constant orbital shaking at 300 rpm. Then, 1 ml of the obtained suspension was serially diluted until dilution factor of  $10^{10}$ . A volume of 100  $\mu$ l from all dilutions was plated on marine agar and KAA plates. The cultured petri dishes were incubated for 72 h at room temperature until the color of colonies was fully developed. The number of colony-forming units was recorded with the naked eyes for *Exiguobacterium* sp. and *Vibrio* sp. and with a magnifying glass for *Enterococcus* sp.

## 2.15 | Statistical analysis

Triplicate samples were analyzed for each experiment. The results were analyzed using SPSS, version 20. Differences in bacterial cell counts between probiotic fed and control animals were identified using the Student's t-test analysis ( $p < 0.05$ ). For other experiments, significant differences among different optimizing factors were detected using ANOVA, Tukey's posthoc test with a  $p$  value set to 0.05.

**TABLE 3** Viscosity of alginate solutions used in the formulation of beads ( $n = 100 \pm \text{SD}$ )

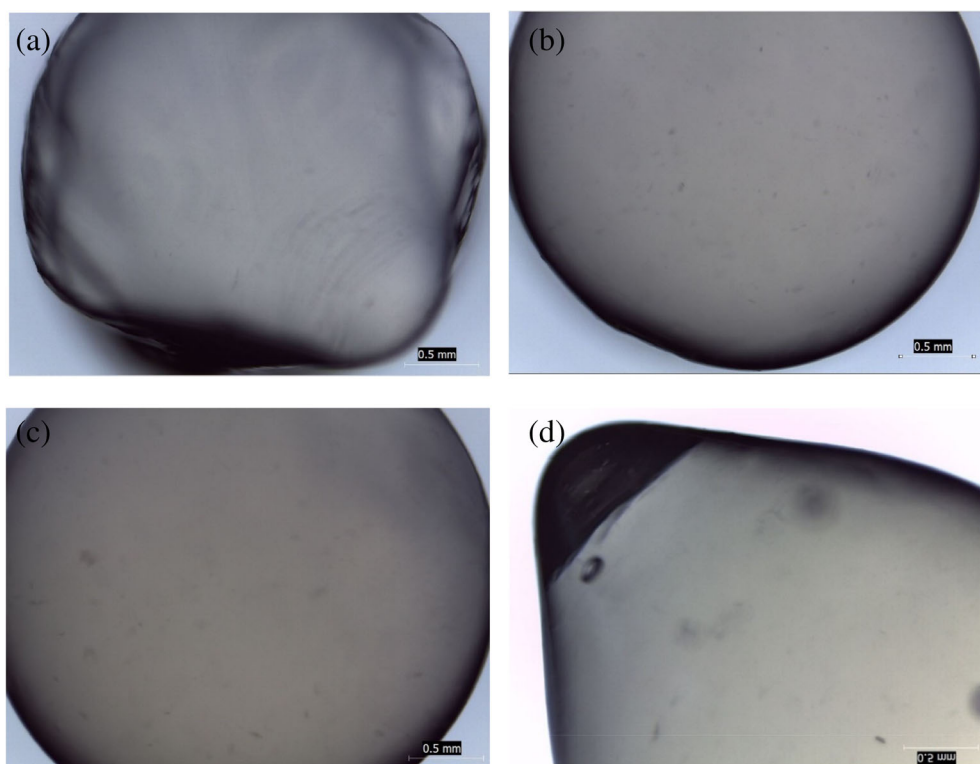
| Formulae code | Concentration of alginate (w/v %) | Viscosity (pa.s) |
|---------------|-----------------------------------|------------------|
| ALG-0.5       | 0.5                               | $0.04 \pm 0.00$  |
| ALG-1.0       | 1.0                               | $0.16 \pm 0.02$  |
| ALG-1.5       | 1.5                               | $0.41 \pm 0.06$  |
| ALG-2.0       | 2.0                               | $0.93 \pm 0.18$  |



**FIGURE 1** Plot of viscosity versus shear rates for different ALG formulations. The concentration of sodium alginate varied from 0.5–2% w/v. conditions: Average of 100 measurements. Time: 120 s, shear rate = 50–400, temp: 26°C [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

**TABLE 4** Measured characteristics of ALG beads ( $n = 50 \pm \text{SD}$  for size,  $n = 10 \pm \text{SD}$  for sinking time)

| Formula code | Shape     | Average size (mm)                | Sinking time of fresh beads (s) | Sinking time of presoaked beads (s) |
|--------------|-----------|----------------------------------|---------------------------------|-------------------------------------|
| ALG-0.5      | Irregular | $3.0 \pm 0.1 \times 2.7 \pm 0.1$ | $473 \pm 12$                    | $164 \pm 32$                        |
| ALG-1.0      | Spherical | $3.2 \pm 0.0$                    | $280 \pm 18$                    | $10 \pm 0.9$                        |
| ALG-1.5      | Spherical | $4.1 \pm 0.1$                    | $157 \pm 37$                    | $6.4 \pm 0.6$                       |
| ALG-2.0      | Pear      | $6.7 \pm 0.3 \times 4.6 \pm 0.2$ | $83 \pm 14$                     | $5.6 \pm 0.6$                       |

**FIGURE 2** Morphology of alginate beads with (a) 0.5, (b) 1, (c) 1.5 and (d) 2% w/v alginate under a light microscope ( $\times 40$ ) [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/app.52626)]

### 3 | RESULTS AND DISCUSSION

#### 3.1 | Phase I: Effect of concentration of sodium alginate on the beads

##### 3.1.1 | Viscosity

While the pH of all alginate formulations remained similar ( $7.05 \pm 0.02$ ), an increase in viscosity of the alginate formulations was observed as alginate concentration increased (Table 3). The apparent viscosity of the alginate formulations was gradually decreased with increasing shear rate (Figure 1). This fluid behavior is a characteristic of non-Newtonian shear-thinning fluids, which can be seen in polymeric systems.<sup>18</sup> The extent of the change in the viscosity of the tested formulations was related to the different concentrations of sodium alginate. As the alginate concentration decreased, viscosity became

independent of the shear rate. This may be due to the internal interactions between the structural units of alginate. Higher alginate concentrations are related to more polymeric chains entangled which offer higher resistance against deformation or flow by demonstrating higher viscosity. These interactions then gradually disappeared at higher shear rates which facilitate flow.

##### 3.1.2 | Bead morphology

The diameter of 50 individual particles was measured and the results are presented as mean diameter  $\pm$  SD in Table 4. The size of alginate particles was increased by increasing the alginate concentration.

The morphologies of alginate beads are illustrated in Figure 2. ALG-0.5 and ALG-2.0 beads were irregular and pear-shaped, respectively. Conversely, ALG-1.0 and ALG-

1.5 formulations produced spherical beads after gelation. The shape of the beads is an important factor that affects the release profile of encapsulated cells. This can happen due to the presence of areas with different thicknesses in nonspherical particles which can generate a unique degradation and release profile due to changes in their shape over time in the release media.<sup>19</sup>

### 3.1.3 | Sinking time in seawater

The sinking time of alginate beads was measured as the time that a particle moved a specific distance through seawater column. The sinking time of the beads is important as they need to be readily accessible to abalone and not be washed away by the water flow in the culturing tanks. The sinking time of alginate beads was measured immediately after the washing step with distilled water following production. There is a lag time before the alginate beads start to sink in seawater where differences in osmotic pressure provoke ion exchanges and water penetration into the beads.<sup>19</sup> As shown in Table 4, beads with faster sinking time can be obtained by increasing the internal alginate concentration of the beads. This may be attributed to the higher density in alginate particles produced with higher alginate concentration.<sup>20</sup>

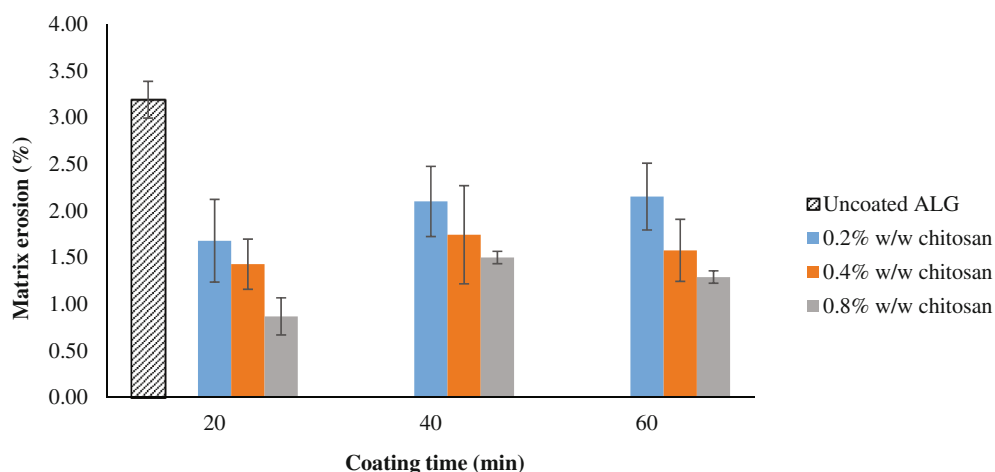
In order to improve the sinking rate, ALG beads could be soaked in seawater for 5 min. The results showed that the presoaking step can reduce the sinking time of the beads. This could be due to a reduction of osmotic pressure gradient between alginate beads and seawater. When seawater-soaked beads are placed in seawater, the internal moiety of the beads is almost isotonic with seawater. Therefore, they exhibited significantly higher sinking rate compared to ALG beads with no soaking step in seawater (Table 4).

### 3.1.4 | Phase I conclusion

The concentration and viscosity of the alginate solution are influential factors that affect the final size and shape of beads.<sup>21</sup> At very low alginate concentrations, alginate droplets may deform when coming in contact with the surface of the cross-linking solution.<sup>22,23</sup> This could explain why at 0.5% w/v alginate concentration, the beads were nonspherical. Similarly, beads containing 2% w/v alginate (ALG-2.0 beads), which had the highest viscosity among the tested formulations, were nonspherical (pear-shaped). Moreover, extrusion of ALG-2.0 into the gelation bath was extremely difficult due to the high viscosity of the formulation. Formulations with 1 and 1.5% w/v alginate (ALG-1.0 and ALG-1.5) produced spherical beads. However, ALG-1.5 beads had alginate with higher viscosity, which could produce better gel strength and stability as suggested by previous studies.<sup>23,24</sup> ALG-1.5 beads also sink faster in seawater, which is ideal for delivering probiotics to bottom feeder abalone. Therefore, 1.5% w/v sodium alginate was selected as the optimized concentration to be used in the next two phases of the optimization process.

## 3.2 | Phase II: Optimization of chitosan coating

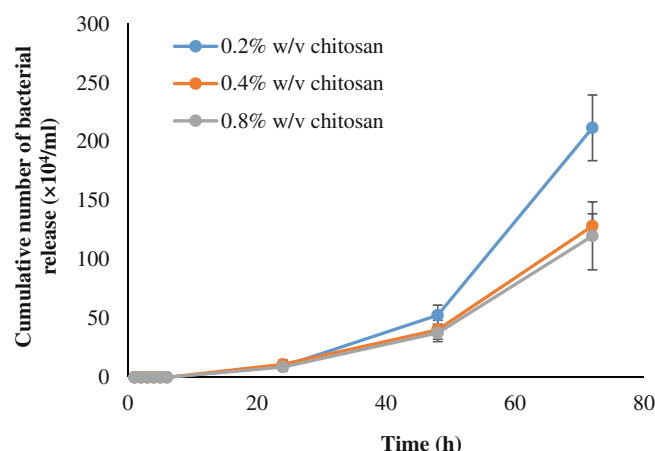
Chitosan coating is proposed to increase the stability of beads in seawater. Therefore, in phase II optimization, the effect of concentration of chitosan and coating time in the formulation of CCALG beads was investigated. Here, three chitosan concentrations (0.2, 0.4, and 0.8% w/v) were used to coat alginate beads for 20, 40, and 60 min.



**FIGURE 3** Percentage of matrix erosion of uncoated ALG and coated CCALG beads with different coating time (20, 40, and 60 min) and chitosan concentration (0.2, 0.4, and 0.8% w/v) 3 days post incubation in seawater ( $n = 3$ ; mean  $\pm$  SD) [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/app.52626)]

### 3.2.1 | Matrix erosion

The percentage matrix erosion for CCALG beads prepared using different concentration of chitosan and coating times is illustrated in Figure 3. A slight reduction (not significant,  $p > 0.05$ ) in erosion was observed when the concentration of chitosan increased. The highest erosion was obtained from uncoated ALG beads with  $3.19\% \pm 0.2$  following by CCALG-0.2-60 with  $2.2\% \pm 0.3$ . CCALG-0.8-20 with  $0.9\% \pm 0.1$  resulted in the lowest matrix erosion after 3 days of incubation in seawater. There was no significant difference in matrix erosion with increasing coating time.



**FIGURE 4** Cumulative release curves of *Exiguobacterium* sp. from CCALG coated with different concentration of chitosan (0.2, 0.4, and 0.8% w/v). Release media was seawater ( $n = 3$ ; mean  $\pm$  SD) [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/app.52626)]

### 3.2.2 | Effect of chitosan coating on the release profile of encapsulated bacteria

To evaluate the effect of chitosan concentration on bacterial release, the following formulations were investigated: CCALG-0.2-20, CCALG-0.4-20, and CCALG-0.8-20. Beads with lower concentration of chitosan showed a faster bacterial release. CCALG-0.2-20 had significantly faster release ( $p < 0.05$ ) after 72 h in seawater. However, there was no significant difference between CCALG-0.4-20 and CCALG-0.8-20 ( $p > 0.05$ ) (Figure 4).

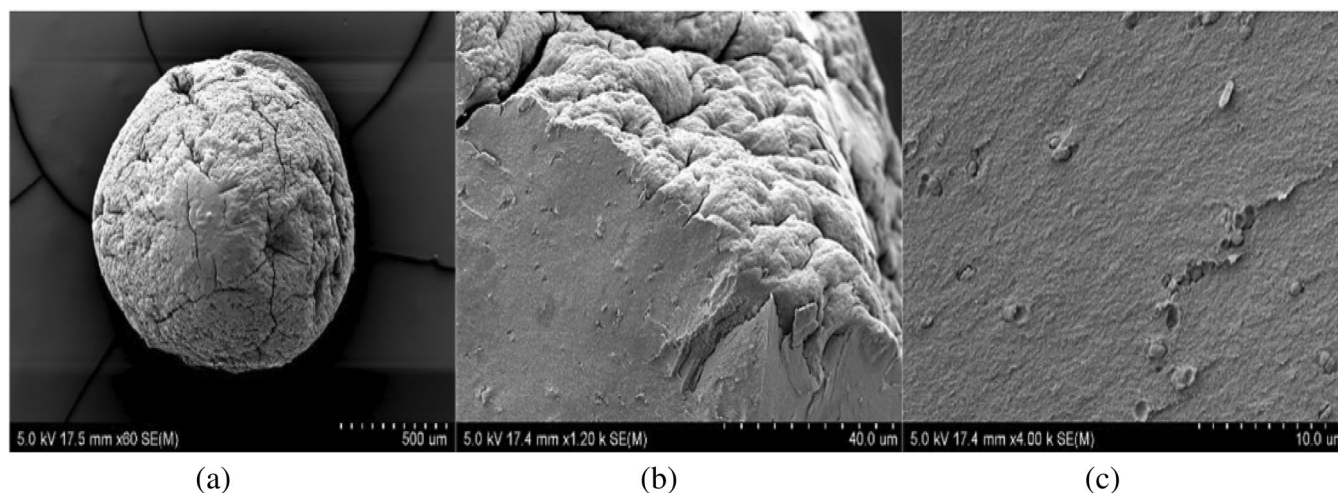
### 3.2.3 | Phase II conclusion

The aim of the second phase of optimization was to attain an optimized formulation with the lowest erosion and release of probiotics in seawater. The lowest percentage of matrix erosion was found with coating time of 20 min, albeit no significant differences were observed with different coating times. Therefore, the lowest coating time was selected. Lower amount of bacterial release was obtained with higher concentrations of chitosan. Based on these results, CCALG-0.8-20 was selected for further characterization due to having a lower bacterial release in seawater and matrix erosion.

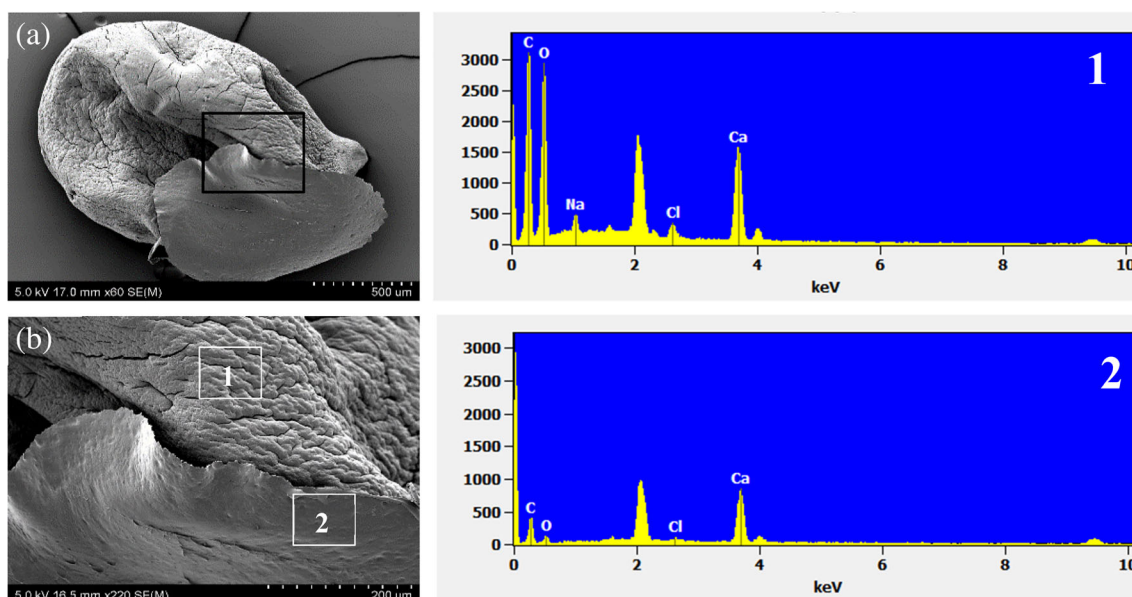
## 3.3 | Characterization of the optimized formulation (CCALG-0.8-20)

### 3.3.1 | Scanning electron microscopy

Scanning electron micrographs of air dried CCALG beads are illustrated in Figure 5. CCALG beads retained their



**FIGURE 5** SEM micrographs of (a) an air-dried CCALG bead, (b) cross-section and surface microstructure, and (c) the encapsulated probiotics within the bead matrix



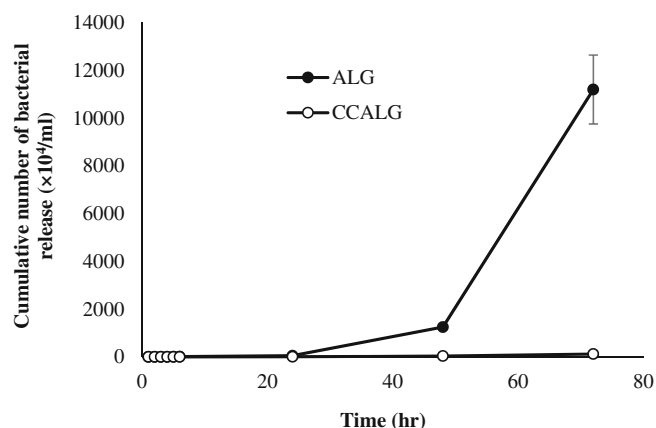
**FIGURE 6** SEM images of (a, b) a CICALG bead and the corresponding EDS graphs of (1) alginate matrix and (2) chitosan coating designated as area 1 and 2 [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/app.52626)]

spherical shape. However, the size of dried beads was notably smaller than the wet beads due to dehydration. The encapsulated probiotics could be observed within the beads.

While examining CICALG beads with SEM, some beads showed a shrunken and separated chitosan coating. In the preparation of samples for SEM, it is possible for the chitosan coat to separate when dehydrated. EDS analysis was used to confirm the presence of coating which showed differences in intensity of calcium, sodium, oxygen and carbon peaks between the matrix and coating identified as area 1 and 2 in Figure 6. The higher amount of carbon and oxygen is related to the presence of carboxyl groups in alginate chemical structure. In addition, the presence of sodium is due to the use of sodium alginate in the formulation, which remained after cross-linking. The higher calcium amount in the matrix can be attributed to the cross-linking of alginate by calcium ions. The EDS results are in agreement with a previous study by Pasparakis & Bouropoulos.<sup>25</sup>

### 3.3.2 | Effect of the encapsulation process on the viability of bacteria

Viability of the three probiotic bacteria encapsulated in CICALG beads were  $81.2 \pm 0.1$ ,  $55.9 \pm 5.8$ , and  $1.6 \pm 0.1$  for *Enterococcus* sp., *Exiguobacterium* sp., and *Vibrio* sp., respectively. The results show that only *Vibrio* sp. exhibited a low encapsulation efficiency of less than 2%. This could be due to the sensitivity of *Vibrio* sp. to



**FIGURE 7** Cumulative release curves of encapsulated *Exiguobacterium* sp. from ALG and CICALG beads. The coating media was 0.8% w/v chitosan. The release medium was seawater ( $n = 3$ ; mean  $\pm$  SD)

chemical and mechanical stressors involved in the encapsulation process which are either detrimental to *Vibrio* sp. or makes this bacterium nonculturable.

### 3.3.3 | Bacterial release from beads

It is critical for the beads to retain probiotics while they are in seawater. The release profiles of encapsulated bacteria from ALG and CICALG beads are shown in Figure 7. The encapsulated probiotics started to release from ALG while no bacteria were

detected in the release media with CCALG after 5 h of incubation in seawater. A higher level of bacterial release was obtained for ALG throughout release period. The number of released bacteria was  $1.1 \times 10^8$   $\text{ml}^{-1}$  and  $1.2 \times 10^6$   $\text{ml}^{-1}$  for ALG and CCALG beads after 72 h of incubation, respectively. This indicates that the addition of chitosan coating provides a barrier to reduce the release of the bacteria into the seawater. The number of viable bacteria retained within CCALG beads after 72 h of incubation in seawater was further determined using viable plate count method. The results showed that approximately  $48 \pm 0.5\%$  of the initial encapsulated bacteria remained viable within the matrix of CCALG even after 72 h of incubation in seawater.

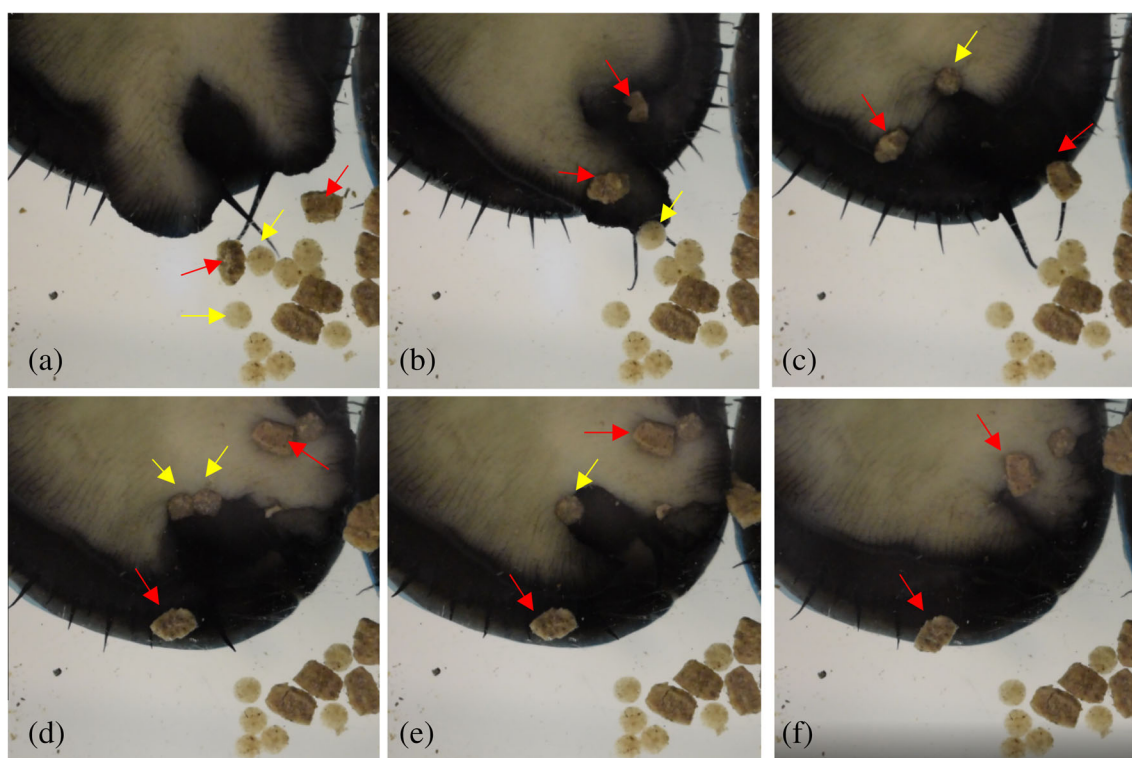
### 3.3.4 | Attractiveness and palatability of the developed beads

The observational study on the attractiveness of CCALG beads to abalone revealed a 100% consumption rate for CCALG beads after 24 h as opposed to less than

50% for commercial feed. Furthermore, this observation showed that abalone use their foot muscles to grab and accumulate particles under their shell before consuming them. A video recording showed that abalone were extremely attracted to CCALG beads compared to the commercial feed. This is understood from their behavior in which they grab both types of presented food, however, the commercial feed remained mostly uneaten as long as beads were available to the animals (Figure 8).

### 3.3.5 | Bacterial load of digestive tract of abalone

The total counts of probiotic bacteria within the GIT of abalone fed with encapsulated probiotic were significantly higher than within the control animals ( $p < 0.05$ ). The average number of *Enterococcus* sp. was five logs higher in probiotics-fed ( $1.3 \times 10^8$  CFU) than the control group ( $6 \times 10^3$  CFU). This indicates that the developed CCALG beads could efficiently deliver viable probiotic bacteria into the GIT of abalone.



**FIGURE 8** A series of captured images showing abalone eating behavior for the two particular feeds. (a) Sensing food including both probiotics encapsulated CCALG beads (indicated by yellow arrows) and commercial feed (indicated by red arrows), (b) 2 commercial feeds and 1 CCALG bead being grabbed by abalone using foot muscle, (c) none of the feeds were consumed by the animal up to this stage, (d) more CCALG beads were captured by this point, (e) abalone consuming beads, (f) a number of CCALG beads were consumed, but the commercial feed remained uneaten. [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/terms-and-conditions)]

| Bacteria                   | Number of encapsulated bacteria ( $\times 10^8$ CFU/g) |                 |                 |
|----------------------------|--------------------------------------------------------|-----------------|-----------------|
|                            | 0 (h)                                                  | 48 (h)          | 96 (h)          |
| <i>Exiguobacterium</i> sp. | 171 $\pm$ 9.8                                          | 19 $\pm$ 0.1    | 26 $\pm$ 1.4    |
| <i>Enterococcus</i> sp.    | 360 $\pm$ 9.8                                          | 22 $\pm$ 1.2    | 53 $\pm$ 2.8    |
| <i>Vibrio</i> sp.          | 2.5 $\pm$ 0.4                                          | 0.12 $\pm$ 0.02 | 0.14 $\pm$ 0.03 |

**TABLE 5** Effect of refrigeration on survivability of encapsulated bacteria ( $n = 3$ ; mean  $\pm$  SD)

**TABLE 6** Average bacterial viable count after freeze drying of encapsulated probiotics ( $n = 3$ ; mean  $\pm$  SD)

| Bacteria                   | Initial viable count (CFU/g) | Viable count after freeze drying (CFU/g) |
|----------------------------|------------------------------|------------------------------------------|
| <i>Exiguobacterium</i> sp. | 119 $\times 10^7$            | 44 $\times 10^5$                         |
| <i>Enterococcus</i> sp.    | 94 $\times 10^9$             | 30 $\times 10^4$                         |
| <i>Vibrio</i> sp.          | 281 $\times 10^8$            | 33 $\times 10^4$                         |

### 3.3.6 | Effect of storage conditions on the viability of encapsulated bacteria

Table 5 summarizes the number of viable bacteria before and after storage at 4°C. The number of viable bacteria was reduced when the probiotic beads were stored at 4°C for 48 h. A slight increase was then observed in the number of viable bacteria when the storage time further increased from 48 to 96 h. Perhaps, the live bacteria can adapt and slowly propagate within the matrix of the beads.

The counts of bacteria after 96 h of refrigeration were different only by one log unit compared to the initial counts at time 0. Approximately  $10^8$ – $10^7$  CFU/g of beads retained viable at the end of the storage period.

Table 6 shows the average bacterial count post-lyophilization. The results indicate that the number of viable bacteria decreased dramatically after freeze drying. The level of viability loss was different for each bacterial strain due to diverse sensitivity to stressors during freeze drying. Among the three probiotic bacteria *Enterococcus* sp. and *Vibrio* sp. were highly sensitive to freeze drying with a 4–5 log reduction in the number of viable bacteria. Approximately,  $10^4$ – $10^5$  CFU/g of viable cells were obtained after the lyophilization. The results of this study indicate that the encapsulated probiotic bacteria in CCALG beads can be stored at 4°C without addition of any growth inducer or protectant. However, further studies are required to enhance the survival of the encapsulated probiotics during lyophilization.

## 4 | OVERALL DISCUSSION

Delivery of probiotics to marine species using natural polymers such as chitosan and alginate were frequently

reported in literature.<sup>11,26</sup> However, understanding the physiochemical properties of polymers and a through optimization process are necessary prior to further investigation through feeding trials. The optimization process ensures a probiotic carrier with ideal morphology, stability in seawater, protection and release profile of the encapsulated probiotics. In this study, the optimized parameters for successful delivery of marine probiotics using chitosan coated alginate particles to farmed abalone were established. A mixture of sodium alginate with probiotics was extruded dropwise into a calcium chloride solution. Calcium chloride is a preferred cross-linker for alginate as it is cheap, nontoxic, readily available, and importantly, a cross-linking process can occur at neutral pH which in turn can maintain the viability of probiotics.<sup>27</sup> A cross-linked layer is formed immediately around spherical droplets as they enter the calcium chloride solution.<sup>28</sup> The cross-linking reaction can then proceed from the surface toward interior regions of the beads as  $\text{Ca}^{2+}$  ions continuously diffuse into the beads.<sup>19</sup>

Alginate beads with distinct shapes and sizes were produced using different concentrations of alginate. A higher viscosity was observed as the alginate concentration increased in bead formulations which influenced the shape of alginate beads. ALG formulation with 1.5% w/v alginate (ALG-1.5) had an optimum viscosity to produce a smooth flow jet from the dripping nozzle, yielded spherical beads compared to other formulations. Beads deformation was observed at low (0.04 pa.s) and high (0.93 pa.s) viscosities during bead formation. This can be explained by the inability of the alginate droplets to overcome the drag force applied to the droplet surface when they entered the gelling bath.<sup>21,22</sup> Pear-shaped alginate beads without a distinct tail were produced using viscous alginate solution (ALG-2.0 beads). This is presumed to be due to the formation of nonspherical droplets at the dripping tip, retaining the shape during dripping distance and finally entering the gelling bath. Furthermore, it should be considered that high viscosity can complicate the encapsulation process due to handling and process difficulties. Some desirable features of delivery systems such as improved mechanical and chemical stability, better esthetic qualities, well-defined size and shape, higher reproducibility and controlled release profiles have been suggested for spherical particles as opposed to particles with nonspherical and irregular shapes.<sup>21,29</sup> Therefore,

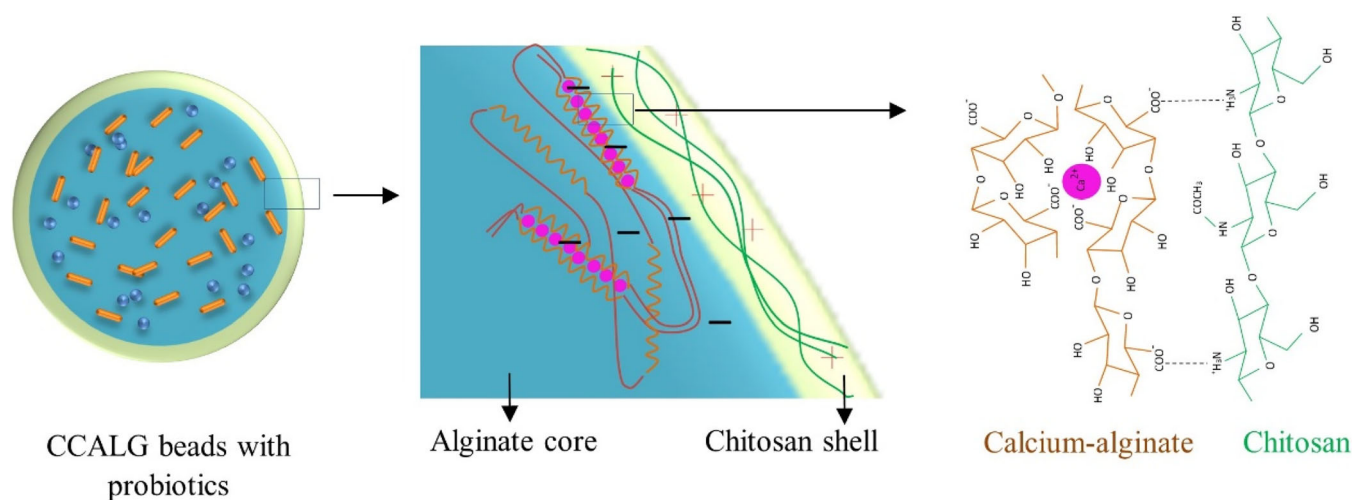
viscosity of the formulation mixture should be examined and adjusted if necessary in order to develop a delivery system with desirable properties.

Sinking properties of a bioactive carrier in seawater is another important factor which will affect the success of a delivery system designed for marine species.<sup>10</sup> The sinking time of ALG beads was inversely proportional to the concentration of alginate due to the higher density of beads as the concentration of alginate increased within the formulations. ALG-1.5 beads require at least 2 min before starting to sink in static seawater. A faster sinking rate can improve the accessibility of beads to abalone, especially in farm condition where the flow of seawater can potentially wash away the beads. The sinking rate of developed ALG beads can be further reduced by pre-soaking of the beads in seawater. The presoaking step can reduce the osmotic pressure gradient between ALG beads and seawater and facilitate sinking within a few seconds. Therefore, it should be noted that adjusting the sinking properties of particles is necessary as it can affect the accessibility of particles to aquatic animals and controlling environmental impact.

Uncoated ALG beads showed a very fast release of probiotics, which is mostly due to the porous structure of alginate particles. The high level of porosity is one of the drawbacks of alginate hydrogels, as it facilitates diffusion of fluids across the alginate matrix leading to a quick release of encapsulated materials.<sup>30</sup> However, the results of this study showed that when the beads were coated with chitosan, the release of probiotics in seawater could be successfully controlled. Dezfooli et al. reported a burst release of encapsulated bacteria from alginate microparticles after 5 h of incubation in seawater which was successfully prolonged by adding chitosan coating to the

formulation.<sup>11</sup> In this study chitosan was used to create a polyelectrolyte complex with alginate to reduce the bacterial release and dissolution of beads in seawater. Chitosan, a cationic polymer derived from the shell of crustaceans, can form a strong complex with alginate. Chitosan possesses positively charged amino groups on its backbone which reacts with negatively charged carboxyl groups of alginate due to electrostatic interactions to form polyelectrolyte complex (Figure 9). A burst release of bioactives from uncoated alginate microparticles was described by different studies while the extent of release depends on the coating time, concentration of chitosan in coating solution and coating method.<sup>31–34</sup>

Chitosan coating delayed the release of probiotics from CCALG bead into seawater where the percentage of matrix erosion in seawater was significantly suppressed. Matrix erosion and bacterial release showed a concentration-dependent relation with chitosan where, a higher concentration of chitosan formed a thicker coating layer around the alginate bead, reducing bead dissolution and release of encapsulated bacteria. Similar reports showed a gradual reduction in release rate of encapsulated materials as the chitosan concentration increased in chitosan coated alginate particles.<sup>32,34,35</sup> On the other hand, coating duration had minimal effect on matrix erosion of the beads. In agreement with the results of this study, Cook et al. reported negligible differences in the thickness of the chitosan coat over 100 min of coating time due to the slow penetration of chitosan into alginate core.<sup>36</sup> Several other factors such as charge density, mixing ratio, order of polymer addition and solution condition such as pH and ionic strength were identified as influential factors on the interaction between the oppositely charged polymers; hence, formation and stability of the chitosan/alginate polyelectrolyte complex.<sup>37–39</sup>



**FIGURE 9** Schematic diagram of CCALG beads with probiotics and the formation of chitosan coated alginate polyelectrolyte complex [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/app.52626)]

In this study, minimum level of probiotic release combined with maximum stability in seawater achieved using CCALG beads with 0.8% w/v chitosan, coated for 20 min (CCALG-0.8-20), hence it was selected as the optimized formulation for delivery of probiotics to abalone. CCALG-0.8-20 beads demonstrated ideal morphological properties, such as spherical shape and smooth surface. The release study showed no bacterial release within the first 6 h of incubating CCALG beads in seawater, which is ideal in terms of minimizing the potential environmental impact. This timeline is satisfactory as these beads had a high consumption rate within the first 24 h of being placed in abalone culture tank further minimizing the risk of environmental contamination. The mechanism of probiotic release from the beads within the gut of abalone can be explained via physical destruction of beads by the radula (teeth-like structures) at the time of consumption and further release of entrapped bacteria from the broken beads via matrix erosion and diffusion.

The developed CCALG beads also showed high encapsulation efficiencies for the tested probiotics. Viability of probiotic bacteria is another important factor which should be considered when developing a delivery system. For the probiotics to confer their benefits, they should be viable when delivered to the site of action. This requires maintaining their viability during the encapsulation process and storage.<sup>40</sup> Bacterial strains show different sensitivity to various stressors. Therefore, it is critical to investigate the survival of bacteria after every step of developing a delivery system including encapsulation and further storage processes. Although the viability of *Exiguobacterium* sp. and *Enterococcus* sp. was relatively high after encapsulation, that of *Vibrio* sp. was less than 2% which could be due to higher sensitivity to calcium in the gelling bath or stirring during encapsulation.<sup>14</sup> Moreover, in order to measure survivability, it was necessary to dissolve beads in sodium citrate, which is a calcium sequestrant that can affect bacterial survivability.<sup>41</sup> A wide range of survival levels have been reported using the same method of encapsulation<sup>14,42</sup> and other encapsulation techniques.<sup>17,43</sup>

The survivability of the encapsulated probiotics during refrigerated storage at 4°C showed only one log reduction in viability of encapsulated probiotics after 96 h of incubation ( $10^7$ – $10^8$  CFU/g of beads). The final viable count of encapsulated probiotics can always be enhanced by increasing the initial number of bacteria before encapsulation. Nevertheless, the obtained probiotic concentration should be sufficient as  $10^7$ – $10^{10}$  viable cells/g of feed is suggested as effective working concentration in different studies on abalone.<sup>44–46</sup>

Lyophilization as an alternative storage option for encapsulated probiotics showed a considerable reduction of between 2 to 5 logs for the three probiotic bacterial

strains. The three bacterial species showed various susceptibility to the freeze drying process as found in other studies which could be due to the differences in the composition of cell membranes.<sup>47,48</sup> It needs to be noted that other additives such as skim milk or sugars can be utilized as cryoprotectants to reduce the chance of cell damage during freeze drying.<sup>5</sup>

## 5 | CONCLUSIONS

Encapsulation techniques have been used to create more efficient and environmentally friendly methods for delivery of bioactives in aquaculture. The application of polymeric capsules for delivery of probiotics to aquatic species has been demonstrated in the literature, while the carrier design was mostly adopted from previous research and minimum efforts was made toward optimizing probiotic carriers before in-vivo testing. This study contributes to the optimization and evaluation of a probiotic delivery system for aquaculture application. The efficacy of spherical CCALG beads in delivering probiotics to abalone was demonstrated. It was shown that different factors, such as concentration of polymers in the core and shell of CCALG beads can impact the probiotic release profile and the animals' accessibility to the beads by changing the morphology, stability and sinking time of the beads in seawater. The low bacterial release in seawater can maintain the quality of culture water when CCALG beads are placed in abalone culture tanks. The demonstrated high consumption rate of CCALG beads by abalone ensures fast delivery of sufficient dosages of probiotics to abalone with potentially less environmental impacts than conventional methods. Further research is required to assess the capability of the developed CCALG beads in delivering nutrients to abalone with a focus on the evaluation of the environmental impact of CCALG beads in aquaculture industrial settings.

## AUTHOR CONTRIBUTIONS

**Seyedehsara Masoomi Dezfooli:** Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (supporting); investigation (lead); methodology (lead); project administration (lead); validation (lead); writing – original draft (lead); writing – review and editing (lead). **Chloe Bonnot:** Data curation (supporting); writing – review and editing (supporting). **Noemi Gutierrez-Maddox:** Supervision (supporting); writing – review and editing (equal). **Andrea C. Alfaro:** Funding acquisition (equal); resources (supporting); supervision (supporting); writing – review and editing (equal). **Ali Seyfoddin:** Conceptualization (lead); funding acquisition (lead); resources (lead); supervision (lead); writing – review and editing (equal).

## ACKNOWLEDGMENTS

This work was supported by AUT Vice Chancellor Doctoral Scholarship to S. Masoomi Dezfooli. The authors would like to thank Moana™ New Zealand for kindly providing abalone for this study. We acknowledge the Ministry for Primary Industries' Sustainable Farming Fund for partially funding this project. Open access publishing facilitated by Auckland University of Technology, as part of the Wiley - Auckland University of Technology agreement via the Council of Australian University Librarians.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ORCID

Seyedehsara Masoomi Dezfooli  <https://orcid.org/0000-0002-2071-5834>

Ali Seyfoddin  <https://orcid.org/0000-0003-4343-9905>

## REFERENCES

- [1] S. H. T. Shefat, *Int. Res. J.* **2018**, 7, 4.
- [2] J. Zhao, Y. Ling, R. Zhang, C. Ke, G. Hong, *Aquaculture* **2018**, 493, 289.
- [3] S. K. Nayak, *Fish Shellfish Immunol.* **2010**, 29, 2.
- [4] K. K. Kumaree, A. Akbar, A. K. Anal, *Ann. Microbiol.* **2014**, 65, 1439.
- [5] K. Pinpimai, C. Rodkhum, N. Chansue, T. Katagiri, M. Maita, N. Pirarat, *Res. Vet. Sci.* **2015**, 102, 103.
- [6] S. Madreseh, H. R. Ghaisari, S. Hosseinzadeh, *Probiotics Anti-microb* **2018**, 19, 1.
- [7] Y. Xia, M. Lu, G. Chen, J. Cao, F. Gao, M. Wang, M. Yi, *Fish Shellfish Immunol.* **2018**, 76, 368.
- [8] G. Darmody, A. P. Maloy, S. A. Lynch, M. Prado-Alvarez, J. Cotterill, T. Wontner-Smith, S. Culloty, *Aquac Int* **2015**, 23, 647.
- [9] N. Pirarat, K. Pinpimai, C. Rodkhum, N. Chansue, E. L. Ooi, T. Katagiri, M. Maita, *Anim. Feed Sci. Technol.* **2015**, 207, 93.
- [10] S. Masoomi Dezfooli, N. Gutierrez-Maddox, A. C. Alfaro, A. Seyfoddin, *Rev. Aquac.* **2018**, 11, 631.
- [11] S. Masoomi Dezfooli, N. Gutierrez-Maddox, A. C. Alfaro, A. Seyfoddin, *Pharm. Dev. Technol.* **2021**, 26, 390.
- [12] K. Y. Lee, T. R. Heo, *Appl. Environ. Microbiol.* **2000**, 66, 869.
- [13] S. Mandal, A. K. Puniya, K. Singh, *Int. Dairy J.* **2006**, 16, 1190.
- [14] P. Rosas-Ledesma, J. M. León-Rubio, F. J. Alarcón, M. A. Moriñigo, M. C. Balebona, *Aquac. Res.* **2012**, 43, 106.
- [15] E. S. Chan, B. B. Lee, P. Ravindra, D. Poncelet, *J. Colloid Interface Sci.* **2009**, 338, 63.
- [16] J. L. Romalde, A. Luzardo-Alvárez, C. Ravelo, A. E. Toranzo, J. Blanco-Méndez, *Aquaculture* **2004**, 236, 119.
- [17] M. J. Martin, F. Lara-Villoslada, M. A. Ruiz, M. E. Morales, *LWT* **2013**, 53, 480.
- [18] F. Belalia, N. E. Djelali, *Rev. Roum. Chim.* **2014**, 59, 135.
- [19] N. M. Velings, M. M. Mestdagh, *Polym Gels Netw* **1995**, 3, 311.
- [20] P. Del Gaudio, P. Colombo, G. Colombo, P. Russo, F. Sonvico, *Int. J. Pharm.* **2005**, 302, 1.
- [21] B. B. Lee, P. Ravindra, E. S. Chan, *Chem Eng Technol* **2013**, 36, 1627.
- [22] D. B. Seifert, J. A. Phillips, *Biotechnol. Prog.* **1997**, 13, 562.
- [23] I. Meiser, S. C. Müller, M. M. Gepp, H. Zimmermann, F. Ehrhart, Proceedings of the 2009 European Conference of the International Federation for Medical and Biological Engineering, Heidelberg: Springer, Berlin 2009.
- [24] W. J. Leo, A. J. Mcloughlin, D. M. Malone, *Biotechnol. Prog.* **1990**, 6, 51.
- [25] G. Pasparakis, N. Bouropoulos, *Int. J. Pharm.* **2006**, 323, 34.
- [26] S. Jia, K. Zhou, R. Pan, J. Wei, Z. Liu, Y. Xu, *Fish Shellfish Immunol.* **2020**, 105, 327.
- [27] J. Foroughi, A. Mirabedini, H. Warren, in *Hydrogels* (Eds: S. Haider, A. Haider), IntechOpen, London, United Kingdom **2018**.
- [28] W. Krasaekoopt, B. Bhandari, H. Deeth, *Int. Dairy J.* **2003**, 13, 3.
- [29] J. W. Woo, H. J. Roh, H. D. Park, C. I. Ji, Y. B. Lee, S. B. Kim, *Food Sci. Biotechnol.* **2007**, 16, 715.
- [30] S. Gouin, *Trends Food Sci Technol* **2004**, 15, 330.
- [31] S. Abbaszadeh, H. Gandomi, A. Misaghi, S. Bokaei, N. Noori, *J. Sci. Food Agric.* **2014**, 94, 2210.
- [32] X. Z. Shu, K. J. Zhu, *Eur. J. Pharm. Biopharm.* **2002**, 53, 193.
- [33] A. J. Ribeiro, C. Silva, D. Ferreira, F. Veiga, *Eur. J. Pharm. Sci.* **2005**, 25, 31.
- [34] Y. Murata, T. Maeda, E. Miyamoto, S. Kawashima, *Int. J. Pharm.* **1993**, 96, 139.
- [35] T. Sahasathian, N. Praphairaksit, N. Muangsinsin, *Arch. Pharm Res.* **2010**, 33, 889.
- [36] M. T. Cook, G. Tzortzis, D. Charalampopoulos, V. V. Khutoryanskiy, *Biomacromolecules* **2011**, 12, 2834.
- [37] R. S. Rabelo, G. M. Tavares, A. S. Prata, M. D. Hubinger, M. D., *Carbohydr. Polym.* **2019**, 223, 115120.
- [38] J. Potaś, E. Szymańska, K. Winnicka, *Eur. Polym. J.* **2020**, 140, 110020.
- [39] X. Yin, H. Xie, R. Li, S. Yan, H. Yin, *Carbohydr. Polym.* **2021**, 255, 117390.
- [40] M. Chávarri, I. Marañón, M. C. Villarán, in *Probiotics* (Ed: E. Cid Rigobelo), InTechOpen, London **2012**.
- [41] I. Camelin, C. Lacroix, C. Paquin, H. Prevost, R. Cachon, C. Divies, *Biotechnol. Prog.* **1993**, 9, 291.
- [42] B. Albertini, B. Vitali, N. Passerini, F. Cruciani, M. Di Sabatino, L. Rodriguez, P. Brigidi, *Eur. J. Pharm. Sci.* **2010**, 40, 359.
- [43] N. T. Annan, A. D. Borza, L. T. Hansen, *Int. Food Res.* **2008**, 41, 184.
- [44] B. M. Macey, V. E. Coyne, *Aquaculture* **2005**, 245, 249.
- [45] K. I. Doeschate, V. E. Coyne, *Aquaculture* **2008**, 284, 174.
- [46] J. A. Hadi, N. Gutierrez, A. C. Alfaro, R. D. Roberts, N. Z. J. Mar. *Freshwater Res.* **2014**, 48, 405.
- [47] S. Rybka, K. Kailasapathy, *Aust. J. Dairy Technol.* **1995**, 50, 51.
- [48] P. Capela, T. K. C. Hay, N. P. Shah, *Int. Food Res.* **2006**, 39, 203.

**How to cite this article:** S. Masoomi Dezfooli, C. Bonnot, N. Gutierrez-Maddox, A. C. Alfaro, A. Seyfoddin, *J. Appl. Polym. Sci.* **2022**, 139(29), e52626. <https://doi.org/10.1002/app.52626>