Application of Encapsulation Technology to Improve the Growth Rate of New Zealand Black-footed Abalone (*Haliotis iris*)

Seyedehsara Masoomi Dezfooli

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Faculty of Health and Environmental Sciences
Auckland University of Technology
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Abstract

Abalone is a marine gastropod mollusc belonging to the family Haliotidae. Black-footed abalone, (*Haliotis iris*) bred and harvested uniquely in New Zealand, has a high market demand due to its high quality and chemical-free farming process. However, the slow growth rate of abalone has limited its sustainable production. Many studies have shown that probiotics can improve the growth rate and health of abalone. However, effective delivery of probiotics to farmed abalone has remained a challenge. Conventional probiotic delivery methods through culture water or as feed supplements may lead to environmental contamination, loss of activity and insufficient dosages. This thesis aims to develop a new probiotic delivery system to enhance health and growth rate of black-footed abalone.

A literature review was conducted to analyse the application of encapsulation technology in developing novel delivery methods for various bioactives including probiotics, nutrients and immunostimulants to farmed aquatic species (Chapter 2). A pre-formulation study was performed to confirm the beneficial characteristics of probiotics previously isolated from healthy abalone. The three bacterial species namely *Exiguobacterium* sp., *Enterococcus* sp. and *Vibrio* sp. were characterised and their abilities to help in feed digestion were confirmed (Chapter 3).

The probiotic bacteria were then encapsulated in chitosan and alginate-based microparticles. The efficiency of microparticles in encapsulating the probiotics and creating a pH-dependent release in the simulated gastric and intestinal fluids of abalone were demonstrated. The developed microparticles displayed floating behaviour in seawater, hence, they were immobilised in alginate beads to improve their accessibility to abalone. Fluorescence microscopy suggested minimum leach of the encapsulated bacteria into seawater followed by the successful delivery of microparticles into abalone's gastrointestinal tract (GIT). The high load of probiotic bacteria in the GIT of probiotic-fed animals illustrated the potential of the developed microparticles as new carriers for oral administration of probiotics to abalone (Chapter 4).

To simplify the production process of encapsulated probiotics, an extrusion method was utilised to develop chitosan-coated alginate beads instead of microparticles. Encapsulated probiotic beads with desired properties including spherical shape, fast

sinking, high palatability, high stability and minimum release in seawater were obtained. The tracking experiment using fluorescent-labelled probiotics confirmed the successful delivery of the encapsulated probiotics into the GIT of probiotic-fed abalone (Chapter 5).

The efficiency of the developed beads to improve health and growth of abalone were assessed by performing a two-month feeding trial. Four different dietary treatments including 1) conventional feed, 2) feed sprayed with probiotic culture, 3) combination of encapsulated probiotics and conventional feed, and 4) encapsulated probiotics containing nutrients were used to feed juvenile black-footed abalone. Diet 4 successfully improved growth performance, feed utilisation, feed conversion rate and reduced feed wastage. Flow cytometric analysis revealed a lower level of oxidative stress associated with diet 4 compared to the other diets. Metabolomics analysis on abalone foot muscle further revealed that abalone fed with diet 4 differentially regulated 31 metabolites mostly composed of free amino acids which may indicate a better delivery of feed. Therefore, chitosan-coated alginate beads can be considered as an effective probiotics and nutrients delivery method to black-footed abalone capable of increasing abalone health and growth performance while minimising feed wastage, environmental impact and hence feed cost (Chapter 6).

In conclusion, an encapsulated system was developed to deliver viable probiotics as well as nutrients to farmed black-footed abalone. The developed system was capable of protecting its contents in seawater, delivering them to the GIT of abalone, enhancing health and growth and achieving high consumption rates as well as minimising wastage. The encapsulated delivery system developed in this thesis can be scaled up for commercial production, and adaptation of such technologies could help to further develop abalone aquaculture industry in New Zealand and abroad in a sustainable manner.

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List of Abbreviations

ASC	Aquaculture Stewardship Council
bp	base pair
CFU	Colony Forming Unit
cm	Centimetre
FCR	Feed Conversion Rate
dNTP	deoxyribonucleotide triphosphate
EDS	Energy Dispersive Spectrometer
e.g	exempli gratia
ET	Encapsulation Technology
FAO	Food and Agriculture Organization
FTIR	Fourier-Transform Infrared Spectroscopy
g	gram
GC	Gas Chromatography
GIT	Gastrointestinal Tract
HLB	Hydrophilic-Lipophilic Balance
KAA	Oxide Kanamycin Esculin Azide Agar
UV	Ultraviolet
kDa	kilo dalton
M	molar concentration
mg	milligram
min	minute
ml	millilitre
µm	micrometre
mm	millimetre
mt	metric tonne
mv	millivolts
MW	Molecular Weight
n	number
NZ	New Zealand
PCR	Polymerase Chain Reaction
PCA	Principle Component Analysis
PLGA	Poly Lactic-co-Glycolic Acid
ROS	Reactive Oxygen Species
rpm	rate per minute
SA	Sodium Alginate
sec	seconds
SEM	Scanning Electron Microscope
SE	Standard Error
SL	Shell Length
THC	Total Haemocyte Count
v/v	volume per volume
VLP	Virus-Like Particle
WHO	World Health Organization
w/v	weight per volume
3 D	Three-dimensional

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgment), nor material which to a substantial extent, has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed

Date 29/10/2019

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Chapter 1

Thesis introduction and framework

“Whatever you do, or dream you can, begin it. Boldness has genius, power and magic in it”.

Johann Wolfgang von Goethe

1.1 Aquaculture and the global food challenge

The world's population is growing continuously and is expected to reach USD 9.3 billion by 2050. Among many challenges of our era, one is to meet the demand for food and water around the globe while also advancing technological development, reducing carbon emissions and protecting the environment. Considering current levels of food consumption, agricultural production will need to produce 60 and 100% more food by 2050 in developed and developing countries, respectively (FAO, 2014). Globally, about 70% of freshwater is used for agriculture (Food and Agriculture Organisation). In New Zealand this value is even higher, reaching 78% out of which 76% is used for irrigation of pastures for the meat and dairy industry (New Zealand Parliament, 2011). With the growing demand for food and water, land-based farming needs to be managed in a more sustainable way. While optimising farm practices can help, alternative food production strategies need to be implemented to reduce the environmental effects of agriculture.

About 71% of the Earth's surface is covered by water, and oceans hold about 96.5% of all Earth's water. Marine life has fed humankind throughout the history and continues to be a major resource. World fisheries and aquaculture play critical roles in supplying food and nutrition to millions of people, with the total combined production of about USD 362 billion in 2016 (FAO, 2018). However, the yields from the seas are diminishing, since world fisheries are under constant pressure and just as freshwater ecosystems, saltwater ecosystems are heading towards a point of no return. Many scientists strongly believe that aquaculture, (i.e. farming food in controlled aquatic environments) can provide a more efficient and sustainable way to feed the world's expanding population.

1.1.1 Moving away from wild catch

The world's fish stocks are diminishing and if drastic measures are not implemented there is a danger that they will be depleted by 2050. This may happen while millions of people depend on fisheries for survival. Depletion of fish stocks has been attributed to the direct impacts of fishing industries. However, severe environmental impacts such as pollution and habitat destruction have also contributed (Gislason, 2003; Kaiser et al.,

2003). Fishing bycatch and discards are other consequences of irresponsible industrial fishing. Around 27 million tonnes of undesired species are dumped annually due to the poor selectivity of fishing practices (Kelleher, 2008).

In response to the environmental and ecological challenges of the fishing industry, fisheries management policies have been created around the world as the first viable solution. Implementation of these new policies, including defined daily limits for catch and regulations on fishing gears such as mesh size of fishing nets have improved the state of fishing and restored fish stocks in some areas. However, for these policies to be effective, the production from catch fisheries need to be reduced or at least maintained at a steady level. Additionally, aquaculture can provide a platform to compensate the problems caused by fishing industry and to respond to the growing demand for fish (New Zealand Government, 2019). The market requirements can also be addressed more reliably as productions from fish farms are mostly constant with minimum fluctuation.

1.1.2 Aquaculture as a sustainable alternative source of protein

At the World Aquaculture Conference in Adelaide in 2014, it was announced that the world would need much higher amount of food in the next thirty years than the total produced in the previous eight thousand years (Connecting Aquaculture Professionals, 2014). This estimation might be overwhelming, yet an interesting opportunity for researchers to come up with innovative methods to feed the growing population without overexploiting our natural resources.

Alongside ongoing population growth, people's attitude toward their diets have changed over the years with more people moving towards consuming more healthy protein sources and less carbohydrates. This has led to a significant increase in seafood consumption, with approximately 20 kg per capita per year, over the last few decades (FAO, 2018). Aquaculture can reduce the gap between high seafood demand and limited wild supply. Since 2014, aquaculture production has exceeded capture fisheries, and as of today, almost half of the global fish production is provided through aquaculture. By

2030, more than 60 percent of fish for human consumption is expected to be produced from aquaculture (World Bank, 2013).

Growth in aquaculture production can improve all the three main pillars of sustainability including environmental, social and economic themes by maintaining biodiversity, compensating excessive harvest from oceans, reducing wastage, improved distribution, providing job opportunities, better incomes and urbanisation (FAO, 2018). Furthermore, lower feed conversion rates in fish farming make aquaculture to be a more sustainable way to produce protein (Figure 1-1).

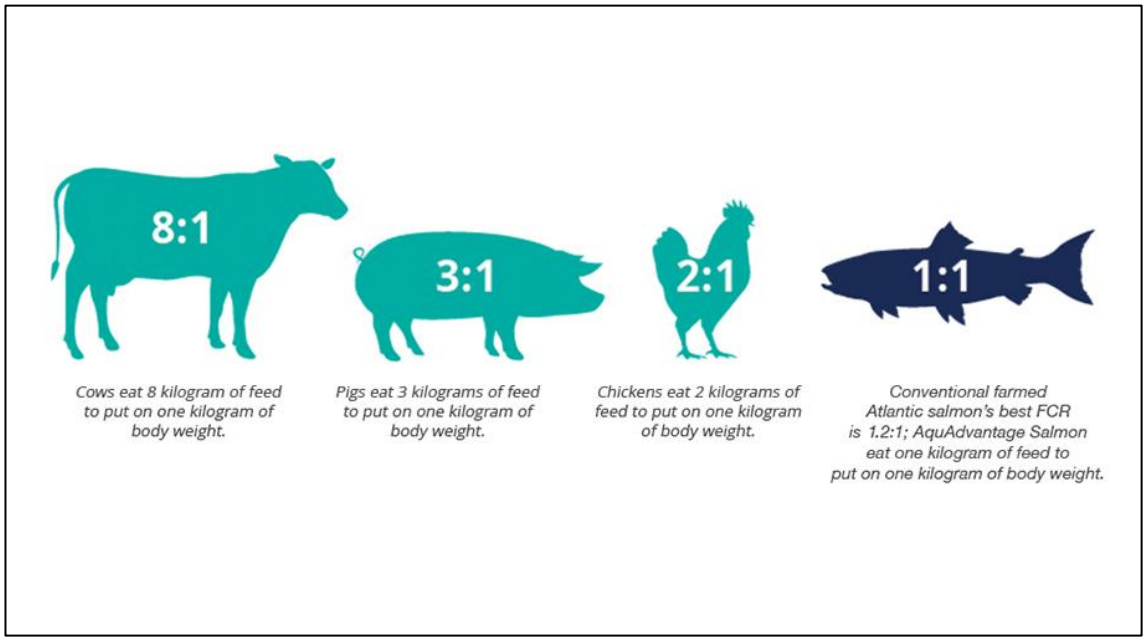


Figure 1-1 Feed conversion rates in some of the most common farmed animals (Sustainable: low impact fish farming, 2019).

1.1.3 Aquaculture in New Zealand context

The history of New Zealand (NZ) aquaculture industry dates back to the 1960s when small operations were set up for oyster and mussel farming. Since then, small farms have expanded their production from domestic to international markets. Farming regulations have been established to manage aquaculture industry in NZ. For example, the Fisheries Act 1983 was administered to prevent poaching of wild stock and possible disease

spread. Furthermore, some regulations for the disposal of farmed fish offal and release of effluent water were defined under the same fisheries act. Since then, further regulations and regional coastal plans, including consent requirements for any farming activity on the seabed and in intertidal areas as well as uptake and discharge of seawater from the coast have been continuously administered by the Ministry of Fisheries in order to steer NZ aquaculture toward sustainable practices. Thereafter, the NZ aquaculture has grown in volume of production with a rate of around 11.7% annually for the last 20 years up to 2005 (Ministry of Environment, 2007; New Zealand Government, 2019).

NZ aquaculture consists of both marine and land-based farms with three main species including the Greenshell™ mussels, the King salmon and the Pacific oysters with a total number of 905 active farms all over NZ (Ministry of Environment, 2007). Farming other species, such as pāua and kingfish are being explored and expanded. A prominent feature of NZ aquaculture is its chemical-free farming approach. This has attracted both investors and consumers over the last 50 years. However, moving toward sustainable farming practices has been an ongoing challenge in aquaculture. Several factors, such as biosecurity threats and pollution have limited total profitability from the aquaculture sector. Disease outbreaks in the farms due to pests and biotoxins can lead to mass mortality and financial losses. Moreover, the value of the NZ dollar compared to the US dollar may persuade aquaculture toward higher production with minimum expansion of farmed areas. This intensification may result in an increase in the aforementioned biosecurity risks. Despite these, NZ aquaculture industries have been committed to produce high-value products to address market demands and maintain their operations as sustainable as possible. The ultimate goal for the NZ aquaculture industry is to reach sale value of $1 billion per year by 2025 (New Zealand Government, 2012) and to $3 billion by 2035 (New Zealand Government, 2019). This has to take place within an environmentally sustainable framework. NZ black-footed abalone farming is a young industry, yet one of the best sustainable farming practices in NZ and around the world. Moana New Zealand is the only abalone farm to receive Aquaculture Stewardship Council (ASC) certification for the best farming practice and environmental sustainability

within NZ. This growing industry endeavours to overcome challenges in order to increase its export revenues ($23 million in 2017) and to occupy a bigger segment in the global abalone market (Willams et al., 2017). As of today (January 2019), there are two more farms being set up in Napier and Bluff, expecting to increase NZ's abalone export to over 600 tonnes per year.

1.1.4 Abalone

Abalone are marine gastropods from Family Haliotidae consisting of 70 different species (Figure 1-2) that inhabit the intertidal and shallow coastal waters with rocky shores around Europe, Africa, Asia, Oceania and North Pacific (Abbott, Dance, & Abbott, 1983; Estes, Lindberg, & Wray, 2005; Adachi et al., 2018). A variety of different colours are characteristic among different species, which also indicates the influence of the diet of animals (Beesley, Ross, & Wells, 1998). Abalone are single-shelled molluscs with shell respiratory apertures which are the prominent features of their genus. Abalone are known to be a good source of protein around the world (Lou, Wang, & Xue, 2013; Latuihamallo & Apituley, 2015). Many bioactive agents, such as anti-microbial, anti-aging, anti-inflammatory and anti-cancer molecules are derived from abalone, and have been frequently reported in the literature (Suleria et al., 2017).

Figure 1-2 Various species of abalone with high shell diversity in terms of shape, colour and pore size.

1.1.5 New Zealand Abalone

The black-footed abalone (*Haliotis iris*) (Figure 1-3) is most commonly found in coastal waters in New Zealand. This species is called pāua by Māori (indigenous people of NZ). Pāua is traditionally important in Māori culture and is considered a high value food. Meat, shell and pearls are the three high value products obtained from this sea snail. The strong blue colour of the exterior of pāua shells and the inner iridescent green-blue layer are distinctive properties of NZ pāua, which provides material for jewelleries and decorative objects.

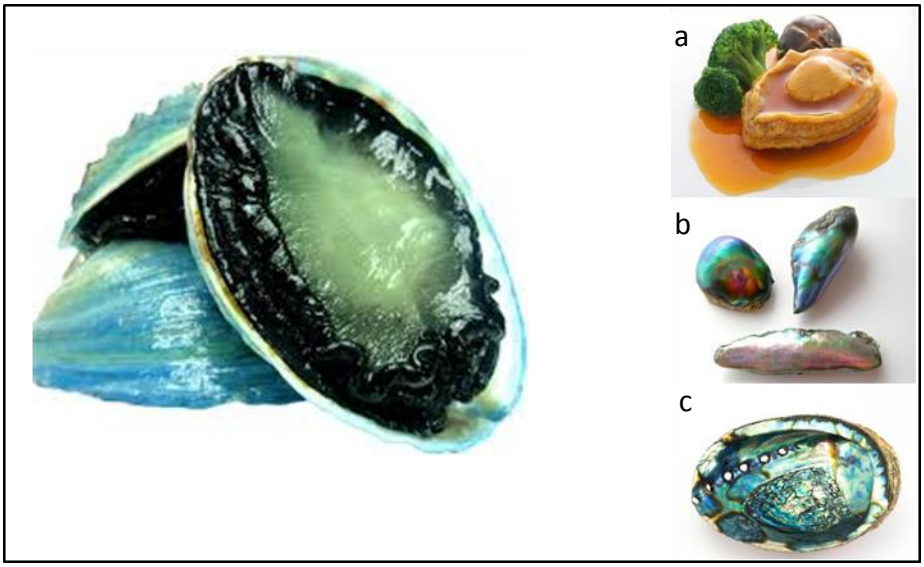


Figure 1-3 Black-footed abalone (*Haliotis. iris*) and the three main pāua products available in the market: (a) pāua meat, (b) pearls and (c) shell.

The New Zealand abalone (*Haliotis iris*) is recognised by the following taxonomic tree (Geiger, 1999).:

Phylum: Mollusca
Class: Gastropoda
Subclass: Prosobranchia
Order: Archaeogastropoda
Superfamily: Pleurotomariacea
Family: Haliotidae
Genus: *Haliotis*
Subgenus: *Pāua*
Species: *Iris*

Pāua can be found in shallow waters between 5 to 20 meters depth in rocky shores of the North and South islands of NZ (Sainsbury, 1982). They usually settle on rocks where there is well-oxygenated water and rapid currents. Adult abalone usually do not move around too much to find food. They await drifting food particles and trap them using their shell and tentacles (Poore, 1972; Allen, Marsden & Ragg, 2001; Ragg, 2003). Teeth-like structures called radula are used to graze on seaweed (Purchon, 1977). Smaller abalone graze on microscopic diatoms (Dutton & Tong, 1981; Garland et al., 1985). It is

thought that wild abalone can live over 10 years and grow up to 180 mm in shell length (Jeffs, 2003; Tung, 2010).

H. iris are slow-growing broadcast spawners. Adult abalone have different maturity rates at different locations, but they normally start to produce mature eggs when they reach about 60 mm shell length, and the spawning happens when they are 4 years old (Poore, 1973). Sperms and mature eggs are released into the water at the same time. Fertilised eggs then develop into small larvae, which can be transferred by sea currents before they settle on a solid surface and start metamorphosis. At the post-larval stage, they feed on microalgae and grow to juveniles. Juvenile abalone live under rocks for 3-5 years to minimise predation (Somerville, 2013). The final stage of the abalone life cycle is adult stage when they can produce mature eggs and sperms and spawn.

1.1.6 Worldwide abalone aquaculture

In recent decades, the demand for abalone products has increased. In the 1970s, the market demand for abalone resulted in a peak worldwide harvest (Gordon & Cook, 2004). At that time, the total production from legal abalone fisheries was around 20,000 metric tonnes (mt), which declined sharply in following years. The wild abalone catch was around 7,900 mt in 2008 (Cook & Gordon, 2010) and only 6,500 mt in 2015 (Cook, 2016) (Figure 1-4). This significant decrease was due to overfishing and poaching as well as disease outbreak, slow growth rate and destruction of natural terrains (Cook & Gordon, 2010).

To sustain abalone production, numerous abalone farms were set up around the world. A 350% increase in farmed abalone production was reported from 2002 to 2008, reaching 30,760 mt in 2008 (Cook & Gordon, 2010). Currently, the highest abalone production is in China with more than 300 abalone farms with the production tonnage of more than 1,000 mt in 2010 and with a total production tonnage of 115,397 mt in 2015 (Cook, 2016) (Figure 1-4). Other countries, such as Korea, South Africa and Australia followed China with their total farmed abalone production in 2015.

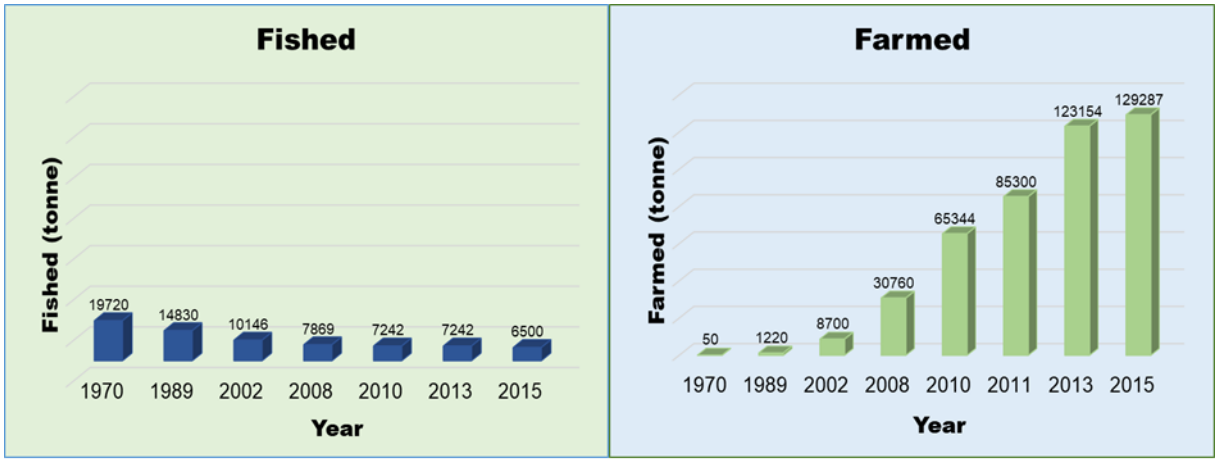


Figure 1-4 Global production of abalone from legal fisheries (left) and farming (right) (Adapted from Cook (2016)).

1.1.7 Abalone aquaculture in New Zealand

Three species of abalone are endemic to New Zealand, including common or black-footed (*H. iris*), the queen or yellow-footed (*H. australis*) and the virgin or white-footed abalone (*H. virginea*) (Tung, 2010) among which *H. iris* is the main species which is farmed for the export market. The pāua industry formed in the 1980s and has been developed since then. Seventeen experimental and pilot abalone farms were operating as land-based facilities in 2003 in both North and South Islands (Jeffs, 2003). Currently, there is only one large land-based pāua farming facility in the North Island producing pāua at commercial scale (Figure 1-5). Among the thirteen influential countries producing farmed abalone, New Zealand holds the ninth position with the production value of around 100 mt in 2015 (Cook, 2016). Since pāua farming in New Zealand is antibiotic/chemical free, pāua products are identified with having a high quality around the world. New Zealand pāua is mainly exported to Hong Kong, Singapore, Taiwan and China.

Figure 1-5 Pāua culturing in Moana New Zealand abalone farm, Ruakaka, NZ. (a, b) Multi-level culture tanks optimised to grow pāua for 3-4 years, (c) culture tanks supplied with tipping buckets designed to provide maximum aeration for Juvenile pāua, (d) Adult abalone (80-100 mm) ready to be harvested.

1.1.7.1 Bottlenecks in abalone culture

Although land-based pāua facilities in NZ has been successful in producing high quality products, there are still challenges and problems being addressed by pāua farmers. The slow growth rate of abalone, which is around 4 to 5 years, is a major bottleneck in pāua farming (Figure 1-6). The long period which abalone requires to reach market size (80-100 mm) can impose serious financial constraints on this growing industry.

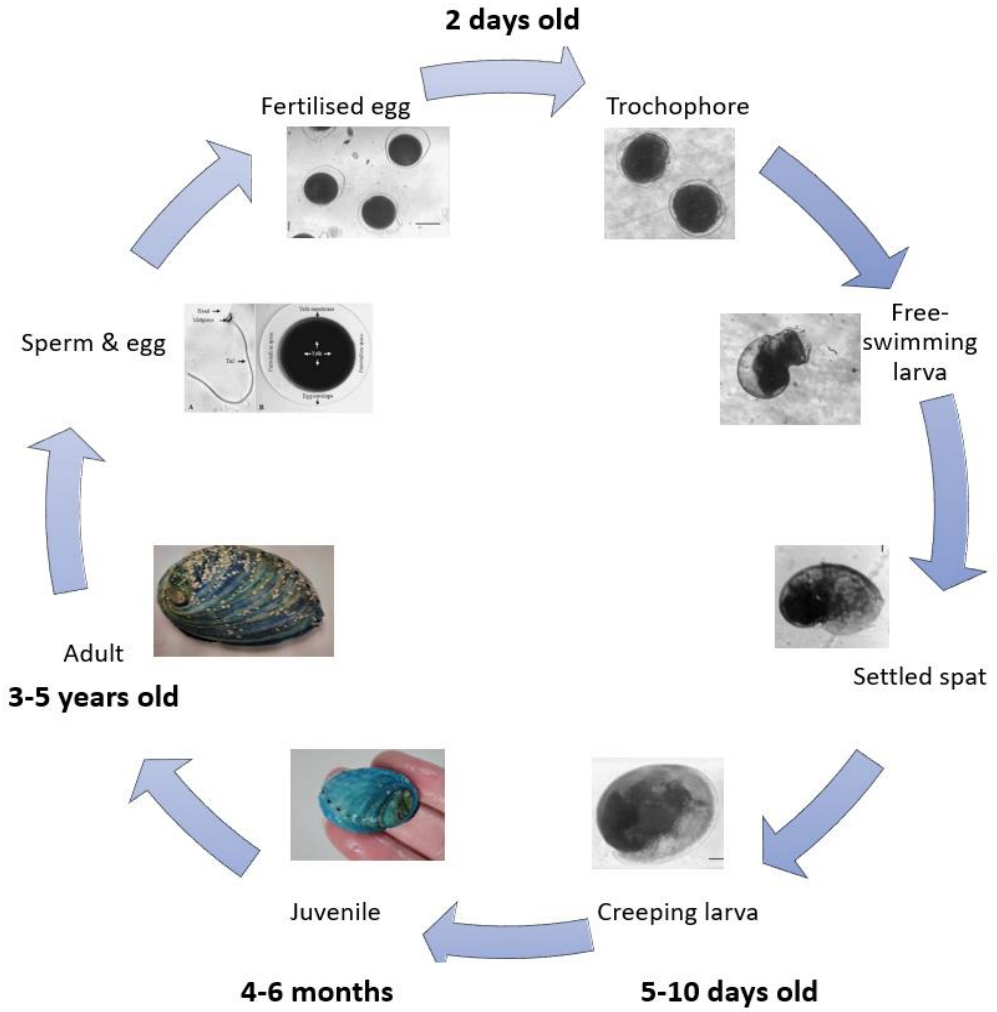


Figure 1-6 Diagram of abalone life cycle indicating different developmental phases and the time required for them to grow from a fertilised egg to an adult (Adopted from Najmudeen & Victor, 2004; Tuterangiwhiu, 2015).

The major factors limiting pāua growth rate are water quality, temperature, health status and lack of a species-specific feed. Pāua are very sensitive to poor water conditions such as pH, temperature, chemical pollution, inadequate oxygen levels, salinity and nitrogenous waste; these directly affect the growth rate of Pāua. Filtering and recirculation systems are used in pāua farms in order to maintain the quality and temperature of seawater. The recirculation system enables farmers to maintain the temperature over winter and summer by controlling seawater intake, thus avoiding water temperature fluctuation in the farm. With increasing summer sea temperatures,

as a result of global warming, some farms utilise a cooling system to avoid massive mortalities. Furthermore, accumulation of faecal matter, uneaten feed and leaching of nutrients, such as protein levels in the recirculated water may increase the amount of nitrogenous compounds and reduce the quality of the culture water. Poor culturing conditions have led to disease outbreaks in NZ pāua commercial facilities in the past. For instance an outbreak of haplosporidosis in juvenile pāua was reported in the summers of 1999-2001 with mortality rates of approximately 90% (Diggles et al., 2002; Diggles & Oliver, 2005). Occasional mass mortalities have also been reported in pāua farms mostly due to algal blooms (Chang, 1999; Chang, Chriswell & Uddstrom, 2001).

Although farming processes have been optimised, the environmental and economic impacts of feed impose a burden on farm development. Farmed abalone are fed with formulated commercial feed containing protein and carbohydrate from sources such as fish meal, kelp and seaweed. Formulated feeds are commercially available from different suppliers. However, none are specifically designed for pāua. Abalone feeds are currently imported to New Zealand, mostly from South Africa and Australia. There is an inherent risk involved with these products as lighter regulations and biosecurity checks may translate into the risk of disease transfer to New Zealand. The food conversion ratios (FCR, weight of given food: increase in body weight) of commercial feeds have been reported to be 3:1 to 4:1 for some farms (Jeffs, 2003) and further improved to up to 1.7:1 for others (Moana New Zealand., personal communication). However, to obtain a better FCR, relatively expensive feeds need to be imported from other countries. This significantly increases the total cost of NZ abalone farming. Moreover, the current commercial pellets imported from South Africa and Australia have low stability in sea water. They become mushy and disintegrate in a short period of time. Apart from nutrient leaching into the water, risks of microorganism growth and low consumption rates, farmers need to feed the animals on daily routines, which results in an increase in labour and production costs. To add to this, about 60% of the feed is wasted, further increasing the cost of production.

1.1.7.2 Feed formulation and sustainable pāua farming

Wild pāua graze on various types of seaweed, especially on red macroalgal species. Some farms breed pāua using seaweed. However, the low feed conversion rate (10:1 to 25:1) results in an increase in the cost for farming. Formulated feeds are available to farmers from different suppliers. In NZ pāua farming, abalone feeds from Marifeed Ltd are used which have been designed and tested on South African abalone (*H. midae*). However, high amounts of feed wastage, nutrient leaching and quick disintegration of feed pellets in seawater are associated with this type of feed. Therefore, artificial feeds are required to be designed specifically based on the species of abalone and culturing system. Lack of a specific formulated feed for farmed pāua is one of the main reasons hampering the growth rates of these animals.

Although substantial research has been carried out on diet composition for abalone, the research has been less focused on the physical quality of feed pellets. These qualities which include palatability, size, uniformity, durability, stability, water absorption and water solubility play a significant role in effectively delivering required nutrient to farmed abalone. The weak structure of current extruded pellets leads to a quick disintegration in seawater. Thus, nutrients leach from the particles before being consumed by animals. Insufficient feed durability and stability in seawater generates higher feed wastage within the farm and contribute to an overall decline in water quality. Therefore, as pāua exports are among the top ten aquaculture products in NZ (Ministry of Fisheries, 2011), it is important to address current problems in order to improve farming and maximise the expected revenues. Research and development in the pāua industry is required to focus on improving feeds and environmental aspects of feeding processes to increase feed consumption, conversion rates and reduce feed wastage, thus, steering the industry toward more environmentally and economically sustainable practices.

1.1.7.3 Use of probiotics in aquaculture

Several strategies have been trialled to enhance abalone growth rates. Design of different culture system (e.g. barrels, cages, tubes) was among the first measures to be

tried (Preece & Mladenov, 1999). Addition of various feed supplements, such as red algae was tested to obtain a better growth performance which showed promising results after three months of feeding *H. iris* (Allen et al., 2006). Selective breeding programmes are also used to produce offspring with desired commercial traits, such as fast growing and late maturing (Elliott, 2000). However, this is usually a long-term endeavour and may create concerns over genetic diversity.

Optimising culture conditions and dietary protein (Tung & Alfaro, 2011ab; Tung & Alfaro, 2012) and use of probiotics (Macey & Coyne, 2005; Hadi et al., 2014) are among the most recent strategies exploited to enhance the growth rate and health of abalone. However, these studies have been limited to small scale laboratory research. Probiotics have been widely tested as a new approach for abalone growth and health improvement in different species (Macey & Coyne, 2005; Doeschate & Coyne, 2008; Wu et al., 2011; Hadi et al., 2014; Faturrahman, Rohyati, & Sukiman, 2015; Huddy & Coyne, 2015; Gao et al., 2018; Venter et al., 2018; Zhao et al., 2018). Probiotics can be used not only to increase the growth rate of animals but also to prevent disease occurrence within a farm. Probiotics can be used as a great alternative for chemical additives or drugs in aquaculture. Although significant improvements in growth rates of abalone have been observed repeatedly since the advent of probiotic application in aquaculture, the probiotic strategy has never stepped further out of laboratories into abalone farms due to environmental, cost and sustainability issues. One of the main obstacles on large scale probiotic application is the lack of an efficient delivery method. Besides that, retaining the viability of probiotics during delivery to farmed animals remains another challenge.

1.1.8 New strategies and future innovation in abalone farming

Aquaculture is rapidly growing around the world which inevitably is accompanied by intensified culturing processes. This will impose risks to the sustainability of aquaculture practices. In order to maximise the profitability of the abalone industry, diet-oriented approaches such as optimising feed ingredients have been mostly adapted. The most common technology for producing abalone feeds is extrusion-based production. This feed production technique uses heat and steam to bind ingredients together and to

extrude feed pellets. The feed pellets may contain optimum dietary requirements. However, less attention has been paid to the physical features of the feed. As aquaculture feeds make up a significant portion of abalone production cost, it is crucial to develop a feed with optimum features, such as 1) good stability in seawater to minimise feed wastage, 2) minimum nutrients leaching from the pellets to ensure delivering specific amounts of nutrients to animals, 3) high palatability and 4) containing optimum dietary requirements. A combination of all these features can accelerate farming productivity, profitability, while assuring long-term sustainability.

New technologies are required in order to develop economical feeds with optimised features. Based on the literature, there is a great potential for application of encapsulation technology (ET) in aquaculture (Dezfooli et al., 2018). This technology is based on enclosing materials in a confined structure. ET has been used to deliver probiotics (Rosas-Ledesma et al., 2012), beneficial yeast cells (Pinpimai et al., 2015), vaccines and immunostimulants (De las Heras, Rodriguez Saint-Jean, & Perez-Prieto, 2010) to different marine species. ET can also be used to entrap feed materials in a bead or capsule structure in order to improve nutrient delivery and to protect valuable ingredients from leaching. Therefore, this technology can provide an opportunity to aquaculture to improve feed quality and thereby farmed-animal performance along with obtaining a significant reduction in feed wastage and enhance the sustainability of farming process.

1.2 Thesis aims

Considering the above, the main aim of this thesis is to develop an encapsulated system that can deliver probiotics in a specific dosage and with a high viable state to farmed abalone. The second aim of this thesis is to investigate the effect of encapsulated probiotics on the growth performance of black-footed abalone. The specific objectives of each chapters are:

i. To characterise three bacterial species (previously isolated from pāua gut in our laboratory) to ensure their ability to hydrolyse nutrients in abalone feed.

ii. To develop and characterise a microencapsulated probiotic carrier using an emulsion technique based on natural polymers for targeted delivery of probiotics to *H. iris*.
iii. To develop and characterise a macroencapsulated probiotic delivery system using extrusion techniques with improved properties such as fast sinking, high palatability and stability in seawater.
iv. To perform a feeding trial to evaluate the effect of the developed encapsulated probiotic and feed on the growth rate and immunity of pāua.

1.3 Thesis structure

This thesis is a multidisciplinary investigation involving microbiology, formulation science, drug delivery, metabolomics, immunology and marine biology in aquaculture.

Chapter one is a general introduction discussing the issues associated with food supply as a challenge in today's world and the role of aquaculture in addressing some of those needs. Sustainability issues regarding wild fisheries were discussed and the role of aquaculture to relieve the pressure from wild fisheries was highlighted. General information about the status of abalone farming worldwide and in New Zealand were provided. Finally, the bottlenecks and technological challenges in abalone aquaculture were discussed and the need for novel technologies to assure sustainable growth of this sector were highlighted. This discussion led to outlining aims and framework for this thesis.

In Chapter two, a broad literature review was conducted on the application of encapsulation technology in aquaculture. Despite the long history of ET usage in pharmaceutical industries, the technology is relatively new in aquaculture. This work was conducted to provide a comprehensive review of previous relevant work to aquaculture and marine scientists. Thus, attention was focused on the potential of encapsulation technology in offering innovative approaches to improve aquaculture practices. This chapter proposed practical considerations and suggestions on selecting

right materials and techniques for designing novel bioactive delivery systems for aquaculture application.

The ultimate goals of the experimental chapters were to develop a capsule compartment with desired physical features and palatability capable of delivering nutrients and bacterial probiotic to farmed NZ abalone.

In Chapter three, the ability of three host-associated bacterial species as beneficial bioactives for NZ abalone was investigated. Biochemical characterisation tests were conducted to ascertain the presence of specific biochemical features for hydrolysing abalone feed nutrients. This chapter further provides information about the three tested microbial species required for development of the encapsulated probiotic supplements in the following chapters.

The next important step was to develop a carrier for delivery of probiotic bacteria to farmed pāua. Two separate chapters worked to address this aim. In Chapter four, microencapsulation was used to encapsulate bacterial cells. The objective of this chapter was to determine whether microparticles generated by an emulsion technique can attain desired features for delivering probiotics to farmed pāua. Emulsification has been widely used for probiotic encapsulation due to retaining high viability of encapsulated probiotics and producing uniform particles. In this chapter, *in-vitro* studies were performed to investigate the release of encapsulated probiotic in seawater and in simulated gastrointestinal fluid of abalone. The fate of microcapsules was also sought within the animal's digestive tract. The efficiency of developed microparticles to obtain a targeted delivery of probiotics to abalone was proved. Despite the successful outcomes of this chapter, an alternative system was developed in Chapter 5 to address issues related to accessibility of microparticles to bottom-feeding abalone and feasibility of scale-up production.

In Chapter five, an extrusion-based technique was used to develop alginate beads. The objective of this chapter was to deploy a simple encapsulation technique feasible for future large-scale production. The palatability of probiotic capsules and their ability to

deliver probiotics to abalone was confirmed in this chapter. In Chapter six, a feeding trial was performed to investigate the efficiency of the new delivery method for feed and probiotics. A general conclusion and discussion constitute the final chapter of this thesis.

1.4 Research outputs from this thesis

Peer-reviewed journal publications

Dezfooli, S. M., Alfaro, A. C., Gutierrez-Maddox, N., Seyfoddin, A. (2018). Encapsulation for delivering bioactives in aquaculture, *Reviews in Aquaculture*, 1-30.

Dezfooli, S. M., Alfaro, A. C., Gutierrez-Maddox, N., Seyfoddin, A. (2019). Abalone aquaculture and current challenges for a sustainable production. Manuscript in preparation.

Dezfooli, S. M., Alfaro, A. C., Gutierrez-Maddox, N., Seyfoddin, A. (2019). Microencapsulation for targeted delivery of probiotics to black-footed abalone (*Haliotis iris*). Manuscript in preparation.

Dezfooli, S. M., Alfaro, A. C., Gutierrez-Maddox, N., Seyfoddin, A. (2019). Development and characterisation of encapsulated probiotic beads for improved delivery of probiotics to farmed abalone. Manuscript in preparation.

Dezfooli, S. M., Alfaro, A. C., Gutierrez-Maddox, N., Seyfoddin, A. (2019). Improved growth rate of black-footed abalone through encapsulated feed-probiotic diet. In preparation. Manuscript in preparation.

Dezfooli, S. M., Alfaro, A. C., Gutierrez-Maddox, N., Seyfoddin, A. (2019). Effect of encapsulated probiotic diet on immune parameters and metabolomics profile of black-footed abalone. Manuscript in preparation.

Conference presentations

Dezfooli, S. M., Gutierrez-Maddox, N., Alfaro, A. C, Seyfoddin, A. (2018). Encapsulated feed for increased growth of farmed pāua and reduced feed wastage. In Australian Malacological Triennial Conference-Molluscs, Wellington, New Zealand.

Dezfooli, S. M., Gutierrez-Maddox, N., Alfaro, A. C, Seyfoddin, A. (2018). Encapsulation technology: A tool to reduce feed wastage in aquaculture. In New Zealand Marine Sciences Society Annual Conference, Napier, New Zealand.

Dezfooli, S. M., Gutierrez-Maddox, N., Alfaro, A. C, Seyfoddin, A. (2018). A new carrier to deliver probiotics to farmed pāua. Poster session presented at New Zealand Marine Sciences Society Annual Conference, Napier, New Zealand.

Dezfooli, S. M., Gutierrez-Maddox, N., Alfaro, A. C, Seyfoddin, A. (2018). Targeted delivery of encapsulated probiotics for increased growth of farmed pāua. In AUT Research Showcase, Auckland University of Technology, Auckland, New Zealand.

Seyfoddin, A., Dezfooli, S. M., Mohammadi, S., Gutierrez-Maddox, N., Alfaro, A. C. (2017). From humans to molluscs: target specific bioactive delivery using microcapsules. In New Zealand Marine Sciences Society Annual Conference, Christchurch, New Zealand.

Dezfooli, S. M., Gutierrez-Maddox, N., Alfaro, A. C, Seyfoddin, A. (2017). Delivery of probiotics to aquaculture: challenges and strategies. In New Zealand Marine Sciences Society Annual Conference, University of Canterbury, Christchurch, New Zealand.

Dezfooli, S. M., Alfaro, A. C., Gutierrez-Maddox, N., Seyfoddin, A. (2017). Encapsulating probiotics for increased growth of farmed abalone. Poster session presented at the 8th Biennial Australian Colloids and Interface Symposium, Coffs Harbour, NSW, Australia.

Seyfoddin, A., Dezfooli, S. M., Mohammadi, S., Gutierrez-Maddox, N., Alfaro, A. C. (2017). Encapsulation techniques for aquaculture application. In the 8th Biennial Australian Colloids and Interface Symposium, Coffs Harbour, NSW, Australia.

Dezfooli, S. M., Gutierrez-Maddox, N., Alfaro, A. C, Seyfoddin, A. (2016). Encapsulation of probiotics for aquaculture applications. In Research Symposium, Auckland University of Technology, Auckland, New Zealand.

Dezfooli, S. M., Gutierrez-Maddox, N., Alfaro, A. C, Seyfoddin, A. (2016). Dietary formulation of encapsulated probiotics for the pāua industry. Poster session presented at the 2nd New Zealand Aquaculture Research Symposium, Nelson, New Zealand.

Chapter 2

Literature review

“The more you know, the more you know you don't know”.

Aristotle

Abstract

The exploitation of wild fisheries has driven the seafood industry to develop more sustainable resources and high-intensity aquaculture. However, high-density aquaculture is prone to outbreaks of diseases. The use of chemicals and antimicrobial drugs in aquaculture is common practice, but such chemicals can have serious detrimental environmental impacts and antibiotics may result in bacterial resistance in many species. The development of non-antibiotic and green strategies, such as using probiotics and immunostimulants, aims for a more sustainable practice for health maintenance in aquaculture. Although the efficacy of such strategies has been demonstrated in cultivations settings, the effective delivery of bioactives remains a challenge. Conventional delivery methods are often ineffective in terms of dosage, stability and species specificity, and can possibly cause widespread environmental contamination. However, recently developed drug delivery systems may provide innovative ways to improve delivery with minimal waste and improved environmental protection. Indeed, such cutting-edge drug delivery systems have already provided measurable benefits in human medicine over the past two decades. New technologies, such as encapsulation and controlled release systems, can be used readily in scaled-up operations to improve the delivery of bioactives and ultimately increase production and profitability in aquaculture. This review critically analyses the use of encapsulation technology with a focus on microparticles for delivery of bioactive agents to farmed aquatic animals. This review discusses various potential encapsulation materials, their properties and a range of methods that can be applied and scaled-up for aquaculture. Finally, practical considerations for designing an efficient delivery system for aquaculture are discussed.

2.1 Introduction

Over-fishing and stock depletion have put wild fisheries under considerable pressure. The results of this pressure are observed as reduced fishery yields and an increased number of threatened species. In response, the industry has moved to develop more sustainable and high-density aquaculture practices. However, high-density farming is prone to health problems, such as bacterial (Birkbeck, Feist & Verner–Jeffreys, 2011; Pridgeon & Klesius, 2012), fungal (Ramaiah, 2006) and viral (Oidtmann, B., & Stentiford, 2011) infections. Billions of dollars have been lost due to disease outbreaks in aquaculture worldwide (Austin & Austin 1993; Hastings et al., 1999; Hill 2002; Murray & Peeler 2005). In many countries, veterinary medicines, such as antibiotics and chemotherapeutics, are regularly used to combat endemic problems. The exposure to these agents has raised serious concerns, especially with regard to antibiotic resistant pathogens and environmental contamination (Smith et al., 1994; Ai et al., 2011).

The development of green strategies, such as utilisation of bioactives (agents having a biological effect) promises a technologically advanced era for aquaculture (Qi et al., 2009; Ringø et al., 2012). Bioactives that act as supplementary nutrients, immunostimulants, vaccines and probiotics can be utilised to 1) provide optimal nutrition to aquaculture species 2) improve their general health 3) enhance farm productivity and management approaches and 4) reduce environmental impacts. Although the efficacy of green strategies has been demonstrated in numerous studies, the delivery of bioactives in aquaculture settings and the scaling up these technologies remains a challenge. The main concerns around the use of such strategies are: 1) effective delivery in terms of dosage, stability and species specificity, 2) risk of environmental contamination 3) the dilution of an administered dose in large tanks and runways, 4) instability and deterioration of bioactive materials in different environmental conditions during processing, storage and delivery and 5) species-specific actions of some bioactives (e.g. probiotics and vaccines) where one bioactive may be beneficial to one species but pathogenic to another. Conventional delivery techniques in aquaculture, such as immersion, injection, diet supplements and enrichment of live

food (De et al., 2014), often deliver insufficient dosages and suffer from uncontrolled release and instability of bioactives during processing and storage (Conway et al., 1987; Anal & Singh 2007). While many bioactives show promising results in research laboratories, it is essential to develop advanced and economical bioactive delivery methods to be able to implement these new advances in a commercial aquaculture scale.

Current aquaculture practices require the use of innovative and environmentally friendly methods which can solve the above problems and maximise the production level and profitability. One of the innovative strategies to improve aquaculture is targeted delivery of bioactives to farmed species. Targeted delivery methods are defined as approaches, formulations and strategies to transport bioactive molecules to their site of action. Novel drug delivery vehicles, such as biocompatible and biodegradable capsules have been used to deliver specific nutrients, probiotics, genes and chemicals in a sustained and controlled manner for human and veterinary applications. Such drug delivery vehicles can also be used to prevent the release of unwanted materials into the environment and extend the shelf life of the encapsulated materials (Zuidam & Shimoni 2010; Huq et al., 2013) and ensures a target-specific delivery of bioactives in a host (Kebary & Hussein 1999; Picot & Lacroix 2004). Delivery of bioactives in aquaculture farms is a new challenge to formulation scientists. Formulating a carrier that can withstand disintegration in seawater whilst maintaining the activity of the entrapped agents is a major challenge. That solved, a programmed released micro- or nano-carrier can be easily designed for various applications.

This chapter aims to highlight the importance and applicability of encapsulation for enhancing the delivery and efficacy of bioactives in aquaculture farming. Potential materials that can be used to prepare microcapsules, their properties and encapsulation methods that can be applied in aquaculture are discussed in detail. Specific case studies are used to illustrate the encapsulation of probiotics, vaccines and immunostimulants. Finally, the feasibility of designing an efficient delivery system for aquaculture is discussed.

2.2 Encapsulation concept in aquaculture

In order to adapt to harsh environmental conditions, many species such as bacteria have formed a protective 'hard shell' around their genetic material. Inspired by these 'hard shells' over the years, encapsulation scientists have created shells using various synthetic and natural materials to encapsulate living organisms for better stability and protection. Encapsulation is a compartmentalisation process that can provide a "unique environment'' that isolates the bioactives from the surrounding environment. The availability of various materials with selective properties, such as solubility in certain pH or temperature has also led to the design of smart delivery systems that target a specific site in the animals' body, such as upper intestine.

In aquaculture, encapsulation can be applied to:

- increase the bioavailability of bioactives in the animal's body by increasing their uptake,
- target a specific delivery site within the animal's body (e.g. stomach, intestine or systemic circulation),
- protect bioactives from unique environmental conditions, such as pH, redox state and ionic strength,
- protect bioactives from processing factors, such as dehydration, exposure to elevated temperatures and extrusion,
- provide effective protection against bioactive degradation,
- increase the viability of live bioactives, such as probiotics,
- provide sustained, delayed or controlled release of bioactives to increase treatment efficacy,
- increase animal species specificity in order to reduce impact on other wild or farmed species,
- provide a practical method to deliver sensitive bioactives, such as probiotics and vaccines in aquaculture farms,

- improve therapeutic efficiency, and therefore, a reduce the dose of materials, such as vaccines, probiotics and immunostimulants required for a bioequivalent effect, and
- deliver DNA, genes and antigens for advanced genomic and proteomic approaches.

While compartmentalisation can be possible following several strategies, such as formulations of multi-layered films, nano- and microparticles, the focus of this review is limited to polymeric spherical microparticles for bioactives delivery. In drug delivery, microparticles are usually referred to spherical particles between 1 to 1000 µm in diameter. They can be produced from a range of natural, synthetic or semisynthetic materials, such as polymers and lipids. Based on their internal structures, microparticles are classified into microspheres and microcapsules. Microcapsules consist of a shell with an empty core as a reservoir to hold the encapsulate or the agent, while microspheres have a solid core and the encapsulated agent is dispersed in the core matrix (Figure 2-1). Both types of microparticles can be further protected by additional coating layers (Zuidam & Shimoni 2009). An extra layer can increase the stability of the particles and allow a better control over the release of the encapsulated agents.

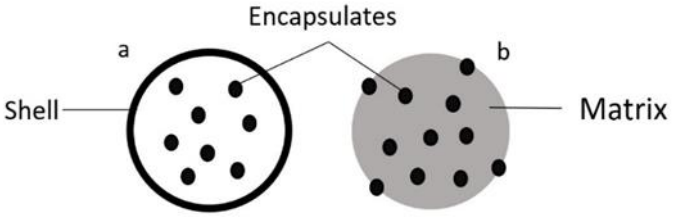


Figure 2-1 Schematic diagram of two types of microparticles: (a) microcapsule and (b) microsphere.

The release mechanism of encapsulates from microparticles can be a complex combination of several internal and external factors deliberately designed to release the encapsulates in a programmed manner. Physical or chemical conditions can break or degrade the shell leading to the release of encapsulated material(s) from microcapsules.

Another potential release mechanism for smaller molecularly sized encapsulates is their diffusion through a semipermeable shell material. Similarly, release from microspheres can result from the erosion of the matrix material in a programmed manner and by diffusion due to osmotic drive or the formation of pores and channels through the matrix. Various polymers and protein-based materials have been used for encapsulation of bioactives in aquaculture, and these are discussed in the following section.

2.2.1 Materials for encapsulation of bioactives for aquaculture applications

Oral delivery of bioactives and nutrients is the preferred method of administration in aquaculture. The advantages of oral delivery systems are that they tend to be cost effective, easy to scale up and less stressful to animals compared to other invasive methods, such as injection. There are two potential barriers to oral bioactive delivery in aquatic animals: 1) the dissolution of the microparticles in water prior to ingestion and 2) the degradation of sensitive bioactive agents in the acidic environment of the stomach before they arrive at their target site, intestine (e.g. probiotics), or can be absorbed into the systemic circulation (e.g. immunostimulants, vaccines and nutrients). In order to design a system that can withstand dissolution prior to ingestion and accurately deliver bioactive agents into the specific sites in the target animal, selecting the right materials for encapsulation is essential (Figure 2-2). When it comes to choosing biomaterials for encapsulation purposes for aquaculture application, one has to consider the fundamental differences among target species, including their physiology, living environment and commercial practicalities. Considering the anatomy and physiology of the target animal is the very first step in designing a bioactive carrier as significant pH differences have been reported for different fish species and shellfishes (McLean 1970; Harris et al., 1998; Krogdahl et al., 2015). The environmental and culturing conditions of the target animal are other important factors to consider when choosing encapsulation materials, especially since the temperature, pH and salt concentration of the culture water can affect the stability and release profile of developed microparticles (

Table 2.1 Conditions to consider for encapsulation of bioactives for delivery to major aquaculture species.

Species	Polymer used for encapsulation	GIT features and pH range	Aquaculture method	Feeding habit	References
Nile tilapia 	- Alginate - Skim-milk alginate chitosan	1.4 - 8	Fresh water biofloc system, tanks, raceways ponds, floating cages and recirculation systems.	Can feed in all level	(FAO, 2017a) (Pinpimai et al., 2015) (Rodrigues *et al.,* 2006) (Leal et al., 2010) (Hlophe *et al.,* 2014)
Goldfish 	Alginate	6.4 - 7.9	Fresh water tanks and ponds	Can feed in all level	(Zhang *et al.,* 2016) (Maurice *et al.,* 2004) (Solovyev *et al.,* 2015)
Japanese flounder 	- Alginate - Poly lactide co-glycolic acid (PLGA) - Chitosan	<4 - 8.5	Seawater land-based culture systems	Bottom feeders	(Rønnestad *et al.,* 2000) (Tian *et al.,* 2008abc)
Channel catfish 	Alginate-gelatine	2 - 8.6	Freshwater ponds, tanks, raceways and cages	Bottom feeders	(Page *et al.,* 1976) (FAO, 2017) (Kumaree *et al.,* 2014)

Table 2.1 Conditions to consider for encapsulation of bioactives for delivery to major aquaculture species.

Species	Polymer used for encapsulation	GIT features and pH range	Aquaculture method	Feeding habit	References
Common carp 	- Alginate-coated chitosan - PLGA - Alginate	6.3 - 7.4	Freshwater cages, irrigation reservoirs, tanks, ponds and in recirculation systems	Bottom feeders	(Joosten *et al.,* 1997) (Behera *et al.,* 2010) (Yun *et al.,* 2017) (Behera & Swain 2014) (Zhang *et al.,* 2016) (Solovyev *et al.,* 2015)
Perch 		3.5 - 6.8	Fresh water tanks, extensive and intensive methods	Bottom feeders	(Moyle *et al.,* 1974)
Gilt-head sea bream 	Alginate	2.6 - 7.9	Saltwater raceway, concrete tanks, sea cages, extensive, semi-intensive and intensive systems	Can feed in all level	(FAO, 2017c) (Deguara *et al.,* 2003) (Cordero *et al.,* 2015)

Table 2.1 Conditions to consider for encapsulation of bioactives for delivery to major aquaculture species.

Species	Polymer used for encapsulation	GIT features and pH range	Aquaculture method	Feeding habit	References
Rainbow trout 	- Alginate - PLGA	2.7 - 8.2	Freshwater monoculture systems, raceway and pounds	Surface feeders	(FAO, 2017b) (Bucking & Wood 2009) (Ghosh, Cain, Nowak, & Bridle, 2016) (Joosten *et al.,* 1997) (Romalde, Luzardo-Alvárez, Ravelo, Toranzo, & Blanco-Méndez, 2004) (Adomako et al., 2012) (De las Heras *et al.,* 2010) (Altun *et al.,* 2009)
Atlantic Salmon 		4.8 - 8.1	Sea cages	Not a bottom feeder	(Krogdahl *et al.,* 2015)
Crayfish 		5 - 7	Seawater ponds or tanks	Bottom feeders	(Brown 1995)

Table 2.1 Conditions to consider for encapsulation of bioactives for delivery to major aquaculture species.

Species	Polymer used for encapsulation	GIT features and pH range	Aquaculture method	Feeding habit	References
Black tiger shrimp 	- β-1,3-D-glucan - VLPs	Feed takes between 48.3 and 90.5 min to pass through the gut, with some feed taking 4 – 6 hrs to pass through the gut	Extensive, semi-intensive and intensive ponds with tidal seawater flow.	Bottom feeders	(FAO, 2017d) (Zhu & Zhang 2012) (Jariyapong *et al.,* 2015) (Beseres *et al.,* 2005)
Abalone 	Alginate	5 - 6.6 Passage through GIT can take a few hours to a few days	Seawater filled tanks, tipping tanks and cages	Bottom feeders	(Poore, 1972) (Harris *et al.,* 1998) (Wu *et al.,* 2011)

).

In addition, the feeding behaviour of the target animal should be examined before designing a release system. For instance, sinking particles are more suitable for shellfish, while floating particles may be more effective for fish. Industrial practicalities are also important to consider, since the controlled release products often need to be cost effective. The compatibility of the additives with the bioactives also need to be considered when selecting biomaterial for encapsulation. For example, adverse effects of chitosan, a commonly used polysaccharide obtained from crustaceans, on lactic acid bacteria was reported to be a constraint in the application of encapsulation of some probiotics (Hyndman et al., 1993). A very low encapsulation efficiency was reported

when siRNA was encapsulated in PLGA due to the very low molecular weight of siRNA and electrostatic repulsion between siRNA backbone and anionic groups in PLGA (Cun et al., 2011). Biomaterial-related features also should be considered since they can influence the encapsulation efficiency. Such factors include solubility of wall materials in a desired solvent, concentration of polymer, interaction between encapsulated and encapsulating materials, solubility and size of entrapped materials (Jyothi et al., 2010). Some of the most commonly used materials that are used in the formulation of microparticles for aquaculture use are discussed in the following section.

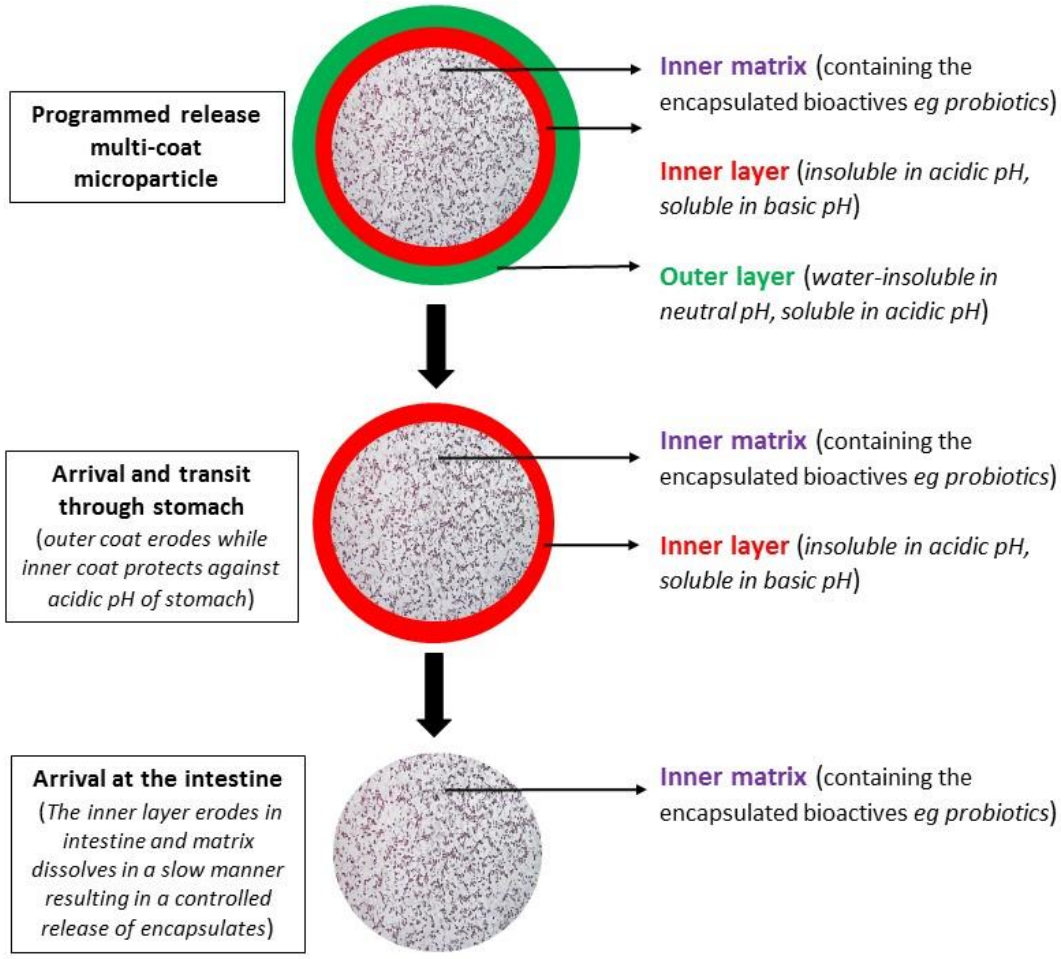


Figure 2-2 Schematic representation of a multi-layered microparticle for delivering bioactives (e.g. probiotics) to the intestine of an aquaculture species.

Table 2.1 Conditions to consider for encapsulation of bioactives for delivery to major aquaculture species.

Species	Polymer used for encapsulation	GIT features and pH range	Aquaculture method	Feeding habit	References
Nile tilapia 	- Alginate - Skim-milk alginate chitosan	1.4 - 8	Fresh water biofloc system, tanks, raceways ponds, floating cages and recirculation systems.	Can feed in all level	(FAO, 2017a) (Pinpimai et al., 2015) (Rodrigues *et al.,* 2006) (Leal et al., 2010) (Hlophe *et al.,* 2014)
Goldfish 	Alginate	6.4 - 7.9	Fresh water tanks and ponds	Can feed in all level	(Zhang *et al.,* 2016) (Maurice *et al.,* 2004) (Solovyev *et al.,* 2015)
Japanese flounder 	- Alginate - Poly lactide co-glycolic acid (PLGA) - Chitosan	<4 - 8.5	Seawater land-based culture systems	Bottom feeders	(Rønnestad *et al.,* 2000) (Tian *et al.,* 2008abc)

Table 2.1 Conditions to consider for encapsulation of bioactives for delivery to major aquaculture species.

Species	Polymer used for encapsulation	GIT features and pH range	Aquaculture method	Feeding habit	References
Channel catfish 	Alginate-gelatine	2 - 8.6	Freshwater ponds, tanks, raceways and cages	Bottom feeders	(Page *et al.,* 1976) (FAO, 2017) (Kumaree *et al.,* 2014)
Common carp 	- Alginate-coated chitosan - PLGA - Alginate	6.3 - 7.4	Freshwater cages, irrigation reservoirs, tanks, ponds and in recirculation systems	Bottom feeders	(Joosten *et al.,* 1997) (Behera *et al.,* 2010) (Yun *et al.,* 2017) (Behera & Swain 2014) (Zhang *et al.,* 2016) (Solovyev *et al.,* 2015)
Perch 		3.5 - 6.8	Fresh water tanks, extensive and intensive methods	Bottom feeders	(Moyle *et al.,* 1974)

Table 2.1 Conditions to consider for encapsulation of bioactives for delivery to major aquaculture species.

Species	Polymer used for encapsulation	GIT features and pH range	Aquaculture method	Feeding habit	References
Gilt-head sea bream 	Alginate	2.6 - 7.9	Saltwater raceway, concrete tanks, sea cages, extensive, semi-intensive and intensive systems	Can feed in all level	(FAO, 2017c) (Deguara *et al.,* 2003) (Cordero *et al.,* 2015)
Rainbow trout 	- Alginate - PLGA	2.7 - 8.2	Freshwater monoculture systems, raceway and pounds	Surface feeders	(FAO, 2017b) (Bucking & Wood 2009) (Ghosh, Cain, Nowak, & Bridle, 2016) (Joosten *et al.,* 1997) (Romalde, Luzardo-Alvárez, Ravelo, Toranzo, & Blanco-Méndez, 2004) (Adomako et al., 2012) (De las Heras *et al.,* 2010) (Altun *et al.,* 2009)

Table 2.1 Conditions to consider for encapsulation of bioactives for delivery to major aquaculture species.

Species	Polymer used for encapsulation	GIT features and pH range	Aquaculture method	Feeding habit	References
Atlantic Salmon 		4.8 - 8.1	Sea cages	Not a bottom feeder	(Krogdahl *et al.,* 2015)
Crayfish 		5 - 7	Seawater ponds or tanks	Bottom feeders	(Brown 1995)
Black tiger shrimp 	- β-1,3-D-glucan - VLPs	Feed takes between 48.3 and 90.5 min to pass through the gut, with some feed taking 4 – 6 hrs to pass through the gut	Extensive, semi-intensive and intensive ponds with tidal seawater flow.	Bottom feeders	(FAO, 2017d) (Zhu & Zhang 2012) (Jariyapong *et al.,* 2015) (Beseres *et al.,* 2005)
Abalone 	Alginate	5 - 6.6 Passage through GIT can take a few hours to a few days	Seawater filled tanks, tipping tanks and cages	Bottom feeders	(Poore, 1972) (Harris *et al.,* 1998) (Wu *et al.,* 2011)

2.2.1.1 Polysaccharides

2.2.1.1.1 Alginate

Alginate is a naturally derived polymer obtained from different species of algae or bacteria, including phaeophyceae, *azotabacter vinelandii* and pseudomonas (Kurt et al., 2005). Alginate is composed of α-L-guluronic acid and β-D-mannuronic acid (Dong et al., 2006; Borgogna et al., 2011) and is the most commonly used encapsulation material in aquaculture (Table 2.2). Alginate offers several advantages, such as simplicity of formulation, biocompatibility, availability and low cost (Krasaekoopt et al., 2003). However, there are certain disadvantages that limit the use of all developed alginate delivery systems, especially during the scale-up process. Temperature and pH are important factors to be considered as the polymer can undergo denaturation during the formulation process (Donati and Paoletti 2009). In acidic pH (lower than 5), acid hydrolysis of glycosidic bonds can result in depolymerisation of alginate (Haug & Larsen 1963). In addition, a decrease in the degree of polymerisation has been reported in alkaline conditions (Hauc et al., 1967). To produce alginate sheets, beads or microcapsules, gelation can be achieved using approaches, such as ionic gelation and covalent cross-linking (Lee & Mooney 2012).

The release profiles of the entrapped bioactive are rather quick due to the porous structure of ionically cross-linked alginate macrostructures (Gouin 2004). Alginate molecules can interact with divalent cations, such as Mg^{2+}, Ca^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}, Cu^{2+} and Pb^{2+}, resulting in hydrogel formation (Haug & Smidsrød 1967; Haug & Smidsrod 1970). The concentration of divalent cross-linking ions is an influential factor on the integrity and stability of the hydrogel. Increasing the cation concentration results in the shrinkage of alginate (Donati and Paoletti 2009), which can be favourable for encapsulation purposes as it leads to enhanced stability and reduced porosity of the alginate (Martinsen et al., 1989; Draget et al., 2001). In applications that require materials to be instantly released, using porous alginate as a core material can be beneficial. However, if longer release profiles are desired, the amount of polymer, divalent cations and gelling time should be optimised in the formulation. Alginate can

also be covalently cross-linked thereby providing even better control over the release profiles. Covalent cross-linking agents, such as poly(ethylene glycol) (PEG) and adipic acid dihydrazide produce increased and stronger cross-links that decrease the swelling and porosity of alginate and in turn slow down bioactive release (Maiti et al., 2009; Lee & Mooney 2012).

Photo cross-linking is an approach for in situ gelation that exploits covalent cross-linking and can be carried out in mild reaction conditions with the appropriate chemical initiators. Alginate, modified with methacrylate and cross-linked by exposure to a laser in the presence of eosin and tri-ethanol amine, forms clear and flexible hydrogels (Lee & Mooney 2012). Photo cross-linking can be the best technique to employ in applications where chemical cross-linking is detrimental to encapsulated cells or deactivates entrapped agents. The total cost and removal of non-reacted photo-initiators can be a major challenge and disadvantage when applied to large-scale aquaculture use.

2.2.1.1.2 Chitosan

Chitosan is a linear, non-toxic and biodegradable polysaccharide derived from chitin through deacetylation. Chitin can be extracted from insects and crustacean or it can be obtained from algae or fungi, such as diatoms, chrysoflagellates and some members of oomycetes (Ruiz-Herrera 1991). Chitosan is a positively charged polysaccharide with a pka of 6.5. The glucosamine structural unit in chitosan can be converted into a soluble form that allows it to be dissolved in acidic solutions (pH lower than 6.5) (Chandy and Sharma 1990).

The application of chitosan for encapsulating probiotics has been limited since it has been reported to have a deleterious effect on lactic acid bacteria (Hyndman et al., 1993). Interactions between positively charged chitosan with negatively charged mucosal surface enable chitosan to have strong mucoadhesive properties (Lehr et al., 1992; Kockisch et al., 2003; Sinha et al., 2004). Moreover, chitosan can facilitate the transport of macromolecules across intestinal epithelial cells (Tian et al., 2008c). It has been

reported that chitosan can open the tight junctions and hence facilitate transport of macromolecules across the epithelium. Enhanced delivery of nasal, ocular and oral drugs have been observed after administration of drugs with chitosan (Lubben et al., 2001; Seyfoddin et al., 2016). Therefore, it is possible that chitosan can be used for enhanced oral delivery of vaccines in aquaculture. Chitosan has been used in aquaculture mostly for encapsulating vaccines and genes (Table 2.2).

Chitosan is available in various molecular weights and deacetylation degrees which are important factors in determining physicochemical characteristics of chitosan microcapsules (Sinha et al., 2004). Chitosan is commercially available in three different molecular weights (Mw) including low (50-190 kDa), medium (190-310 kDa) and high (310 kDa) (Sigma). Chitosan with varying molecular weights has shown various degrees of adsorption to mucin with the medium molecular weight variant having the best mucoadhesive properties (Ribeiro et al., 2014). One study indicated that high Mw chitosan formed microparticles with smaller size, higher mucoadhesive properties, lower drug release rates and stronger molecular bonding compared to medium and low Mw chitosan (Honary et al., 2009). Also, encapsulation efficiency is reported to increase when the Mw is increased from low to medium and high (Gan and Wang 2007).

2.2.1.1.3 Cellulose acetate phthalate

Cellulose acetate phthalate (CAP) is another polymer, soluble in pH 6 or higher, which is commonly used as a coating material (Malm et al., 1951). CAP is known as an enteric coating agent, which is physiologically inert (Rao et al., 1989). CAP has been used as a protective shell for probiotics and drugs to treat gastric conditions (Albertini et al., 2010). In aquaculture, CAP can be used to protect embedded materials against the acidic pH of upper sections of animal gut followed by a controlled release in intestine where pH is higher than 6. However, for oral delivery of CAP-coated particles, the basic pH of water used in farms could dissolve the particles. The current lack of CAP application in aquaculture is possibly due to its easy disintegration (before ingestion by aquatic animals) when placed in water/seawater.

2.2.1.1.4 Gellan gum

Gellan gum is an anionic microbial exopolysaccharide produced by *Pseudomonas elodea* (Morris et al., 2012). Two residues of glucose and one residue of rhamnose and glucuronic acid form its repeating structural units. Gellan gum can withstand acidic conditions and high temperatures and therefore can be used as a thermo-resistant agent to protect embedded materials, such as probiotics against heat (Chen et al., 2007). L-glycerol and acetyl are acyl substituents in native gellan gum (Chandrasekaran et al., 1992). Both residues can be removed by alkaline hydrolysis to generate deacetylated- or low acetyl gellan gum (Kang et al., 1982).

The gelation process of gellan gum is temperature dependent and can be performed in the presence of mono-, di- and trivalent cations (Mao et al., 2000; Maiti et al., 2011). Several factors, such as acetyl content, type and concentration of cations, pH and addition of hydrophilic compounds influence gel strength. In natural forms of gellan gum, the acyl groups can prevent interaction between polymer chains thereby resulting in a weak, elastic and thermo-reversible gel. Conversely, deacetylated gellan gum produces a more rigid gel (Mao et al., 2000). Calcium, potassium, sodium and magnesium ions can be used for gelation of gellan gum with divalent cations leading to the formation of a more rigid gel (Huang et al., 2004).

Gellan gum and its composites have been used for encapsulation of bioactive agents, including probiotics, nutrients and enzymes (Table 2.2). Gellan gum can be particularly useful for retaining the encapsulates in seawater due to its high stability in basic pH and also in NaCl concentrations up to 50 g/L (Ashtaputre & Shah 1995). The stability of gellan gum against a range of enzymes, such as pectinase, amylase, cellulase, papain and lipase (Kang et al., 1982), makes it ideal for protecting bioactives against harsh digestive conditions. Therefore, addition of gellan gum to a desired formulation can extend the stability of produced particles in seawater and gastrointestinal tract (GIT) of a target animal.

2.2.1.2 Protein-based materials

A variety of protein-based materials have been employed for encapsulation of bacteria or genes for aquaculture applications (Table 2.2). Gelatine, pea protein, whey protein and dairy proteins are among the protein-based materials that have been used for encapsulation purposes (Dong et al., 2013). Protein-based materials have been used as core (Heidebach et al., 2009a; Heidebach et al., 2009b) and shell (Annan et al., 2008; Gerez et al., 2012) materials to protect bioactive agents against adverse environmental conditions as the buffering capacity of proteins is an effective factor in protecting encapsulates against extreme acidic or basic conditions (Heidebach et al., 2009b). Dairy proteins commonly act as a shell, which has a high solubility and low viscosity in a solution, good emulsion, and film-forming properties (Picot & Lacroix 2004). Denatured whey protein can provide protection against thermal stress (Doherty et al., 2010). Other proteins, such as soy proteins that require heat treatment for gelation might not be useful for encapsulation of thermolabile materials, such as viable cells.

2.2.1.2.1 Gelatine

Gelatine is a widely available by-product of the meat and leather industry that is of nutritional value and is also suitable as an encapsulation material. The use of gelatine in the aquaculture industry as a measure to increase sustainability has been encouraged. In aquaculture, gelatine has been used in combination with alginate to encapsulate probiotics for catfish (Kumaree et al., 2014). Its amphoteric characteristic facilitates interaction with other anionic polysaccharides (King 1995). Gelatine is a cheap, non-toxic and biodegradable encapsulating agent. However, it is thermo-reversible with low stability to heat and insoluble in cold water. The small difference in melting and gelling temperature usually limits its application as a protective agent in encapsulation (Estevinho et al., 2013).

The release from gelatine-based delivery systems is rather quick and therefore cross-linking with agents, such as genipin is required to produce rigid gels. Even with cross-linking, gelatine microparticles have been shown to release their contents quickly when in contact with gastric mediums. To combat this, a second protective coating, such as

alginate is often required. Being negatively charged, (due to proportional and sequential arrangements of D-mannuronic and L-guluronic units along the polymer backbone in aqueous solutions) alginate can form polyion complexation with positively charged gelatine polymers (Annan et al., 2008). Gelatine can also be methacrylated to produce a hydrogel allowing it to be a potentially attractive material which is stable at 37˚C. The mechanical and swelling properties of produced hydrogel can be manipulated by using different gel concentrations and optimising the degree of methacrylation to achieve the desired characteristic for certain application (Nichol et al., 2010).

2.2.1.2.2 Whey protein

Whey protein is a globular and amphiphilic molecule derived from milk that has mostly been used as an emulsifier. The interface between two immiscible phases is the functioning zone for whey protein to undertake a partial unfolding to form hydrophobic bonds (Funtenberger et al., 1995; Damodaran 1996). Whey protein has amphoteric features that can interact with negatively charged polysaccharides under pH condition under its isoelectric point (Gbassi & Vandamme 2012). Whey protein has been used for encapsulation of probiotics, such as Bifidobacterium Bb-12 by heat-induced gelation via spray-drying. A better survival was reported for whey-encapsulated bacteria in simulated gastric conditions (De Castro-Cislaghi et al., 2012). Other similar studies indicate the efficiency of whey protein in protecting probiotics against simulated gastric digestion (Doherty et al., 2011; Doherty et al., 2012). In aquaculture, whey protein has been utilised in combination with pectin to protect probiotics for the delivery to pacu larvae (Rodrigues et al., 2014).

2.2.1.2.3 Viral protein-cages

Viral protein cages refer to virus-like particles (VLP), which can be used as biomaterials for nanoencapsulation. VLPs are composed of a proteinaceous shell that resembles an authentic native virus but without any genetic material (Figure 2-3) (Roldão et al., 2010).These nanocarriers can encapsulate various cargoes, such as protein (Minten et al., 2009), enzymes, genes (Young et al., 2008), imaging tags (Shen et al., 2015) and drugs (Biabanikhankahdani et al., 2016) to create a controlled drug delivery system.

VLPs can be modified chemically and genetically to produce engineered programmed nano-containers having the ability to deliver desired agents to a specific target site. In addition, their disassembly and reassembly in different pHs makes them suitable for creating a pH-responsive programmed release system (Minten et al., 2009). Other advantages of VLPs include nano-size (10 to 2000 nm), structural uniformity for each type of VLP, high resolution three-dimensional structure, easy manipulation of construction and high production yield in various hosts (Lee et al., 2009; Zeltins 2013).

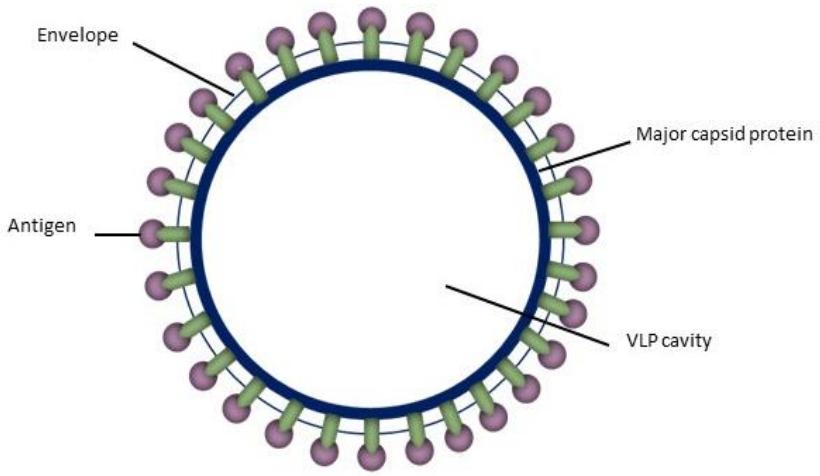


Figure 2-3 Schematic diagram of a recombinant enveloped virus-like particle with heterologous antigens presented on its surface.

2.2.1.3 Poly Lactic-co-Glycolic Acid (PLGA)

PLGA is a widely used material in drug delivery and is favoured due to its long clinical experience, favourable degradation characteristics and possibilities for sustained drug delivery (Allison 2008). Additionally, PLGA contains an asymmetric α-carbon which allows it to have enantiomeric forms. PLGA can be processed into any shape and size and can encapsulate molecules of any size. PLGA absorbs less water and thus, degrades more slowly (Makadia & Siegel 2011). “Drugs formulated in such polymers are released either by diffusion through the polymer barrier, or by erosion of the polymer material,

or by a combination of both diffusion and erosion mechanisms." (Anderson & Shive 2012). PLGA used to deliver plasmid DNA and antigens to fish has been shown to result in an enhanced immune response in treated animals (Table 2.2). However, PLGA should be used cautiously as organic solvents are required during encapsulation processes (Jariyapong et al., 2015).

2.2.2 Methods of encapsulation

With respect to encapsulation for aquaculture applications, emulsion, extrusion, spray drying, and freeze-drying are the most commonly used methods. A variety of factors should be considered when selecting the most suitable method of encapsulation. Retaining the structural stability and biological activity of embedded materials is crucial to achieve efficient encapsulation. For example, the three-dimensional (3D) structure of proteins should be preserved during encapsulation to obtain biologically active proteins after encapsulation. Encapsulation efficiency is also important in order to maximise the entrapment of target materials during the encapsulation process, leading to lesser amounts of required material and final cost. Some techniques, such as spray drying can result in a low encapsulation efficiency as high temperatures may have detrimental effects on the added bioactives. Manufacturing costs and the shelf life of the encapsulation product should also be considered when choosing the best technique for the encapsulation process.

2.2.2.1 Emulsification

Emulsification has been used for encapsulation of probiotics, plasmid DNA and antigens in aquaculture (Table 2.2). A mixture of two immiscible aqueous phases stabilised by an emulsifier can form an emulsion. Based on the nature of the core phase, emulsions can be formed as water-in-oil (W/O) and oil-in-water (O/W) (Figure 2-4). Emulsions are generated based on the interactions between continuous and discontinuous phases. Microparticles can then be produced by adding an emulsifier and a solidifying agent to the emulsion (Chen et al., 2007; Kailasapathy 2009; De Vos et al., 2010). Emulsification has been consistently used to produce particles to retain the viability of bioactives, such as probiotics against harsh environmental conditions (Hou et al., 2003).

In aquaculture, W/O emulsion has been commonly used for encapsulation to protect probiotics and oral vaccines against the acidic condition of the gastrointestinal tract in fish (Tian et al., 2008a; Tian et al., 2008b). Double emulsion is another type of W/O where simple particles are entrapped in a hydrophilic shell to form a water-in-oil-in-water (W/O/W) emulsion. A significant increase in the viability of probiotics was reported after entrapment of *Lactobacillus rhamnosus* in the aqueous core of W/O/W particles that protect against acidic and bile salt conditions (Pimentel-González et al., 2009). Tian et al. (2008 abc) utilised this technique to encapsulate plasmid DNA and successfully delivered vaccine microparticles to Japanese flounder. In addition to simple and scalable production techniques, emulsification allows for a wide variety of particle size ranging from a few micrometre to millimetre (Chávarri et al., 2012) (Table 2.2).

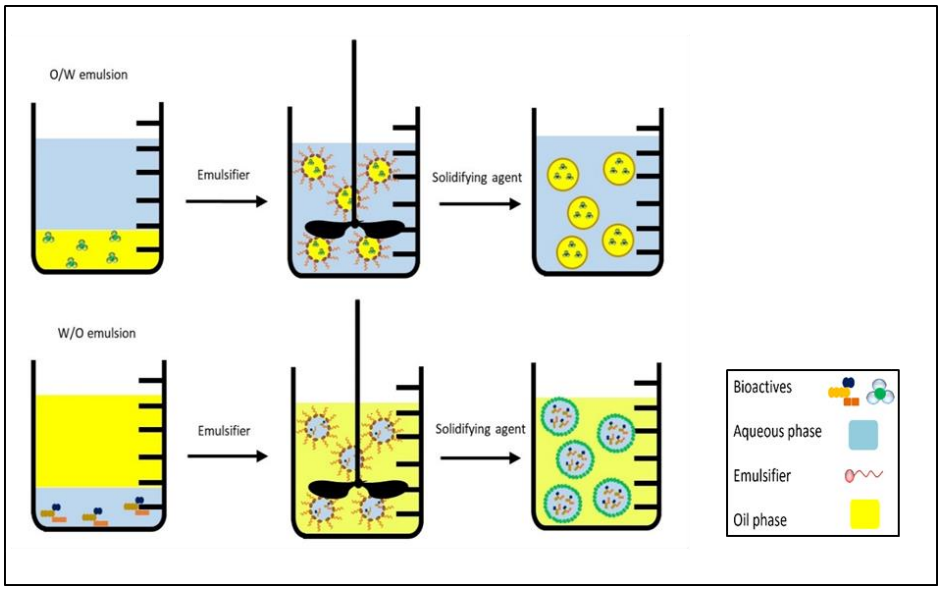


Figure 2-4 Schematic diagram of O/W and W/O emulsion techniques. The aqueous phase and oil phase are mixed, and the produced spheres are stabilised by an emulsifier. Addition of a solidifying agent results in polymerisation of wall or matrix of spheres to form particle.

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
-Alginate - Alginate-bentonite	Extrusion	Enzyme	2.7 - 3 mm 2.9 - 3.1 mm	Oral	Nile tilapia	-Improve protection of the encapsulated enzymes in acidic pH -Successful delivery of enzymes to fish intestine	(Rodriguez et al., 2018)
Alginate-chitosan	Extrusion	Probiotic bacteria (*Bacillus vireti)*		Oral	Freshwater prawn	Increase in immunological parameters and antioxidant activities	(Vidhya Hindu et al., 2018)
Alginate	Extrusion	Bacterial probiotic (*Lactobacillus fermentum*) and prebiotic (Lactulose)		Oral (feed additives)	Rainbow Trout (Oncorhynchus)	-Improve in growth performance -Exclude absorption and accumulation of heavy metals	(Madreseh, Ghaisari, & Hosseinzadeh, 2018)

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
Alginate-skim milk	Extrusion	*Saccharomyces cerevisiae*	1 - 1.5 mm	Oral	Nile tilapia	- High viability of encapsulated yeast in simulated acidic and bile salt conditions - Improve in growth performance of tilapia - Lower mortality after bacterial challenge	(Pinpimai *et al.,* 2015)
- Alginate - Chitosan-coated alginate - Poly-L- lysin coated alginate	Extrusion	Styrene-maleic acid (SMA)	50 - 100 µm	Water rout	Oyster	SMA microbeads delivered in different tissues such as digestive tubules, gill and mantle	(Darmody *et al.,* 2015)

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
Alginate-gelatin	Extrusion	*Lactobacillus plantarum* isolated from catfish	1 - 2 mm	Oral	Catfish	Improvement in viability of probiotic	(Kumaree *et al.,* 2014)
Alginate	Extrusion	*Shewanella putrefaciens Pdp11* isolated from skin of *Sparus aurata*		Oral	Gilthead seabream	Improvement in some of the innate immunity parameters	(Cordero *et al.,* 2015)
1-Alginate 2-Skim-milk alginate	Extrusion	*Lactobacillus rhamnosus GG* from human source	1 - 1.5 mm	Oral	Tilapia	- Higher cell viability in simulated gastric juice and during storage - Enhancement in growth performance of animal	(Pirarat *et al.,* 2015)

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
Alginate coated chitosan	Extrusion	Antigen (Formalin-killed whole cell)	1101 ± 10nm	Oral (feed additives)	Indian major carp	- Protection of the entrapped antigen against acidity of the treated fish GIT - Enhancement of immune response	(Behera & Swain 2014)
Alginate	Extrusion	Formalin-inactivated *Lactococcus. garvieae*	30 µm	Oral	Rainbow trout	Protection to rain bow trout (RPS of %50)	(Romalde *et al.,* 2004)
Alginate	Extrusion	Recombinant protein		Oral (Intubation)	Goldfish	Increase in serum antibody titres	(Maurice *et al.,* 2004)
Alginate	Extrusion	*Shewanella putrefaciens* isolated from Gilthead sea bream	2.4 - 3.2 mm	Oral	Senegalese sole	Enhancement in protecting probiotics in digestive crude extract and during storage	(Rosas-Ledesma *et al.,* 2012)

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
Alginate	Emulsion (W/O)	Plasmid DNA (pDNA)	< 10 µm	Oral (intubation)	Japanese flounder	Increase in antibody production	(Tian *et al.,* 2008a)
Alginate	Emulsion (O/W)	*Enterobacter* sp. isolated from Rainbow trout	22.7 ± 0.1 µm	Oral/Intraperitoneal injection	Rainbow trout	Increase in viability of probiotics Improvement in survival rate of animals after bacterial challenge	(Ghosh *et al.,* 2016)
Alginate	Emulsion	Inactivated *Aeromonas hydrophila*	< 50 µm	In vitro (simulated gastric condition of Nile tilapia)	Nile tilapia	Protection against gastrointestinal condition	(Rodrigues *et al.,* 2006)
PLGA	Emulsion (w/o/w)	pDNA	< 10 µm	Oral (intubation)	Japanese flounder	Increase in antibody level against lymphocystis disease virus (LCDV)	(Tian *et al.,* 2008b)

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
Chitosan	Emulsion (W/O)	Plasmid encoding major capsid protein of LCDV	< 10 µm	Oral (intubation)	Japanese flounder	- RNA of the protein was detected in gills, kidney, spleen and intestine - Higher antibody level against LCDV	(Tian *et al.,* 2008c)
Alginate	Emulsion	pDNA	< 10 µm	Oral (intubation)	- Brown trout - Rainbow trout	- Protection for pDNA - Increase in expression of pDNA resulted in antibody and interferon production - Higher survival of animal after viral challenge	(De las Heras *et al.,* 2010)

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
Alginate	Emulsion	Bacterin	unknown	- Oral - Intramuscular (i.m) - Intraperitoneal (i.p)	Nile tilapia	- No immune response for oral delivery of alginate-bacterin - Significant humoral immune response for i.p and i.m delivery methods	(Leal *et al.,* 2010)
PLGA	Double emulsion	pDNA	200 - 1000 nm	Oral	Rainbow trout	- Nanoparticles absorbed to lower intestinal tract - A slight increase in survival at 6 weeks post-vaccination after a virus challenge	(Adomako *et al.,* 2012)
PLGA	Double-emulsion	Antigen (formalin-killed cells)	36 µm	intraperitoneal injection	- Cyprinid loaches - Common carp	- Higher survival for antigen encapsulates fed animals in both species after bacterial challenge - Longer and better immune responses	(Yun *et al.,* 2017)

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
PLGA	Double emulsion	Antigen	1 µm	Injection	Indian major carp	Enhancement in innate and adaptive immune parameters	(Behera *et al.,* 2010)
Chitosan-cyclodextrin	Ionic gelation	Vitamin C	<300 nm	Oral (intubation)	- Solea larvae - Rotifers	- Improve in protection of encapsulates against basic pH and salinity of seawater - Penetration of nanoparticles into fish intestinal epithelium - Significantly higher ascorbic acid level	(Jiménez-fernández *et al.,* 2014)
Pectin-oil-whey protein	Ionic gelation	*Lactobacillus acidophilus*	225.8 ± 41 263.2 ± 10 µm	Oral	Pacu larvae	Enhancement in growth rate of animal	(Rodrigues *et al.,* 2014)

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
β-1,3-D-glucan	Ionic gelation	Anti-viral siRNA		Injection	Shrimp	- Successful delivery of siRNA in target site (hemocytes) - Improve in antiviral activity - Improve in survivability of RNA encapsulates fed animal	(Zhu & Zhang 2012)
Alginate	Ionic gelation	Formalin killed bacterin		Oral (feed additives)	Rainbow trout	RPS of 53.48% after bacterial challenge	(Altun *et al.,* 2009)
Alginate	Spray drying	Anti-vibrio egg yolk immunoglobulins (IgY)		Oral (feed additives)	Abalone	- Improve in protection of encapsulated IgY against inactivation in GIT of abalone - Higher immunity and survival rate in encapsulated IgY fed abalone	(Wu *et al.,* 2011)
PLGA	Spray-drying	Formalin killed bacterin		Oral (feed additives)	Rainbow trout	RPS of 62.79% after bacterial challenge	(Altun *et al.,* 2009)

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
Chitosan	Coacervation	Plasmid DNA encoding gene of V. anguillarum outer membrane protein (OMP)		Oral (Feed additive)	Asian sea bass	- Expression of OMP in liver, kidney, spleen and intestine - Partial protection against bacterial challenge	(Rajesh Kumar *et al.,* 2008)
Chitosan	Complex-coacervation	DNA		Oral (feed additives)	Fishpond black tilapia	Expression of encapsulated gene in spleen, stomach and gills	(Ramos *et al.,* 2005)
VLPs	Recombinant bacterial expression system	double-stranded RNA	30 ± 3 nm	Injection	Shrimp	- MrNv-VLP encapsulation improved VP28 dsRNA efficiency against white spot syndrome (WSSV) - Higher survival rate of 16.7% in shrimp	(Jariyapong *et al.,* 2015)

Table 2.2 Encapsulation technologies used in aquaculture.

Encapsulation material	Encapsulation technique	Encapsulated materials	Diameter of particles	Delivering method	Animal	Effect of encapsulation on viability of probiotic/animals' growth and/or immunity in treated compared to control groups	Reference
- Lecithin - α-tocopherol	High-pressure homogenisation	Cinnamaldehyde		Immersion	Zebrafish	- Improve in the survival rate of treated animals during bacterial challenge - Several immune factors such interleukins, tumour necrosis factor and interferon found in higher level	(Ning *et al.,* 2014)
Alginate		*Vibrio anguillarum* bacterin	5 µm	Oral (mixed with food)	- Carp - Rainbow trout	- Higher protection of antigens - Antibody production	(Joosten *et al.,* 1997)

2.2.2.2 Extrusion

Extrusion is another method of encapsulation in which a mixture of encapsulated material is pressed through an extruder, such as a syringe. The generated droplets are then dropped into a gelling solution whilst stirring to form microbeads. This technique has been used to encapsulate bacterial cells, vaccines and antigens in aquaculture (Table 2.2). Particles as small as a few micrometre can be produced using extrusion methods (Romalde et al., 2004). The nature of the gelling solution and the distance between the extruder nozzle and the gelling solution determine the shape and size of produced beads (Gbassi & Vandamme 2012). In extrusion methods, where gravity is the main force of dripping, weight and surface tension of droplet and nozzle diameter are determining factors for droplets size. However, the size of generated beads is often large, and the process is long and not efficient enough at the industrial level (Chávarri et al., 2012). In order to have more control over the size and formation of microbeads, some modifications, such as pulsation or vibration of extruder nozzle and use of electrostatic fields have been implemented with some success (Kailasapathy, 2002).

2.2.2.3 Spray drying

Spray drying is a drying technique that is commonly used for encapsulation. A suspension of active and encapsulating agents is atomised in a vessel followed by an evaporation step with hot air flow to remove the solvent (Figure 2-5). The applied suspension is retrieved in the powder form containing a protective layer around the active agent. Spray drying is economical and can protect various materials efficiently. However, spray drying is generally not suitable for viable agents due to the hot drying process (Burgain et al., 2011). Therefore, the viability of encapsulated cells using this method depends on the cell species, temperature, duration of drying, and characteristics of shell material (Chávarri et al., 2012). Some studies have reported the efficiency of spray drying for use in probiotic encapsulation (Kitamura et al., 2009; Riveros et al., 2009). Prebiotics, such as aguamiel and inulin (Rodríguez-Huezo et al., 2007; Fritzen-Freire et al., 2012) and thermal-resistant materials, such as gellan gum

(Chen et al., 2007; Anal & Singh 2007) can be used to improve the viability of encapsulated cells during the drying process.

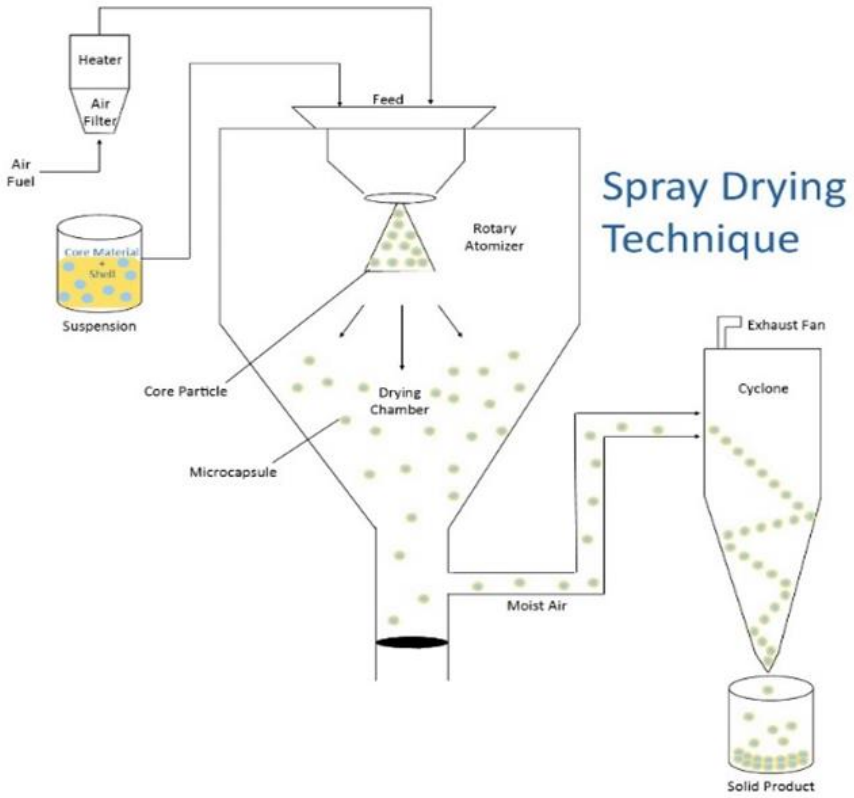


Figure 2-5 Schematic diagram of a typical spray drying technique.

2.2.2.4 Freeze drying

Freeze drying is a drying technique that includes two main steps of freezing and sublimation. The material is rapidly frozen followed by reducing pressure until the frozen water in the sample directly transits from a solid to a gaseous state (

Figure 2-6). The addition of carrier materials and cryoprotectants is important especially in case of probiotic samples in order to maintain their viability and stability during processing and storage. Vacuum drying is a technique similar to freeze drying, which

requires a higher temperature and is more economical and less damaging for probiotics (Chávarri et al., 2012).

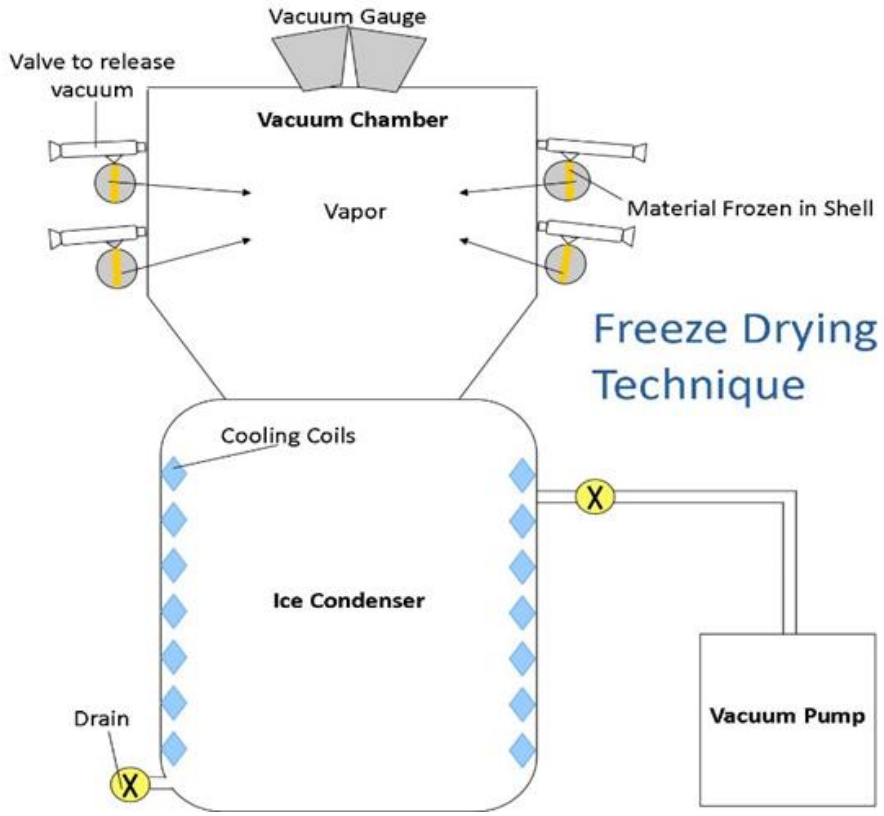


Figure 2-6 Schematic diagram of a typical freeze drying technique.

2.2.2.5 Genetic engineering

Protein recombinant technology can be used to produce heterogeneous and homogeneous viral cages. VLPs can act as a protein shell enclosing bioactive agents for delivery of active agents. Cloning of a desired gene expressing VLPs structural protein(s) is the main step for recombinant viral protein production. The cloned genes are then expressed in a suitable expression system, such as bacterial, yeast, insect, plant and mammalian system. The produced recombinant proteins have the ability to self-assemble. However, it is worth mentioning that not all viral proteins are able to form

VLPs. Readers are directed to a thorough review published on VLPs and their construction and characterisation by Zeltins (2013).

2.2.3 Applications of encapsulation technology in aquaculture - case studies

2.2.3.1 Encapsulation of probiotics

The use of probiotics is one of the strategies that FAO has approved for aquaculture (Subasinghe et al., 2003). Probiotics are live bacteria and yeast cells that can confer health benefits to a host when they are ingested in adequate amounts (World Health Organization, 2001). Probiotics used in aquaculture are isolated from the gastrointestinal tract, skin and mucous of aquatic animals or their environments. However, a human derived probiotic has also shown to be suitable for fish (Pirarat et al., 2015). Many studies have reported that probiotics effectively increase the growth rate and immunity and improve the general health and survival rate of aquatic species (Irianto & Austin 2002; Hadi et al., 2014; Hai 2015; Talukder Shefat, 2018; Xia et al., 2018). Therefore, probiotics provide an alternative treatment that can replace or minimise the use of antibiotics and chemotherapeutics in aquaculture (Rekiel et al., 2007).

The delivery method of probiotics in aquaculture is a key factor in order to prevent the contamination of the aquatic environment with high concentrations of bacteria and to avoid producing negative effects on other aquatic animals. Probiotics have conventionally been administered in the form of feed additives or released into water (Moriarty 1998; Skjermo & Vadstein 1999). However, the direct use of probiotics is not desirable due to the possibility of contaminating the aquatic environment and reduced viability of probiotics. Furthermore, it may lead to uptake of probiotics in insufficient dosages. A large number of probiotics are sensitive to the harsh processing and gastrointestinal conditions, such as low pH, salinity, temperature or oxidative stress (Mattila-Sandholm et al., 2002). Therefore, the preparation and delivery of probiotics should be designed cautiously in order to maintain the viability of probiotics during the process of preparation, storage and consumption (Chávarri et al., 2012). This makes the development of advanced techniques to retain probiotic viability an important and

urgent need. To this end, several strategies have been introduced to increase probiotic viability, such as selecting tolerant probiotic strains, adapting probiotics to stressful conditions, using protective materials and employing encapsulation methods (Sarkar 2010). Among these, the encapsulation technique is considered one of the most promising techniques for preserving probiotics against detrimental environmental conditions (Dong et al., 2013).

Although the use and effectiveness of probiotics in aquaculture is widely accepted, little research has been carried out on encapsulating probiotics for aquaculture application (Table 2.2). Alginate is the most common polymer that has been researched for immobilising and encapsulating probiotics. This natural polymer is well-known for its simplicity, biocompatibility and low cost (Krasaekoopt et al., 2003). Alginate can protect probiotics from the acidic pH of an animal's gut until they are released into the intestine (Morinigo et al., 2008). Up to 95% improvement in probiotic survival rate has been reported after encapsulating probiotics with alginate (Mandal et al., 2006). Alginate has also been used to enhance the shelf life of encapsulated probiotics. Rosas-Ledesma et al., (2012) produced alginate-encapsulated probiotics for oral delivery to fish. The stability of probiotic was maintained above 90% for one month at 4°C (Rosas-Ledesma et al., 2012). Calcium ions are commonly used to produce alginate hydrogels (Krasaekoopt et al., 2003). However, it must be noted that calcium can affect the viability of probiotics in a concentration dependent manner (Rosas-Ledesma et al., 2012). In order to enhance the protection efficiency of alginate encapsulates, other additives, such as skim milk have been used. A combination of alginate-skim milk has been shown to improve the viability of *Saccharomyces cerevisiae* after freeze-drying and incubation at room temperature (Pinpimai et al., 2015) and resulted in an improvement in the shelf life of the encapsulates. Skim milk can act as a cryoprotectant and can maintain the viability of bacteria during lyophilisation through stabilising the cell membrane (Castro et al., 1995; Li et al., 2011).

An improvement in growth performance and protection against bacterial challenge was observed in tilapia fed with both alginate and skim milk-alginate capsules (Pirarat et al.,

2015). However, Skim milk-alginate capsules were reported to provide a significantly better protection after freeze drying and storage at room temperature compared to alginate alone (Pinpimai et al., 2015). Kumaree et al. (2014) encapsulated a probiotic species isolated from the gut of catfish using a combination of alginate and gelatine. Viability of encapsulated probiotics remained significantly high after 90 min incubation in acidic condition, whereas free cells lost their viability within 30 min incubation in acidic condition (pH:2). The survival of encapsulated probiotics was reported to be significantly higher than free cells when incubated at 50°C for 60 min. Moreover, a complete deactivation of free cells was seen after a thermal challenge at 50°C for 60 min (Kumaree et al., 2015). A mixture of alginate, calcium chloride and canola oil was utilised to produce microencapsulated *Enterobacter* sp. via a W/O emulsion technique. A bacterial challenge test performed on rainbow trout fed with encapsulates showed that the percentage of cumulative mortality was lower in fish fed with microencapsulated probiotic than blank-oral (empty microcapsules) and control (untreated) animals after 28 days (Ghosh et al., 2016). The mode of action of alginate-encapsulated probiotics in gilthead sea bream was investigated by Cordero et al. (2015). They found an increase in some of the innate immunity parameters, such as serum peroxidase activity during a four-week time period and serum immunoglobulin M level within the second week in gilthead sea bream fed with alginate-encapsulated probiotics.

Selecting efficient biomaterials that protect bacteria against harsh conditions during the preparation and ensure delivery and release into target sites is a critical step for encapsulation of probiotics. All materials selected for encapsulation of probiotics should be non-toxic and biocompatible (Gbassi & Vandamme 2012). Extensive research is required before selecting biomaterials for encapsulation due to differences in probiotics' characteristics and conditions involved in their delivery and release into target sites within the animals' body. The stability of probiotics in acidic and basic pH and the ability to digest certain biopolymers are important factors to consider. Material selection also depends on the physiology of the animals' gut tract. Formulation of probiotics can be a challenging task, as a successful probiotic delivery system needs to

remain intact in sea water and in the animal's gut after ingestion before releasing its content into the intestine (Ai et al., 2011).

2.2.3.2 Encapsulation of vaccines

Vaccines are an effective way to prevent diseases in high-density aquatic cultures, and have been delivered through different methods including injection, immersion, ultrasound and oral vaccination. Effective and long-term health protection is possible using these techniques. However, there are several constraints of using vaccines, which include high cost, difficulty and the need for extensive animal handling (Mitchell, 1995; Frenkel et al., 2000; Rocha & Coll 2001; Zhou et al., 2002; Navot et al., 2004; Plant & LaPatra, 2011).

Oral vaccination is the most practical method for large scale vaccination in aquaculture. With oral vaccines, the main concern is to maintain the stability and activity of vaccines during transition through an animal's gut tract until final delivery to the absorption site (Quentel & Vigneulle 1996). In order to improve oral delivery of vaccines, encapsulation of antigens with natural or biocompatible polymers can be a promising approach.

Alginate microcapsules have been produced for oral vaccination of fish species. Alginate is an excellent encapsulation polymer for vaccination since it also has the ability to induce immune response in fish (Fujiki et al., 1994). Joosten et al. (1997) were among the first to show the use of alginate microparticles for oral delivery of vaccines to aquatic animals (Joosten et al., 1997). Antigens were detected in enterocytes, which implied the uptake of encapsulated antigens in the posterior section of digestive tract in fish. The release of the antigen from the microparticles was thought to be due to the swelling of alginate microcapsules in alkaline pH (Joosten et al., 1997). A relative percent survival (RPS) of about 50% was reported in rainbow trout after oral administration of an alginate-encapsulated vaccine (Romalde et al., 2004). The protein-alginate formula has also been fed to gold fish, and high antibody titres were achieved (Maurice et al., 2004). Rodrigues et al. (2006) produced alginate microparticles smaller than 50 µm containing inactivated bacteria for oral fish vaccination that provided protection against simulated

conditions in the digestive tract of Nile tilapia. Alginate microspheres also have been used for encapsulating plasmid DNA (pDNA) for protection against aggressive gastrointestinal conditions in trout and was detected in kidney, spleen, liver and pyloric caeca. Moreover, vaccinated animals showed a high survival rate after a viral challenge (De las Heras et al., 2010).

Chitosan is another polymer that has been used for vaccination. This mucoadhesive natural polymer facilitates transport of macromolecules across the epithelial cells (Van der Lubben et al., 2001; Ouji et al., 2002). Chitosan has been used to encapsulate DNA-based vaccines for aquaculture applications. In study on Japanese flounder, it was reported that the concentration of antibodies was elevated within four weeks after oral administration of chitosan-loaded vaccines (Tian et al., 2008c).

PLGA is another polymer that has been used for delivery of inactivated bacterin (Altun et al., 2009) and macromolecules, such as peptides (Partidos et al., 1997), nucleic acids (Sourabhan et al., 2009) and proteins (O'donnell et al., 1996). PLGA may not be the best option for live cells due to the necessity to use organic solvents for dissolving it. Behera et al. (2010) showed the potential of PLGA for encapsulating antigens and the boosting effect of PLGA-encapsulated antigen on innate and adaptive immunity of Indian carp. The intestinal absorption of PLGA nanoparticles was studied by Adomako et al. (2012). The nanoparticles were coated on to fish feed and were orally administered and subsequently detected in epithelial cells in lower intestine of trout. Maximum DNA release from PLGA nanoparticles was achieved within 7 days at pH 8.4, whereas a more prolonged liberation with lower amount of DNA was obtained at pH 4 in vitro.

Altun et al. (2009) compared the efficiency of PLGA and alginate as an oral immunising procedure to deliver bacterin to rainbow trout. Both alginate and PLGA could efficiently protect rainbow trout against bacterial challenge with no significant differences. However, alginate is a more cost-effective polymer to be used in large scale production (Altun et al., 2009). Tian et al. (2008 abc) utilised three different polymers including alginate, chitosan and PLGA to encapsulate a plasmid DNA for oral vaccine delivery to

Japanese flounder. pDNA from all three encapsulates was expressed and induced an immune response by producing antibodies in Japanese flounder although there was a difference in time frame of antibody production.

VLPs can be used as a novel approach for vaccination in aquaculture. The ability of *Macrobrachium rosenbergii* nodavirus-like particles (MrNv-VLPs) to deliver therapeutic agents in shrimp has been demonstrated (Jariyapong et al., 2015). The produced VLP was able to penetrate several tissues in shrimp and increased the survival rate against pathogenic infection by 16.7% compared to non-encapsulated therapeutic agent. Self-assembly, accessibility to various shrimp tissues and immune stimulant features of the developed VLPs has rendered them an efficient drug delivery system in shrimp (Jariyapong et al., 2015). Other studies have also produced recombinant MrNv-VLPs in prokaryotic (Goh et al., 2011) and eukaryotic (Kueh et al., 2017) systems and proved the capability of VLPs in packaging both RNA and DNA molecules. Moreover, high pH (2 to 12) and thermal (up to 45˚C) stability were reported for recombinant MrNv-VLPs produced in a eukaryotic system (Kueh et al., 2017). Other recombinant VLPs structures have been produced in eukaryotic systems (Lin et al., 2001) but require more studies for further application as a nano-carrier of therapeutic agents in aquaculture. Although accurately programmed and controlled release bioactive carriers can be achieved using genetically modified VLPs, application of VLPs as nanocarriers is a new domain in aquaculture and more investigations are required to with regards to environmental issues and the economy of large-scale production.

2.2.3.3 Encapsulation of nutrients and immunostimulants

Enrichment of feed with nutrients and immunostimulants has been widely used in aquaculture as a protection strategy against various stress factors and diseases (Ringø et al., 2012). A wide variety of compounds such as β-glucan, alginate, se-chitosan, vitamins and minerals can induce an immune response in aquatic animals (Volman et al., 2008; Victor et al., 2019). β-glucan is the most commonly used immunostimulants in aquaculture and is composed of glucose residues. It can be extracted from yeast, fungi and some bacterial sources (Volman et al., 2008). The immunomodulating activity of β-

glucan has been reported by various studies after in vitro and in vivo investigation in humans and animals (Volman et al., 2008; Ringø et al., 2012). Macrograd® is a β-glucan-based feed supplement that boosts immunity and improves the growth rate and feed conversion in aquatic animals (Supamattaya & Pongmaneerat 2000; Sealey et al., 2008; Soltanian et al., 2009). Nucleotide supplemented feed can improve growth rate, modulate immunity and enhance resistance against stress and pathogen insults (Sakai et al., 2001; Li et al., 2007). The use of *S. cerevisiae* as a feed additive to aquatic animals has been shown to improve growth rate, innate and adaptive immune responses by providing various nutrients, such as β-glucan, nucleotides and oligosaccharides (Oliva-Teles, 2001; Ortuño et al., 2002; Cuesta & Meseguer, 2004).

Immunostimulants have been delivered to aquatic animals either orally as feed supplements (Pal et al., 2007) or by injection (Misra et al., 2006). Oral delivery of immunostimulants is a more efficient method as it requires less time and labour and does not cause stress to aquatic animals. However, oral delivery does require a high concentration of materials to be delivered in large-scale farming. Furthermore, chemicals, nutrients and immunostimulants may lose their activity during storage or in environmental conditions before and after being consumed by animals. Encapsulation of nutrients and immunostimulants would offer advantages over direct delivery via feed or water especially for larvae culture as the level of capture and ingestion of nutrients by larvae depends on the physical characteristics of diet, such as size, shape and colour (Kvåle et al., 2006). Encapsulation technology can be used to design a larvae's specific diet to enhance their growth and survival. Alginate could be a suitable carrier for this purpose due to its immune stimulatory properties. Alginate and alginate with high content of mannuronic acid (High-M alginate) have been used to induce immunity in fish larvae (Skjermo et al., 1995; Vadstein 1997b) when administered in the form of feed additives and bio-encapsulated in artemia. In one study, alginate microparticles ranging from 2-30 µm in size delivered to fish after being bio-encapsulated in artemia resulted in 38% reduction in mortality in Halibut larvae (Skjermo & Bergh 2004). Bio-

encapsulating in live feed like artemia and rotifers does, however, impose an extra cost when considered for industrial use.

The distribution of microparticles in various tissues can be affected by the type of coating polymer. Darmody et al., (2015) compared the effect of alginate coating on styrene-maleic acid (SMA) microbeads. They found that alginate capsules, regardless of coating material (chitosan and Poly-L-lysine), could be taken up by oysters. The microbeads encapsulated with chitosan-alginate were mostly observed in the gill tissues and poly-L-lysine capsules-alginate encapsulated beads were mostly found in digestive gland and mantle tissue (Darmody et al., 2015).

2.3 Bioactives delivery in aquaculture: practical considerations

The proper selection of formulation excipients is a crucial step for ensuring the protection and controlled release of embedded bioactives into a target site. For example, alginate can provide protection against the acidic pH of the gut. The solubility profile of polymers is another important formulation factor. For example, gellan gum only dissolves at high temperatures and therefore may not be suitable for probiotic formulations. The required release profile and dosage of embedded materials are other important determining factors in bio-encapsulation. Understanding the pH conditions in the digestive tract of the encapsulate-receiving host is important as the shell materials of the encapsulates should remain intact after being consumed by animals. Ideally, formulation of encapsulates should be optimised based on the animal gastrointestinal tract conditions, including pH, length, transport time and uptake capacity (McLean & Donaldson 1990; Joosten et al., 1997).

The method of encapsulation can also directly affect the activity and survival of encapsulated materials and the most suitable technique should be determined based on the type of material to be encapsulated, target size of particles and production scale. In the case of probiotics, the encapsulating technique should not exert deleterious effects on cells survival and activity. Drying procedures involved in the formulation of capsules, such as freeze drying, can reduce cell survival and affect capsule performance

in the gastrointestinal tract (Lankaputhra & Shah 1995). Nevertheless, dried products are preferred for commercial and industrial use due to ease of transport and a prolonged shelf life. The addition of protective substances, such as skim milk before freeze drying should be considered in order to increase cell protection (Rokka & Rantamäki 2010). Although, from an industrial point of view, spray drying is more cost effective than freeze drying (Chávez & Ledeboer 2007; Petrović et al., 2007) the high temperatures used can reduce viability of cells (Teixeira et al., 1997). Several approaches, such as the use of cryoprotectants, growth promoting factors and hydrocolloids can minimise the effect of spray drying on cell survival (Conrad et al., 2000; Crittenden et al., 2001; Desmond et al., 2002; Picot & Lacroix 2003).

Extrusion and emulsification are the most commonly used techniques for encapsulating probiotics, vaccines and bioactive agents in aquaculture. Both methods involve simple procedures and usually do not cause deleterious effects on bioactives (Krasaekoopt et al., 2003; Burgain et al., 2011). A probiotic survival rate of about 80-95% has been reported for both techniques (Krasaekoopt et al., 2003). Emulsions are particularly suitable for industrial applications. However, continuous stirring and use of surfactants during preparation could potentially have unfavourable effects on encapsulated probiotics (Burgain et al., 2011; Gbassi & Vandamme 2012).

Regardless of the encapsulation technique and material used, the size of particles plays a key role in uptake by aquatic animals (Ouji et al., 2002; Bhavsar & Amiji 2007). Particles with smaller size have been reported to be assimilated more easily (Dang & Leong 2006). However, a wide range of particle size from less than 10 µm (Joosten et al., 1997; Tian et al., 2008a; Li et al., 2011) to a few millimetre (Rosas-Ledesma et al., 2012; Pinpimai et al., 2015; Pirarat et al., 2015) have been used successfully to deliver plasmid DNA, bacterin and probiotics to aquatic species. Research by Winkel and Davids (2006) has shown that the particle size selection by the mantle cavity and stomach was within the range of 15 - 50 µm with smaller and bigger sized particles being rejected and excreted in zebra mussel pseudo-faeces. However, this selection mechanism did not occur in starved animals. With regards to live encapsulates, other factors including shape,

density and chemical clues, such as extracellular metabolites are important factors in particle selection (Pales Espinosa et al., 2007; Gosling 2008). Particle size can also affect the viability and survival of encapsulated probiotics, with larger particles providing better protection (Hansen et al., 2002; Mandal et al., 2006). Furthermore, the coating material of the particle can influence particle selection due to the possibility of interaction between the particle surface and mucus (Emmanuelle et al., 2009).

2.4 Conclusion

In conclusion, encapsulation technology is a promising approach for the development of sustainable aquaculture strategies. This technology offers several advantages over current aquaculture practices including (1) precise delivery of nutrients, vaccines, immunostimulants and probiotics to fish, crustacean and molluscs, (2) better control over water quality, (3) environment-friendliness, (4) reduction in concentration of materials resulting from less material loss in water and a more cost-effective aqua-farming process, (5) controlled release of encapsulated compounds into target sites within an animal and (6) protection of embedded materials during preparation, storage and delivery. Despite the benefits associated with this technology at the laboratory scale, it is yet to be up scaled in the aquaculture industry. Future research and development should focus on:

- development of control experiments to measure the real benefits of encapsulation in aquaculture,
- development of techniques for tracking microcapsules in the animal's body through direct/indirect measurements,
- evaluation of the impact of implementing encapsulation on farm productivity and management,
- evaluation of cost benefits to aquaculture industry in long term, and
- development of effective strategies to implement the research outcomes in aquaculture farms.

Chapter 3

Characterisation of probiotics isolated from *Haliotis iris*

"We cannot fathom the marvelous complexity of an organic being; but on the hypothesis here advanced this complexity is much increased. Each living creature must be looked at as a microcosm--a little universe, formed of a host of self-propagating organisms, inconceivably minute and as numerous as the stars in heaven". Charles Darwin

Abstract

The high market demand for abalone has encouraged farmers to grow abalone to the full potential of land-based farms. However, the long grow-out period of abalone is a limiting factor on maintaining an economically sustainable production annually. The use of beneficial bacteria can improve abalone growth rate, health status and sustain environmentally friendly aquaculture practices. Three bacterial strains were previously isolated in our lab from healthy abalone demonstrated beneficial effects on growth performance of black-footed abalone. However, at the time of starting this thesis, a significant concern existed over the presence of beneficial characteristics in the isolated bacteria after a long-chilled storage at -80˚C. In this study, an investigation was carried out to understand the bacterial qualities that may have been retained or lost during chilled storage. The ability of *Exiguobacterium* sp., *Vibrio* sp., and *Enterococcus* sp. to hydrolyse some of the abalone feed nutrients (such as protein, starch and alginate), produce organic acid and grow in a wide range of pHs was examined. Identification of the isolated bacteria was carried out by sequencing 16s rRNA amplicon. This information can determine whether the bacterial isolates possess desired characteristics as required in potential probiotics for pāua. Screening of the three bacterial species confirmed the presence of beneficial traits which potentially can enhance the digestibility of current feed used in the farm. Such properties include protein, starch and alginate hydrolysis. The three probiotic species showed some level of sensitivity to different pH conditions that they may encounter during delivery to abalone. In summary, this chapter confirms that the three bacterial species retained the desired properties even after a prolonged chilled storage.

3.1 Introduction

A growing global demand for seafood has resulted in depletion of fisheries and raised the need for high density farming. Intensified aquaculture can increase the risk of disease outbreaks within the farms. Improper use and overexploitation of various chemical additives and antibiotics has led to the emergence of antibacterial resistant pathogens (Bachere, 2000). This process has a substantial impacts on environment and public health (Romero, Feijoó, & Navarrete, 2012).

The advent of non-antibiotic and green strategies is of value for health maintenance in aquaculture (Qi et al., 2009). For example, addition of nutrients and immunostimulants to promote health and welfare of aquatic animals. Oligosaccharides, vitamins and minerals are compounds that have been used as immunostimulants in aquaculture (Ringø et al., 2012; Widanarni et al., 2018; Nedaei et al., 2019). Recently, probiotics have been introduced to aquaculture industry as an alternative for antibiotics and chemical agents. Probiotics are defined as live micro-organisms that confirm a health benefit to the host when administered in adequate amounts (FAO/WHO, 2001). Probiotics have been widely used to improve growth and health of aquatic animals (Hai, 2015; Talukder Shefat, 2018; Xia et al., 2018). For example, *Bacillus pumilus* (Aly et al., 2008) and *Lactobacillus rhamnosus* (Pirarat et al., 2015) reportedly improved health and growth of Nile tilapia. Other studies reported improved survival and immunity after administration of *Bacillus* sp. to shrimp (*Litopenaeus vannamei*)(Rengpipat et al., 2000; Li, Tan, & Mai, 2009).

Different mechanisms have been introduced as mode of action for probiotics. The first pathway is excluding pathogens by means of competing for receptors within intestinal mucus. In other words, probiotics can reside and colonise within the host GIT where no adherence sites will be available for pathogens (Verschuer et al., 2000; Balcázar et al., 2007). Some probiotics possess antagonistic activity against pathogenic bacteria. They do this by producing inhibitory substances such as sulphur containing compounds or acidic molecules (Bruhn, 2007). Some of the secreted molecules can act as immunostimulants inducing cellular and humoral immune defence (Sahu et al., 2008).

Assisting food digestion is another mechanism that can be achieved by the probiotics' enzymes (Krishnaprakash et al., 2009). In addition, improvement of water quality was reported for some probiotics used in aquaculture (Dalmin, Kathiresan, & Purushothaman, 2001; Wang, Xu, & Xia, 2005; dong Li, Tian, & Dong, 2019). Probiotics for aquaculture have been isolated from various sources such as gastrointestinal tract (GIT) of marine animals (Leyva-Madrigal et al, 2011; Luis-Villasenor et al., 2011; Ramesh et al., 2015) and fish mucus (Tapia-Paniagua et al., 2012). Water, sediments and other aquatic resources have been also identified as potential sources for isolating useful bacteria (Hai, Fotedar, & Buller, 2007; Del'duca et al., 2013).

Several endogenous cells including different species of bacteria and yeasts have been isolated as natural microflora in the GIT of *Haliotis midae* (Erasmus, 1996; Macey & Coyne, 2005; Goosen, 2007). It has been suggested that these bacteria can help in degradation of agar, alginate, carrageenan and laminarin in abalone's feed by secreting extracellular enzymes (Erasmus, Cook, & Coyne, 1997). More than 70% of enzymatic activity in *H. midae* is attributed to extracellular enzymes which are released by endogenous bacteria within the animal GIT (Doeschate & Coyne, 2008). The most beneficial bacterium detected in three species of Japanese abalone (*H. discus, H. diversicoloraquatilis, H. diversicolordiversicolor*) and one species of African abalone (*H. Midae*) was recognised to be *Vibrio halioticoli* which contributes to feed digestion (Tanaka et al., 2003). This non-motile *Vibrio* species secretes polysaccharolytic enzymes that can improve digestion within abalone's gut (El-Shanshoury et al., 1994). Another species of *vibrio* namely "*Vibrio midae''* was reported to act as a probiotic for *H. midae* (Macey & Coyne, 2005; Doeschate & Coyne, 2008). Probiotics used as feed supplements for *H. midae* led to improved growth rates (Doeschate & Coyne, 2008). In another study *Vibrio midae* and two yeast species, which were reported to have proteolytic and amylolytic activities, increased the level of digestibility of molecules such as proteins and amylose. After eight months of feeding trial, the enhanced enzymatic activity resulted in an increased growth rate by 8% and 34% in small and large abalone respectively (Macey & Coyne, 2005). *Pseudoalteromonas* C4 was another bacterium that was used

as feed supplements for *H. midae*. This probiotic was able to enhance the growth rate of *H. midae* significantly in laboratory and farm feeding trial. The 163-day feeding trial resulted in 38% and 39% improvement in shell length and animals' weight, respectively. The ability of *Pseudoalteromonas* C4 for digesting alginate was reported as a possible reason for these significant improvements (Doeschate & Coyne, 2008).

In our lab, Hadi et al. (2014) used three different bacterial species namely *Exiguobacterium* sp.*, Vibrio* sp. and *Enterococcus* sp. as probiotics for *Haliotis iris* (black-footed abalone). The three bacterial species were isolated from the gut of healthy and fast-growing adult abalone. The probiotic-supplemented feed improved the growth rates by 20.9% and 19.8% in shell length and wet weight gain, respectively. Moreover, reduced mortality was reported in probiotic-fed animals by five folds compared to a control group (Hadi et al., 2014). These bacterial species were stored at -80˚C and sub-cultured multiple times for a few years after being isolated from animal source. There was a need to confirm the quality of these probiotic bacteria since their activity could have been affected by the long-term storage at -80˚C. Therefore, this study attempted to characterise the three bacterial species using quantitative and qualitative microbial tests in order to re-confirm their effectiveness. The specific objectives of this chapter are:

- to check the ability of the bacterial isolates to hydrolyse protein,
- to investigate the amylolytic activity,
- to investigate the alginolytic activity,
- to test the bacterial resistance in a wide range of pHs (2-8), and
- to identify the isolated bacteria by 16s rRNA gene sequencing.

3.2 Materials and methods

3.2.1 Materials

Bacterial isolates were kindly provided by Aquaculture and Biotechnology Research Group at Auckland University of Technology (Auckland, New Zealand). The strains were maintained in Marine broth supplemented with 15% v/v glycerol stored in -80˚C. Marine broth and agar were obtained from Difco, France. Kanamycin Esculin Azide Agar and kanamycin supplement were purchased from Oxide Ltd. UK. GasPak EZ anaerobe container system was used to provide anaerobic condition when required (Becton Dickinson, USA). All the other materials were of analytical grade. The cultures were subcultured weekly and maintained on Marine agar supplemented with 1% w/v yeast extract at 4˚C.

3.2.2 Probiotics culture

All the three probiotic bacteria namely *Exiguobacterium* sp.*, Vibrio* sp.*,* and *Enterococcus* sp. were revived from glycerol stocks and sub-cultured at least three times before performing experiments. The multiple passaging was done to allow the revived bacteria regain their full strength and activities. *Exiguobacterium* sp. and *Vibrio* sp. were cultured in marine broth containing yeast extract (1% w/v). The cultures were incubated at room temperature for 18 hrs under agitation at 100 rpm. *Enterococcus* sp. was cultured in the same media for 18 hrs under anaerobic condition at room temperature. A selective media, Oxide Kanamycin Esculin Azide Agar (KAA), was used to selectively culture *Enterococcus* sp. when required.

3.2.3 Assessment of probiotics' ability to digest nutrients

3.2.3.1 Protein hydrolysis

Casein agar plates were prepared by mixing sodium caseinate (0.2% w/v), glucose (0.1% w/v), technical agar (1.5% w/v), magnesium sulphate (0.02% w/v), ferrous sulphate (0.001% w/v), di-potassium phosphate (0.02% w/v) and deionised water (Laboratory manual by Gutierrez-Maddox 2002; Hadi, 2012). The well-mixed suspension was sterilised by autoclaving at 121˚C for 15 min. The casein plates were then prepared in

aseptic conditions. Probiotics were cultured on casein agar plates (0.2% w/v) and incubated for 48 hrs at room temperature. The experiment was performed in triplicates for each probiotic species. The cultured casein plates were investigated for casein hydrolysis after the incubation time. The presence of a clear zone around colonies will indicate casein hydrolysis. In order to facilitate observation of a clear zone around the growth area, a low concentration of HCl (1% v/v) was added to the plate so that non-degraded casein precipitates inside the plates.

3.2.3.2 Starch hydrolysis

In order to understand the ability of probiotics to use starch as a carbon and energy source, starch plates were prepared following laboratory manual by Gutierrez-Maddox (2002). Starch agar solution containing starch powder (1% w/v), nutrient agar and NaCl (1.9% w/v) was prepared and subsequently autoclaved at 121˚C for 15 min. The suspension was collected and poured into petri dishes under aseptic conditions. Probiotics were spread on plates and incubated for 48 hrs at room temperature until the colonies were developed. Iodine solution was added to flood the surface of each plate. The experiment was performed in triplicates for each probiotic species.

3.2.3.3 Alginate hydrolysis

Probiotic species were inoculated in sodium alginate broth (10 ml, sodium alginate 0.1% w/v, peptone 0.5% w/v, yeast extract 0.1% w/v and NaCl 3.0% w/v, (pH:7.6)). Three replicates were assigned for each bacterium. Tubes were incubated at room temperature for *Exiguobacterium* sp. and *Vibrio* sp. *Enterococcus* sp. was incubated anaerobically. The level of alginate hydrolysis was tested every 24 hrs using a spectrophotometric method (Kitamikado et al., 1990; Hadi, 2012). After incubation time, 1 ml of each bacterial culture was withdrawn and centrifuged (4000 rpm, 15 min) to remove bacterial cells. The supernatant was separated and mixed with acidic bovine albumin solution (Bovin albumin (0.1% w/v), sodium acetate (0.32 % w/v), glacial acetic acid (0.45% v/v), pH: 3.72-3.78) (ratio 1:1). Percentage of transmission at 600 nm was measured using a spectrophotometer (Ultrospec™2100 pro UV/Visible) after 15 min incubation time at room temperature. The same sodium alginate broth without

probiotics was treated the same way as test samples, added to acidic albumin, and used as blank during the measurement.

3.2.3.4 Acid production

The three probiotics were tested for acid production following American Dry Milk Institute (1971) guidelines. Marine broth containing glucose (5% w/v) was prepared and sterilised at 121˚C for 15 min. Probiotics were inoculated into marine broth separately and incubated for 48 hrs at room temperature. The media was then centrifuged (4000 rpm, 15 min) and supernatant was transferred into 125 ml flask. Phenolphthalein was used as colour indicator. Titration was performed using 0.1 M Sodium hydroxide (NaOH) as a titrant. In order to find the endpoint, NaOH was gradually added until a very light pink colour was developed in the media. Marine broth containing glucose (5% w/v) with no bacteria was used as the negative control in this experiment. The consumed volume of Sodium hydroxide was recorded to calculate the concentration of the acid produced inside cultured marine broth using equation 1:

$$\text{Acid (\%)} = \frac{(\text{Vol of NaOH (ml)} \times \text{M NaOH} \times \text{mol. weight lactic acid} \times 100)}{(\text{Vol of analyte (ml)})}$$

3.2.4 pH tolerance of probiotics

Four different pH values (2, 5, 6.5 and 8) were selected based on the possible pH conditions that probiotics might be exposed to, during encapsulation and delivery process. In order to examine the pH stability of the three probiotics, marine broth was prepared by dissolving broth powder in distilled water. The media was incubated on a magnetic stirrer for 1 hour. The pH of the media was adjusted by adding NaOH or HCl. The volume was adjusted after the final pH adjustment using distilled water. Marine broth without any pH adjustment was used as control. The pH of control media was around 7.6 according to the manufacturer's protocol. The media was sterilised using autoclave at 121˚C for 15 min and kept in fridge until use. The prepared media was brought to room temperature before the start of the experiment. A volume equal to 10 ml of each media was transferred into each test tube and inoculated with an overnight

bacterial culture (all pHs were tested in duplicate for each bacterium). The inoculated media was incubated for 3 hrs at room temperature. The samples were serially diluted and plated out on KAA and Marine agar with a standard plating procedure (plates were cultured in duplicate for each dilution).

3.2.5 Identification of probiotic bacteria

Amplification of the genomic 16S rRNA was carried out in order to identify the probiotic bacteria down to the species level. To prepare genomic DNA, one colony of an overnight culture was added to nuclease free water (250 µl). The mixture was vortexed briefly and microwaved for 10-15 sec to break the cells. The mixture was then centrifuged at 10,000 rpm for 1 min to precipitate all the heavy organelles, broken cell wall and membrane. The supernatant containing DNA was used as DNA stock to prepare the Polymerase Chain Reaction (PCR) mixture. In order to amplify 16S rRNA, a PCR mixture was prepared by mixing KAPA SYBR® FAST master mix (12.5 µl) (containing Taq polymerase, Mg^{2+} and dNTPs), 5 µl of forward primer 5'-AGAGTTTGATCMTGGCTCAG-3', Sigma), 5 µl of reverse primer 5'- CGGTTACCTTGTTACGACTT-3', Sigma) and 2.5 µl of prepared DNA stock. PCR was performed with the initial denaturation at 95˚C for 5 min. The next 30 cycles were carried out using following conditions: denaturation at 95˚C for 30 sec, annealing at 51˚C for 30 sec, extension at 72˚C for 2 min and final extension at 72˚C for 10 min. PCR products were analysed on 1% w/v agarose gel in TBE (Trisboric acid) buffer at 120 V for 60 min. Ready-to-load DNA ladder (100 bp, Solis BioDyne) was used to detect desired bands on agarose gel. The target band was purified using a gel extraction kit. The nucleotide sequence of the PCR product was then determined by Sanger's sequencing method (Sanger and Coulson, 1975). A comparative analysis was then performed by aligning acquired nucleotide sequences using BLAST tool in NCBI genome database.

3.3 Results and discussion

3.3.1 Colony morphology

The colony morphology of the three probiotic bacteria is illustrated in (Figure 3-1). Variations in colony morphology were clearly apparent on KAA and Marine agar.

Exiguobacterium was revealed as circular light-yellowish colonies after 24 hrs. However, a change in colony colour was observed when incubated longer (more than 48 hrs). Smooth surface with entire margin was noticed for *Exiguobacterium* sp. colonies. *Vibrio* sp. was identified with large white colonies with smooth surfaces and entire edges. *Enterococcus* sp. colonies were significantly smaller compared to the other two probiotic bacteria. Raised colony, smooth surface and entire margin were some of the phenotypic characteristics of *Enterococcus* sp. colonies.

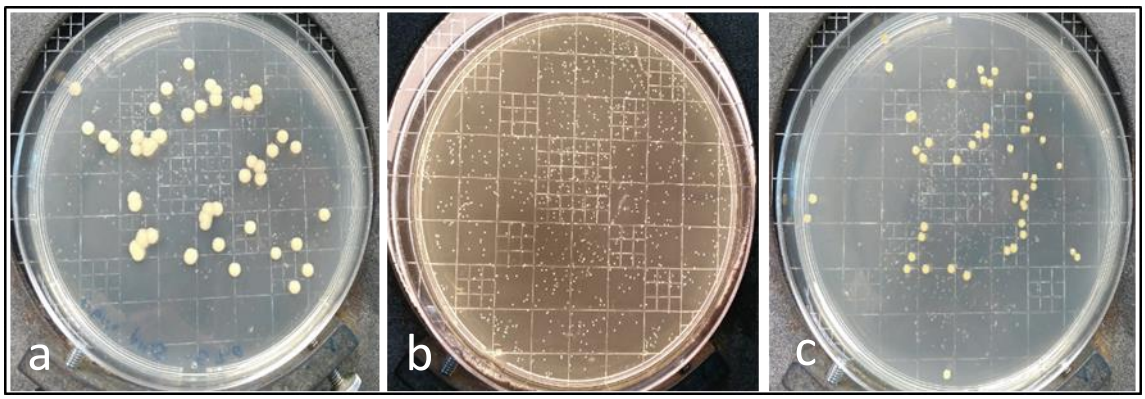


Figure 3-1 Bacterial culture of (a) *Vibrio* sp., (b) *Enterococcus* sp. and (c) *Exiguobacterium* sp..

3.3.2 Assessment of probiotics ability to digest nutrients

3.3.2.1 Protein hydrolysis

The ability of probiotic bacteria to hydrolyse protein was screened using agar plates containing casein (0.2% w/v). When probiotics produce the extracellular enzyme, casease, clear zones are generated around bacterial colonies indicating the hydrolysis of existed casein inside the plate. Among the three probiotic bacteria only *Exiguobacterium* was identified with the ability to hydrolyse milk protein (Figure 3-2).

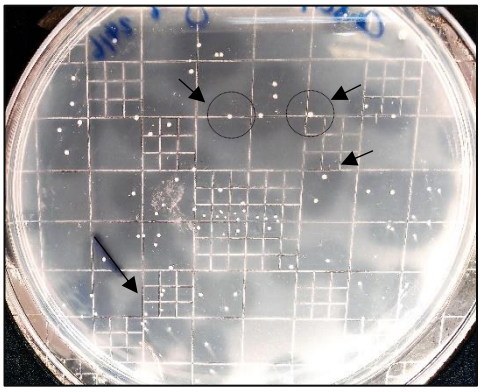


Figure 3-2 Clear zones indicated by arrows produced by *Exiguobacterium* colonies through casein hydrolysis.

3.3.2.2 Starch hydrolysis

Three probiotic bacteria were screened for starch hydrolysis by culturing them on starch agar plates. Non-digested starch in the media forms complexes with iodine reagent developing a dark blue colour where clear zones around bacterial colonies indicate the production of alpha amylase by bacteria and therefore digestion of starch molecules in the surrounding medium. Clear zones were developed around the area of growth of *Exiguobacterium* sp. and *Vibrio* sp. (Figure 3-3). Starch agar cultured with *Enterococcus* sp. developed a dark blue colour which indicates the inability of *Enterococcus* sp. to digest starch.

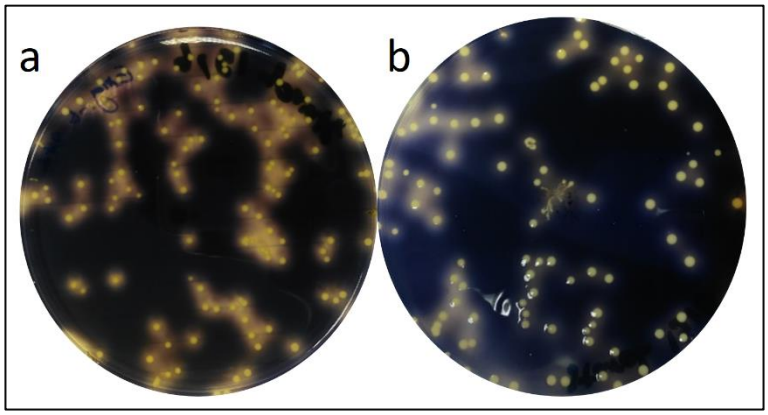


Figure 3-3 Clear zones of starch hydrolysis for (a) *Exiguobacterium* sp. and (b) *Vibrio* sp..

3.3.2.3 Alginate hydrolysis

The ability of probiotic bacteria to degrade alginate was measured using an accurate turbidity method. In this method, acidic polysaccharides such as alginic acid can combine with bovine albumin under acidic condition forming a white turbidity. Bacteria capable of alginate degradation will produce enzymatic products that do not develop any turbidity (Figure 3-4). Alginate-hydrolysing cultures are identified by the transparency of the test solution (*Vibrio* sp.), and the non- or low-alginate-degrading culture by the turbidity of the test solution (*Enterococcus* sp. and *Exiguobacterium* sp.). The percentage of turbidity for each bacterium was quantified and summarised in Table 3.1. All the three bacterial species were able to digest alginate; however, *Vibrio* sp. showed the highest alginate hydrolysis over the three days incubation. The negative control was the test solution with no inoculated bacteria.

Table 3.1 Alginate hydrolysis of the three probiotic bacteria (n=3; mean ± SD).

Bacterium	Transmission (%) at 600 nm		
	24 hrs	48 hrs	72 hrs
Exiguobacterium sp.	4.5 ± 0.2	7.4 ± 3.8	13.8 ± 6.7
Vibrio sp.	81.6 ± 5.9	86.7 ± 1.4	88.3 ± 1.4
Enterococcus sp.	7.7 ± 0.8	8.1 ± 2.9	19.6 ± 3.6

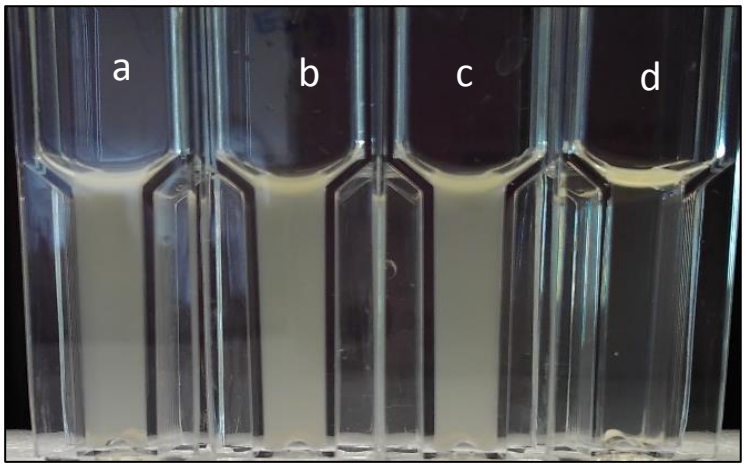


Figure 3-4 Alginate hydrolysis test for (a) negative control, (b) *Exiguobacterium* sp., (c) *Enterococcus* sp. and (d) *Vibrio* sp. Only *Vibrio* sp. showed a clear solution after 72 hrs incubation in medium containing alginate.

3.3.2.4 Acid production

Producing acid by probiotics can improve the host's adaptive and innate immunity as well as providing anti-pathogenic activity (Ringø et al., 2012). Acid production was tested for the three probiotic bacteria using a titration method. *Enterococcus* sp. demonstrated the highest ability to produce acid where *Exiguobacterium* sp. and *Vibrio* sp. showed lower abilities (Figure 3-5).

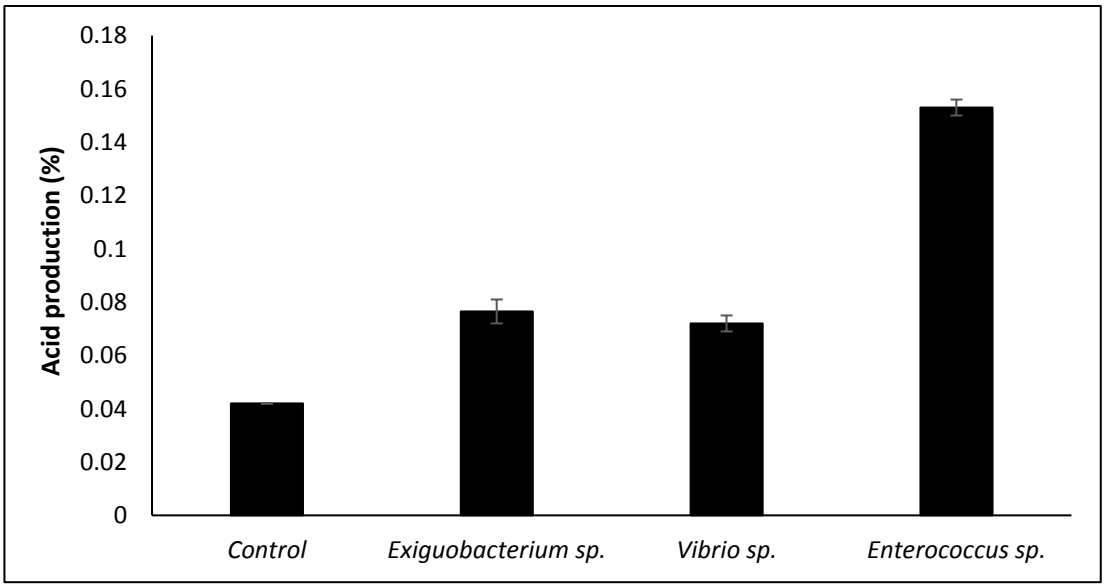


Figure 3-5 Comparison of the amount of acid produced by probiotic bacteria (n=4; mean ± SD).

3.3.3 pH tolerance of probiotics

The viability of the three probiotics was measured at four different pHs of 2, 5, 6.5 and 8. Media with pH: 7.6 was the control. The viable counts of *Enterococcus* sp. (Figure 3-6) and *Exiguobacterium* sp. (Figure 3-7) remained almost stable or slightly increased at pHs between 5 to 8. While the number of viable *Vibrio* sp. cells slightly decreased at acidic pH of 5 (Figure 3-8). None of the probiotic bacteria remained viable at pH 2.

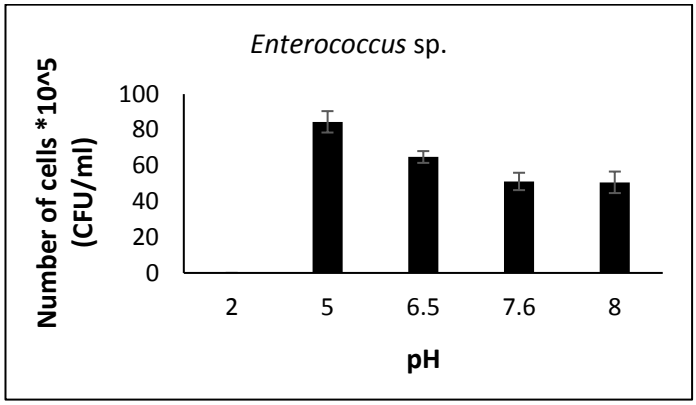


Figure 3-6 pH tolerance of *Enterococcus* sp. with initial count of approximately 45× 10^5 CFU/ml after 3 hrs incubation (n=3; mean ± SD).

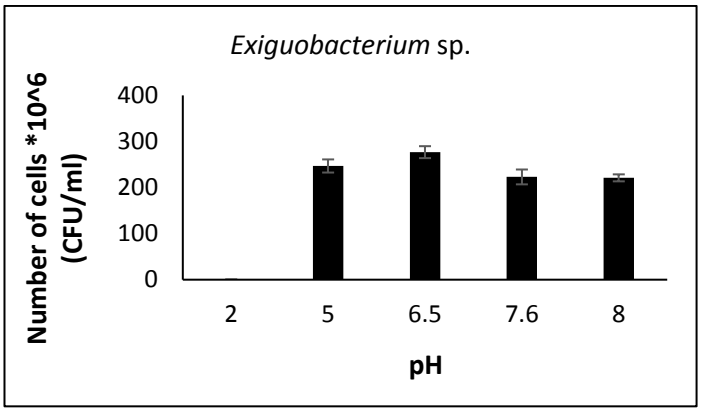


Figure 3-7 pH tolerance of *Exiguobacterium* sp. with initial count of approximately 217× 10^6 CFU/ml after 3 hrs incubation (n=3; mean ± SD).

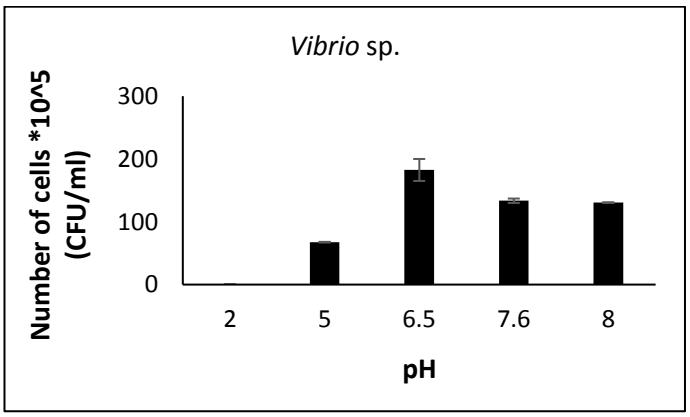


Figure 3-8 pH tolerance of *Vibrio* sp. with initial count of approximately 139× 10^5 CFU/ml after 3 hrs incubation (n=3; mean ± SD).

3.3.4 Identification of probiotic bacteria with PCR and sequencing techniques

The 16s rRNA nucleotide sequences related to each probiotic bacterium were analysed by NCBI BLAST. Query BLAST for *Exiguobacterium* with 100% query coverage resulted in 99.85% similarity to both *Exiguobacterium indicum* and *Exiguobacterium acetylicum* in NCBI GenBank. *Enterococcus* DNA isolate shared 100% identity with *Enterococcus hirae* with 100% query coverage. The closest genus to the *Vibrio* sp. on NCBI GenBank was an unknown *Vibrio* species isolated from digestive tract of *Haliotis rufescens* abalone in Chile which showed 99.54% (Accession number: GQ861541.1) (Appendix 1). Therefore, further experiments such as DNA-DNA hybridization and %G+C content are required to determine the species of *Vibrio* sp. and *Exiguobacterium* sp. used in this study.

3.4 Overall discussion

Three bacterial species were previously isolated from healthy adult abalone as probiotics. The three probiotics were identified as *Exiguobacterium* sp., *Vibrio* sp. and *Enterococcus* sp. based on phenotypic and biochemical characteristics (Hadi et al., 2014). This chapter aimed to reassure the presence of features that the isolates were initially selected for. This re-assuring step is critical since the probiotic bacteria were isolated four years before the commencement of this study and undergone a long storage at -80˚C. Chilled storage of bacteria may lead to loss of bacterial viability and activity (Fonseca, Marin, & Morris, 2006; Succi et al., 2007). The loss of biological activity depends on the type of bacteria and culture condition before and during chilled storage (Fonseca, Béal, & Corrieu, 2000, 2002). Screening the properties of the three probiotic bacteria showed that they all retained their abilities to digest at least one of the nutrients present in abalone commercial feed such as alginate, protein and starch. The results of starch hydrolysis test is in agreement with those of Hadi et al. (2014). However, two major differences were observed in the results of other assays. First, Hadi et al. 2014 reported the capability of *Vibrio* sp. in casein hydrolysis which seems to be lost after reviving *Vibrio* species from -80˚C storage. The loss of casein hydrolysis in *Vibrio* species could be due to the long storage time at -80˚C. Also, a slight alginolytic activity was observed for *Enterococcus* sp. which wasn't observed in the previous study. Appearance

of alginolytic activity in *Enterococcus* sp. could be due to the technical differences such as difference in alginate source. Although a few changes were observed in probiotic characteristics, probably due to the long-chilled storage and technical variations, all three species retained their critical features for which they were initially selected. These prominent features are high alginolytic activity of the *Vibrio* sp., protein and starch hydrolysis ability of *Exiguobacterium* sp. and acid producing ability of *Enterococcus* sp. Therefore, a cocktail of *Exiguobacterium* sp., *Vibrio* sp. and *Enterococcus* sp. can improve animals' food digestion through digesting starch and alginate and producing organic acid.

Further, pH tolerance of probiotics was investigated to assess the ability of each probiotic bacteria to survive various pH conditions. This study was necessary to understand the effect of acidic pH within abalone's gut and basic pH of seawater on the viability of probiotics. The results indicated that *Vibrio* sp. was the least tolerant to acidic pH of 5 representing abalone's gut environment (Harris et al., 1998; Ragg, 2003; Hadi, 2012). Hadi et al. (2014) also reported one log unit reduction in viable count of *Vibrio* at pH: 5. All the three probiotics were completely inhibited in acidic pH of 2. It is anticipated that providing a protective layer around probiotic bacteria in the form of a carrier can maintain the viability of probiotics during delivery. The ability of *Enterococcus* sp. to produce organic acid can help abalone in food digestion and enhance their immunity by excluding acid-sensitive pathogens (Ringø et al., 2012).

Gene sequencing was also carried out to identify the species of the three probiotic bacteria. The ribosomal RNA segment is commonly used to study bacterial phylogeny and taxonomy due to several reasons including 1) being highly conserved during evolution, 2) being present in almost all bacteria and 3) the 16s rRNA gene being large enough (1500 bp) for informatics analysis. Since the sequence of 16s rRNA is highly conserved during evolution, by determining this for an unknown bacterium and comparing it with known sequences, the species of the working bacteria can be identified. *Enterococcus hirae* with 100% query coverage and E value of 0.0 had 100% similarity with our working *Enterococcus* species. Marine resources including fish

processing waste was reported as a source for the *E. hirae* which can strengthen the likelihood of *E. hirae* to be matched with the working *Enterococcus* species in this study. Furthermore, the results of 16S rRNA analysis suggested 99.85% similarity between the working *Exiguobacterium* and both *Exiguobacterium indicum* and *Exiguobacterium acetylicum*. *Vibrio* sp. showed 99.54% similarity to DNA amplicon of an unknown *Vibrio* species. For identification of *Exiguobacterium* and *Vibrio* species, difficulties for species identification using 16s rRNA gene sequencing have to be considered as inaccuracy may arise from this method due to 1) lack of enough sequences in gene database, 2) species sharing similar and/or identical 16s rRNA sequences and 3) nomenclature problem associated with assigning multiple name to single species (Janda and Abbott, 2007). Therefore, further experiments are required to identify the *Vibrio* and *Exiguobacterium* probiotic isolates to the species level.

3.5 Conclusion

The three probiotic species were capable of digesting some of the nutrients in abalone food such as alginate, protein and starch. This may result in a better food digestibility, leading up to an improved nutrient absorption in probiotic-fed pāua. The probiotic bacteria retained their original biochemical characteristics for which they were initially selected. Therefore, a combination of the three probiotic bacteria potentially can benefit pāua in terms of food digestion. The three probiotics can grow in a wide range of pHs with an exception of *Vibrio* being sensitive to acidic pH. This implies the necessity of considering further protection in order to efficiently deliver viable probiotics to black-footed abalone. In the next chapter, encapsulation technique is utilised to develop a carrier to deliver probiotics to *H. iris*.

Chapter 4

Development of microencapsulated probiotic delivery system for *Haliotis iris* using an emulsion technique

"Nothing is particularly hard if you divide it into small jobs".

Henry Ford

Abstract

Probiotics have been used widely to confer health benefits to farmed aquatic animals. However, conventional methods have not been efficient to deliver probiotics in high viable number to specific target sites with minimum environmental contamination. This study aims to exploit microencapsulation approach to deliver probiotics to black-footed abalone (*Haliotis iris*). Emulsion technique was used to encapsulate probiotic bacteria within double-layered chitosan-coated alginate microparticles (CALG). The interaction between chitosan and alginate was assessed by Fourier-transform infrared (FTIR). The efficacy of CALG microparticles in delivery of probiotics to abalone was explored using *in-vitro* and *in-vivo* studies. Spherical CALG microparticles with the mean diameter of 113 µm and encapsulation efficiency of more than 75% were formed using internal gelation. The *in-vitro* release studies revealed a lack of probiotic discharge in the first six hrs of incubating CALG in seawater followed by a slight bacterial release within the next 42 hrs. The exposure of CALG microparticles to simulated gastric and intestinal media exhibited a significantly higher release of encapsulated bacteria in simulated intestinal medium. Fluorescence microscopy of consumed microparticles within abalone gastrointestinal tract (GIT) showed a successful delivery of microparticles to the abalone's gut. The number of probiotic bacteria in probiotic-fed abalone was significantly greater than control animals. The results suggest that the CALG microparticles could be potentially used as carriers to deliver viable probiotic bacteria to the abalone's GIT.

4.1 Introduction

Abalone are one of the highest value seafood around the world with almost 95% of production is derived from aquaculture. Almost 160,662 metric tonnes of abalone was produced from farms globally in 2016/17 (Cook, 2019). The high price of abalone products including meat and pearl has made abalone industry attractive in many regions. Nutritional requirements of abalone are met via formulated feed or cultured seaweed which takes 3-5 years of farming until a market-sized abalone is produced. This can limit the development and reliable continuation of abalone production as mortalities may occur due to unwanted changes in culture conditions (e.g. seasonal temperature fluctuations) and possible disease outbreaks.

In recent years, considerable attention has been given to reduce this time frame using various measures such as utilising probiotics to increase the growth rates of abalone through better feed digestion (Macey & Coyne, 2005; Doeschate & Coyne, 2008; Silva-Aciares et al., 2011; Hadi et al., 2014). Using probiotics as a beneficial measure has been tested to improve growth and health of black-footed abalone (Hadi et al., 2014). Probiotics can provide a mechanism to reduce abalone grow-out period leading to a better economically sustainable abalone industry. This requires the development of an environmentally friendly method to deliver live microorganism to farmed abalone.

Probiotics are conventionally added to the culture water, but this approach can lead to environmental issues and inefficient delivery. Introducing high concentration of probiotics into culture water may contaminate environment via the farm's water effluent. Moreover, the probiotic viability may be lost before they can even reach to the possible target site within the abalone gastrointestinal tract (GIT). Therefore, there is a need to develop a delivery method that can retain the probiotics' viability and prevent probiotic discharge into the environment.

Microencapsulation technology has been used in aquaculture to obtain a controlled release targeted delivery and to retain innate characteristics of encapsulated bioactives such as nutrients, probiotics (Pirarat et al., 2006; Kumaree et al., 2014; Pinpimai et al.,

2015; Madreseh et al., 2018; Xia et al., 2018), vaccines and immunostimulants (Adomako et al., 2012; Vidhya Hindu et al., 2018). It has been reported that encapsulation can improve probiotics viability (Zhang, Lin, & Zhong, 2015) as well as providing a better control over the dosage and site of release (Rodriguez et al., 2018).

The aim of this chapter is to develop a novel system for delivering probiotics to the gastrointestinal tract of abalone. Thereby, the major objectives are:

- to develop and characterise microparticles as carriers for *Exiguobacterium sp*., *Vibrio sp*. and *Enteroccocus sp*.,
- to study the release of encapsulated probiotics in seawater, simulated gastric and intestinal fluid of abalone, and
- to investigate the fate of microparticles within abalone gastrointestinal tract.

4.2 Materials and Methods

4.2.1 Materials

Sodium alginate was purchased from AcrosOrganics (Beijing, China). Medium molecular weight chitosan and Span 80 were acquired from Sigma (St. Louis, USA). Calcium carbobonate ($CaCO_3$) was obtained from Omya (Orgon, France). Calcium chloride ($CaCl_2$) was purchased from Ajax Finechem (NSW, Australia). LIVE/DEAD Bacterial Viability Kit (L7012) was purchased from BacLight (Oregon, USA). Sunflower oil was purchased from local market. All the other chemicals used in this study were of analytical grades. Juvenile black-footed abalone (average shell length 30 mm) were provided by Moana New Zealand abalone farm (Ruakaka, New Zealand). Bacterial isolates were kindly provided by Aquaculture and Biotechnology Research Group at Auckland University of Technology (Auckland, New Zealand).

4.2.2 Preparation of cell suspension

Pure cultures of *Exiguobacterium* sp., *Vibrio* sp. and *Enterococcus* sp. were revived by streaking bacterial glycerol stock on marine agar. A well-isolated colony was then inoculated in marine broth containing yeast extract (1% w/v). Bacterial strains were then activated by two successive transfers in broth media. *Exiguobacterium* sp. and *Vibrio* sp. were cultivated overnight while shaking at 100 rpm. *Enterococcus* sp. was cultured under anaerobic condition. 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 encapsulation.

4.2.3 Microencapsulation of probiotics by emulsification

Alginate solution was prepared by dissolving sodium alginate powder (2% w/v) in distilled water. The mixture was then stirred for 2 hrs at 400 rpm and incubated overnight to eliminate bubbles and obtain a uniform solution.

An emulsion technique was used to produce alginate microparticles using sunflower oil as continuous phase. The protocol suggested by Song et al. (2013) was principally followed with some modifications. Alginate (4 ml) was mixed with calcium carbonate ($CaCO_3$, 0.2 g) and the mixture was stirred at 200 rpm for 45 min. A mixture of the three probiotic bacteria were then added to the alginate and stirred for another 10 min. Sunflower oil (20 ml) was then added to $CaCO_3$-alginate–cell mixture for emulsification and stirred for 20 min at 1500 rpm. Span 80 (1% v/v) was added to the mixture and incubated for 30 min at the same stirring condition. Glacial acetic acid (0.2% v/v) was then added to initiate the gelation. The emulsion obtained was incubated on a stirrer at 1500 rpm for another 30 min to allow capsule formation. Alginate microparticles (ALG) were then collected by centrifugation for 5 min at 2000 rpm. The top layer (oil) was removed and the microparticles were washed three times with total 45 ml of distilled water under the same centrifugation condition. The produced ALG microparticles were coated by incubation in 20 mL of chitosan solution (0.8% w/v in lactic acid 1%v/v, pH: 5.7). The suspension was stirred at 100 rpm for 20 min followed by a brief centrifugation (1000 rpm, 5 min) and washed twice with $CaCl_2$ (0.2 M) solution (Figure 4-1). The

produced chitosan-coated alginate particles (CALG) were collected by centrifugation at 1000 rpm for 5 min.

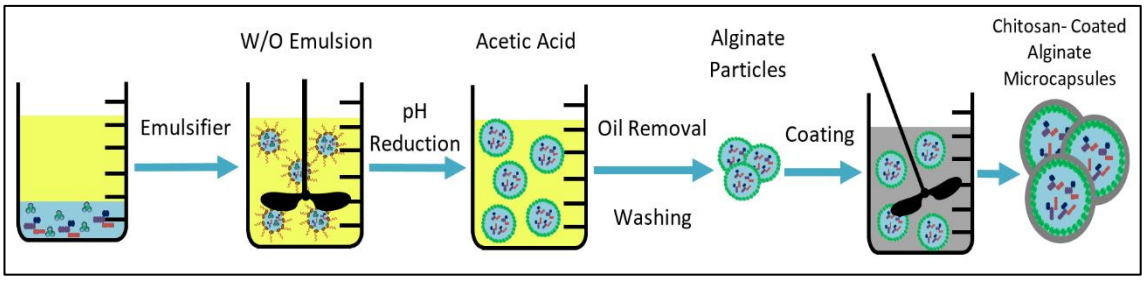


Figure 4-1 Schematic diagram of the encapsulation process.

4.2.4 Surface charge and size distribution of microparticles

Zeta potential of the produced particles was measured with a Malvern zeta sizer (3000-HS, UK) at 25˚C. The size distribution of the beads was determined by a laser diffraction method using a Malvern equipment (Mastersizer 2000, England). Distilled water was used as dispersion medium. The microcapsule suspension was slowly introduced to a dispersion unit until the percentage of laser obstruction was more than 14%. Span factor was calculated using the below formula to report the size distribution (Lefebvre, 1993),

$$Span\ factor = \frac{D(90) - D(10)}{D(50)}$$

where D (90), D (10) and D (50) are the diameters at 90%, 10% and 50% of the cumulative volume, respectively.

4.2.5 Evaluation of the viability/encapsulation efficiency of encapsulated probiotics

The viability of encapsulated cells was determined immediately after preparation of microparticles. All microparticles produced from 4 ml of alginate were dissolved in sodium hydrogen carbonate (4% w/v, 10 ml). The suspension of the fully dissolved microparticles was serially diluted and cultured on appropriate agar medium. Viable plate count was used to determine bacterial numbers by a 10-fold serial dilution

followed by spreading 100 µl of each dilution on marine agar and kanamycin esculin azide agar (KAA) plates. Experiments were carried out in triplicates. The encapsulation efficiency was calculated according to below equation (Martin et al., 2013):

$$EE\% = \frac{Number\ of\ viable\ cells\ released\ from\ capsules}{Initial\ number\ of\ viable\ bacteria\ added\ to\ the\ formulation}$$

4.2.6 Morphological properties of microparticles

The morphology of the produced microparticles was examined using optical light microscopy (Olympus CX31, Philippines) and scanning electron microscopy (SEM) (Hitachi SU-70, Japan). SEM analysis was performed on air-dried microparticles. The dried microparticles were dispersed on electrically conductive carbon film placed on a specimen stub. The samples were then coated with platinum with an ion sputter coater (Hitachi E-1045, Japan) for 100 sec before SEM imaging.

4.2.7 Spectroscopic analysis

Fourier-transform infrared (FTIR) spectra of ALG and CALG were obtained using a spectrometer (Nicolet™ iS10, USA) equipped with a horizontal attenuated total reflectance (ATR) crystal. Pure powders of alginate, chitosan and $CaCO_3$ were used to obtain references. An average of 32 scans at 4 cm^{-1} resolution were recorded within the wave number range of 400-4000 cm^{-1}.

4.2.8 Bacterial staining with fluorescent dyes

Marine broth (100 ml) containing yeast extract (1% w/v) was inoculated with a single colony of *Exiguobacterium* then incubated on a shaker (100 rpm) at room temperature for 18 hrs. The bacterial culture was divided into two separate falcon tubes and then centrifuged at 4000 rpm for 15 min to collect the bacterial cells. Bacterial pellets were washed two times with 20 ml of a washing solution (NaCl 0.85% w/v). LIVE/DEAD Bacterial Viability Kit was used for the staining of bacteria. The kit contains SYTO9 and propidium iodide dyes with 485⁄498 and 535⁄617 nm excitation/emission wavelengths, respectively. Both fluorescence dyes were incubated at room temperature until thawed. A volume equal to 30 µL of each dye were mixed together in a dark centrifuge tube. Half

of the mixture was mixed with the recovered bacterial pellets in each falcon tube 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.

4.2.9 Bacterial release in seawater

Exiguobacterium sp. was used as the model bacterium to study the release of encapsulated probiotics in seawater. Fluorescent-labelled bacteria were used to produce chitosan-coated- and uncoated microparticles as described in Section 4.2.3. The encapsulated bacteria (0.4 g) were placed in a falcon tube with 4 ml of filtered seawater (0.22 µm). The tubes were kept at 15˚C and 40 µl of the seawater was withdrawn at various time intervals (1, 2, 3, 4, 5, 6, 24, 48 hrs). Samples were centrifuged at 500 rpm for 5 min. Supernatant (10 µl) was placed on a counting chamber to count the number of released bacteria under fluorescence microscope. The experiment was performed in triplicates for each formulation.

4.2.10 Tracking microparticles within the GIT of abalone

4.2.10.1 Preparation of feed

Exiguobacterium sp. was used as the model bacterium to track the encapsulated bacteria within the GIT of abalone. Fluorescent-labelled bacterial cells were added to alginate formulation to produce microparticles (Section 4.2.3). The produced microparticles containing the fluorescein-labelled bacteria were then used to produce feed pellets for abalone. The produced microparticles containing probiotic were then immobilised in alginate beads before delivering to abalone. The immobilisation process was performed by mixing the produced microparticles with 10 ml alginate (1.5% w/v) and dropping the mixture into a 100 ml of $CaCl_2$ cross-linking solution (0.1 M) using a syringe. Alginate beads were immediately formed as droplets entered the cross-linking bath. Particles were collected after 30 min using a sieve and rinsed with 200 ml of distilled water (Figure 4-2).

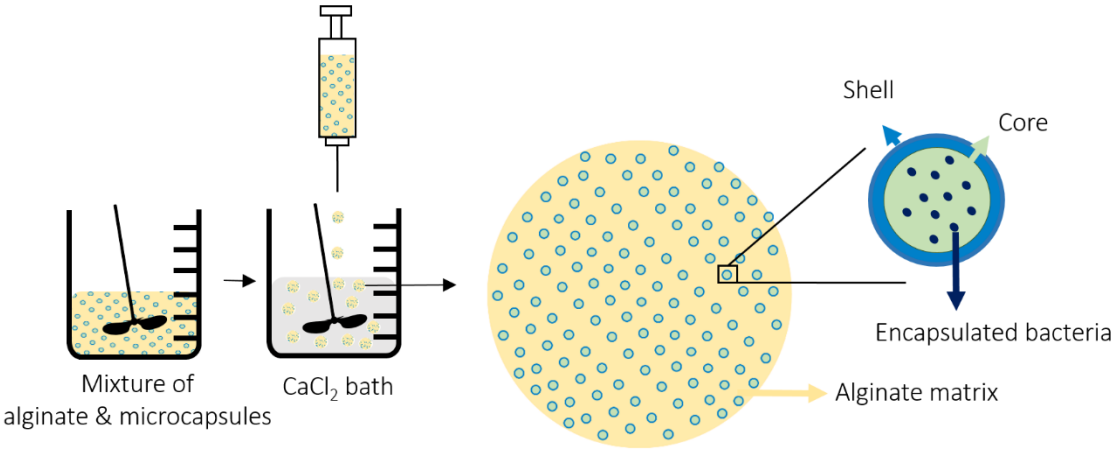


Figure 4-2 Schematic diagram of steps for producing alginate beads containing encapsulated probiotics.

4.2.10.2 Feeding experiment

Eight juvenile abalone (25-35 mm length) were not fed for two days prior to this experimentation to maximise feeding within the first few hrs of introducing encapsulated probiotic particles to the animals. The animals were kept in 10 L of seawater at 15˚C. The seawater in the maintenance tank was filtered (50 µm), sterilised by UV and aerated using air stones. Animals were fed once with 4 gr of immobilised microparticles dropped inside the tank. After 12 hrs the remaining microparticles were removed and fresh seawater was replaced. Two animals were then removed from the tank for dissection at 12, 24, 48 and 72 hrs after feeding. Microparticles containing non-stained probiotics and microsections of abalone fed with conventional feed were observed with fluorescence microscope to ensure the lack of fluorescent signals from the samples prior to the experiment.

4.2.10.3 Cryo-sectioning and microscopy

Two animals collected at each time point were dissected to remove the gastrointestinal tract. The recovered tissues were then frozen by a tissue freezing medium (Leica, UK) and Surgipath Frostbite rapid coolant (Leica, UK) and cut using a Leica CM1850 cryostat (UK) (Figure 4-3). Thin sections (40 µm) from various layers were collected on a glass slide and kept in the dark at -25°C until microscopic analysis. Fluorescence images were

obtained using Olympus BX51 microscope equipped with a camera (Optronics, Japan) and MagniFire imaging software (DP12, Olympus, Japan).

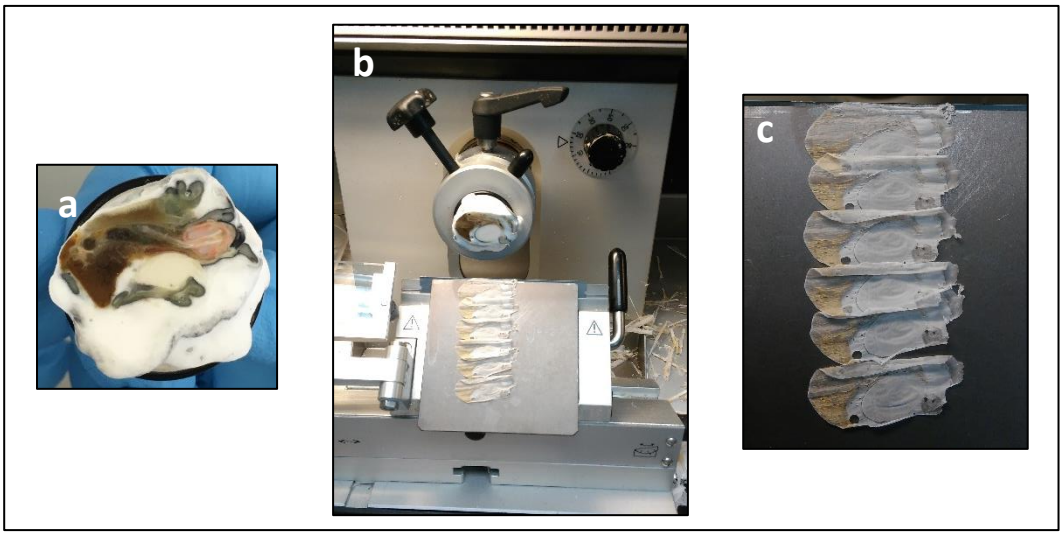


Figure 4-3 Images of (a) a frozen block of abalone tissue, (b) sectioning process using Leica cryotom and (c) thin sections of abalone's GIT.

4.2.11 *In-vitro* bacterial release into simulated gastric and intestinal media

4.2.11.1 Preparation of simulated gastric (SGM) and intestinal (SIM) media

Three juvenile abalone (30-35 mm shell length) were dissected for this purpose and the stomach and intestine were removed and kept on ice separately. The stomach of three animals were pooled together. Sterilised cold saline (0.85% w/v, 13 ml, pH: 5.2) was added to the tissues before homogenising with a stomacher (Seward laboratory, USA) for 4 min to extract crude enzymes. The homogenate was centrifuged at 4000 rpm for 10 min at 4˚C. The supernatant was recovered and used as SGM. To produce SIM, sterilised cold saline (0.85% w/v, 13 ml, pH: 6.3) was used instead to homogenise intestinal sections.

Microparticles containing stained bacteria were prepared as described in Sections 4.2.3 and 4.2.8. The produced microparticles (0.4 g wet weight) were immersed in 4 ml of SGM and SIM. The release experiments were carried out at 15˚C in the dark to resemble abalone culture conditions. Samples (40 µl) were collected at different time points (0.5,

1, 2, 3, 4, 5, 6, 24, and 48 hrs) and centrifuged at 500 rpm for 5 min. The number of released bacteria was counted using a counting chamber under fluorescence microscope. The experiment was repeated thrice.

4.2.12 Delivery of probiotics to abalone

Probiotic culture and microparticles were prepared as explained in Sections 4.2.2 and 4.2.3, respectively. The diet was prepared according to Section 4.2.10.1. Seawater was collected from Okahu bay, Auckland. Juvenile black-footed abalone (30-35 mm SL) were kept at 15˚C in black tanks containing 15 L of filtered (50 µm) seawater (three tanks for each treatment). Animal holding tanks were aerated vigorously and seawater was checked regularly for dissolved oxygen (100%), pH (8-8.3), ammonia (0-0.25 ppm) and nitrite (0-0.25 ppm) concentrations.

Enterococcus sp. was used as the model bacterium to investigate the efficacy of microparticles in delivering probiotic to abalone. Feeding, tank cleaning and water exchange were carried out every two days. A group of 6 abalone were fed with encapsulated probiotics (approximately 10^{12} CFU of *Enterococcus* sp. at each feeding time) and another group with commercial pellets (control group). The bacterial load of control and probiotic-fed animals was quantified after four days to determine whether the encapsulated bacteria were released from the microparticles into the abalone gastrointestinal tract.

All the 12 animals in the feeding experiments were dissected and the content of their gastrointestinal tract were homogenised in 10 ml of marine broth using a Stomacher (Seward laboratory, USA) for 4 min. Serial dilutions of homogenised mixture were subsequently prepared. KAA agar plates were used to culture 100 µl from each dilution (each dilution was assayed in triplicate). The colonies were counted after 48 hrs of incubating cultured plates in an anaerobic jar.

4.3 Results and Discussion

4.3.1 Surface charge and size distribution of microparticles

Size and surface charge of the produced ALG and CALG microparticles were tested using a laser scattering technique (Table 4.1). The size and surface charge of microparticles were measured in distilled water. ALG and CALG microparticles had a weighted residual of 0.51% and 0.59%, respectively. The percentage of residual below 1% for both formulations indicates that the calculated data by software was perfectly fitted to the measured data. A normal size distribution was found for both ALG and CALG microparticles. The size of the microparticles was summarised in Table 4.1. The mean diameter of ALG and CALG microparticles ranged from 69 and 113 µm. The higher mean diameter of CALG could be due to the presence of coating around alginate microspheres and microparticles clustering. Higher span value of 4.6 compared to 2.3 in ALG shows higher polydispersity of CALG formulation. This could be due to the zeta potential of CALG being close to zero which may have caused slight agglomeration of microparticles. However, samples were sonicated right before the measurements in order to deagglomerate the microparticles. Microparticles were washed with calcium chloride solution in order to increase the surface charge of microparticles and minimise agglomeration. The zeta potential of CALG was higher than that of ALG microspheres which confirms the success of coating process.

Table 4.1 Size and surface properties of the produced microparticles (n=3; mean ± SD).

Formulation code	Mean diameter (µm)	Span factor	ZP (mV)
ALG	69.2 ± 1.0	2.3	-45.8
CALG	113.4 ± 3.9	4.6	0.20

4.3.2 Evaluation of the viability of encapsulated probiotics in microparticles

The viable amount of encapsulated probiotic was evaluated as a combined measure of entrapment efficacy and survivability of cells during encapsulation process for each probiotic strain. High encapsulation efficiency was found for all the three probiotics

using the emulsion technique (Table 4.2). The viable cell counts above 75% for all the three probiotic bacteria implies that the applied emulsion procedure did not impose a significant detrimental effect on viability of *Exiguobacterium* sp., *Enterococcus* sp. and *Vibrio* sp.

Table 4.2 Viability of encapsulated probiotics in microparticles (n=3; mean ± SD).

Bacterial species	EE%
Enterococcus sp.	85.7 ± 1.4
Exiguobacterium sp.	75.9 ± 4.1
Vibrio sp.	75.3 ± 2.5

4.3.3 Morphological properties of microparticles

Optical micrographs of wet alginate and chitosan-coated alginate microparticles are illustrated in Figure 4-4. Spherical microparticles with a wide size range were observed. SEM images are shown in Figure 4-5a. Alginate microspheres showed a rough surface which might be due to the water loss during the drying process. The presence of bacteria within the matrix of microparticles can be clearly seen in SEM micrographs (Figure 4-5b).

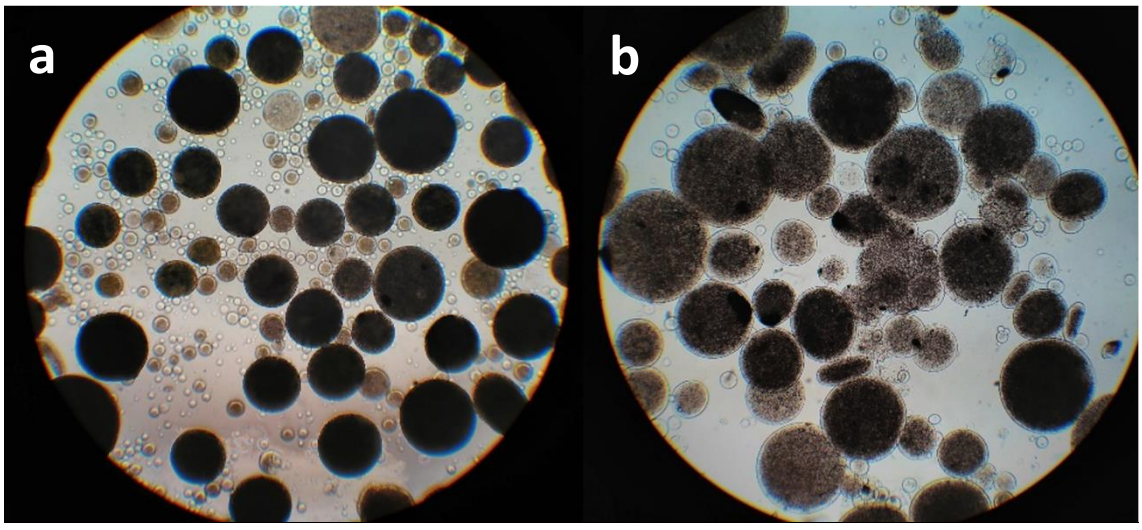


Figure 4-4 Optical microscope images of (a) ALG and (b) CALG microspheres with magnification of x400, produced by emulsification.

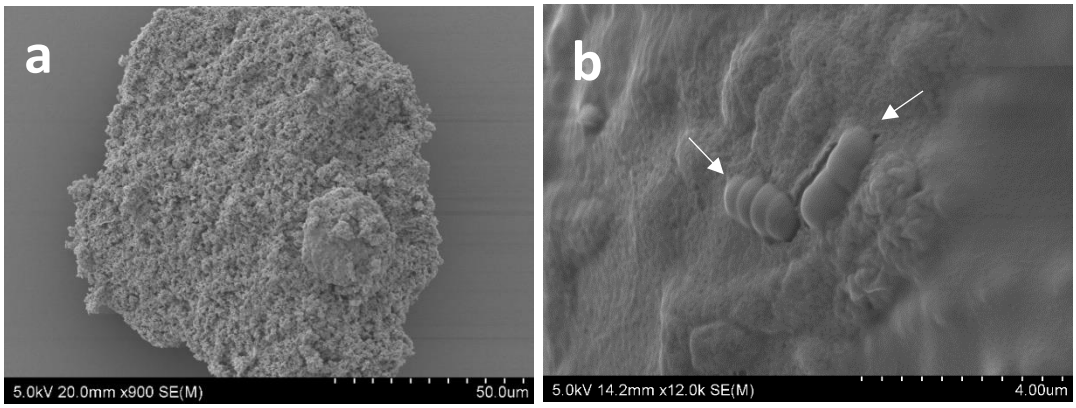


Figure 4-5 SEM image of (a) probiotic loaded alginate microparticle and (b) the encapsulated probiotics indicated by arrows.

4.3.4 Spectroscopic analysis

FTIR spectra of samples were obtained to confirm the presence of chitosan coating in CALG microparticles and to identify potential interactions between chitosan and alginate. Infrared spectrum intensity of ALG and CALG microspheres, alginate, chitosan, $CaCO_3$ powder, and a physical mixture of them are shown in Figure 4-6. The assignment of functional groups to the major IR peaks are summarised in Table 4.3.

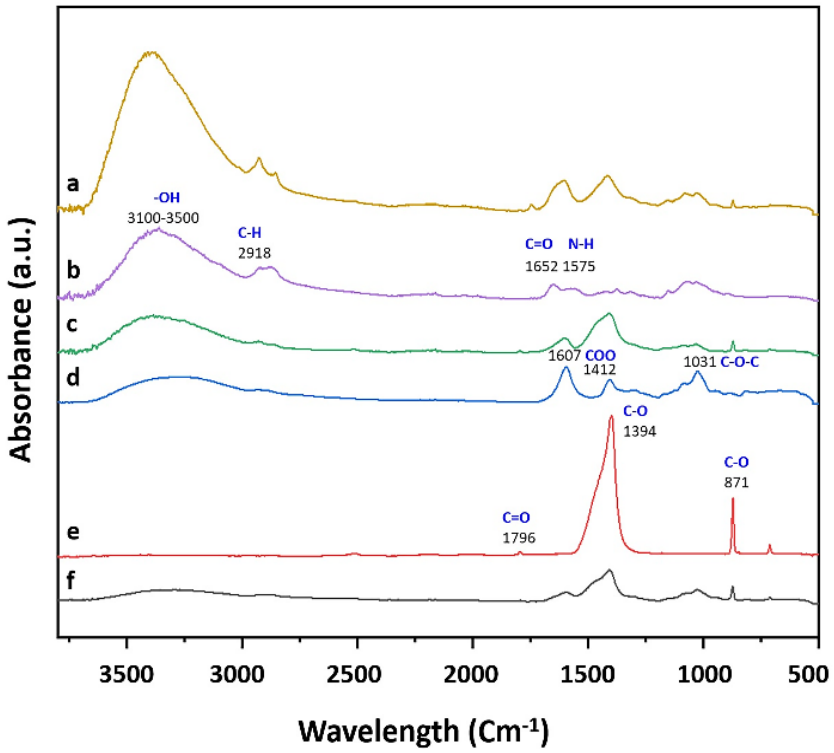


Figure 4-6 The infrared spectra of (a) CALG microparticles, (b) chitosan, (c) ALG microspheres, (d) sodium alginate, (e) $CaCO_3$ and (f) physical mixture of sodium alginate, chitosan and $CaCO_3$.

Two characteristic peaks were observed for alginate at 1607 cm^{-1} and 1412 cm^{-1} corresponding to the carboxylate group in its structure (Figure 4-7). The third distinctive peak presenting the C-O-C bond was at 1031 cm^{-1} which is related to saccharide structure of alginate (Figure 4-6d)(Figure 4-8)(Lawrie et al., 2007; Li et al., 2008; Papageorgiou et al., 2010).

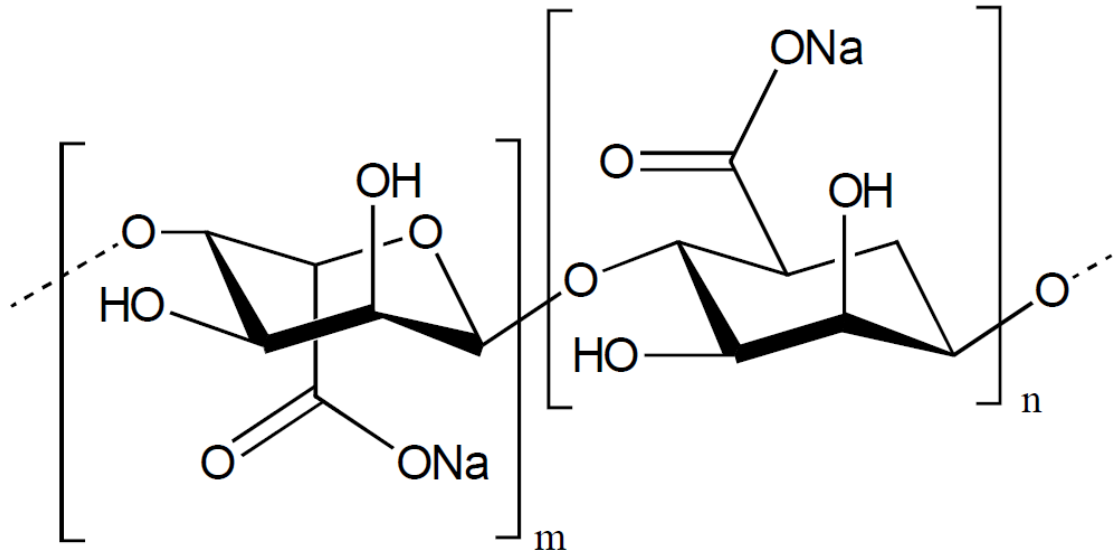


Figure 4-7 Chemical structure of sodium alginate.

In the spectra of $CaCO_3$, the band at 1796 cm^{-1} corresponds to C=O bonds. Other absorption bands of single bonded C-O and Ca-O bonds appeared at 1394, 871 and 721 cm^{-1} (Table 4.3). The use of $CaCO_3$ has contributed to absorption band of symmetric vibration of carboxylate groups which resulted in a shift from 1412 cm^{-1} in alginate powder to 1409 cm^{-1} in IR spectrum of alginate microspheres. The presence of wider peak at 1409 cm^{-1} with an adjacent shoulder is indicative of symmetric stretching vibration of carboxylate groups (1412 cm^{-1}) in alginate and C-O band (1394 cm^{-1}) of $CaCO_3$ (Galván-Ruiz et al., 2009). Furthermore, the presence of other absorption peaks including 712, 1796 and 871 cm^{-1} indicates the presence of $CaCO_3$ within ALG microspheres.

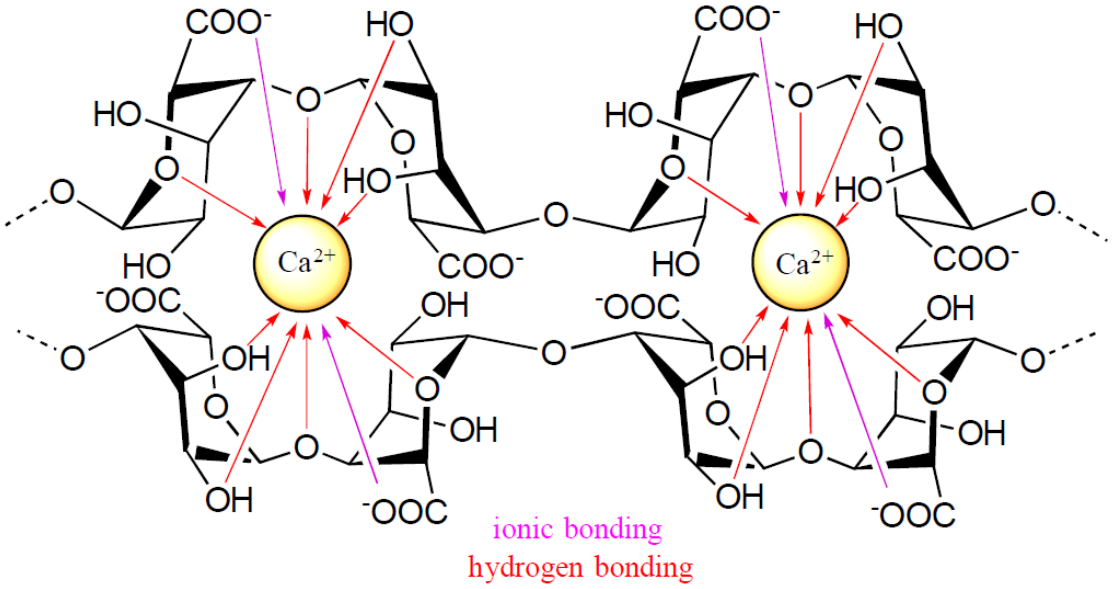


Figure 4-8 Schematic diagram of egg-box structure of calcium alginate gelation.

In the spectra of chitosan, four major peaks are distinguishable at 1575, 1652, 2873 and 2918 cm^{-1} (Figure 4-6b). The absorption band of 2918 cm^{-1} is related to the C-H stretching in chemical structure of chitosan (Figure 4-9). The peak at 2873 corresponds to the –OH stretching. The absorption bands of 1575 and 1652 cm^{-1} have been assigned to the vibration of amide II and amide I, respectively (Li et al., 2008; Wang, Zhu, & Zhou, 2011). The remaining peaks are summarised in Table 4.3.

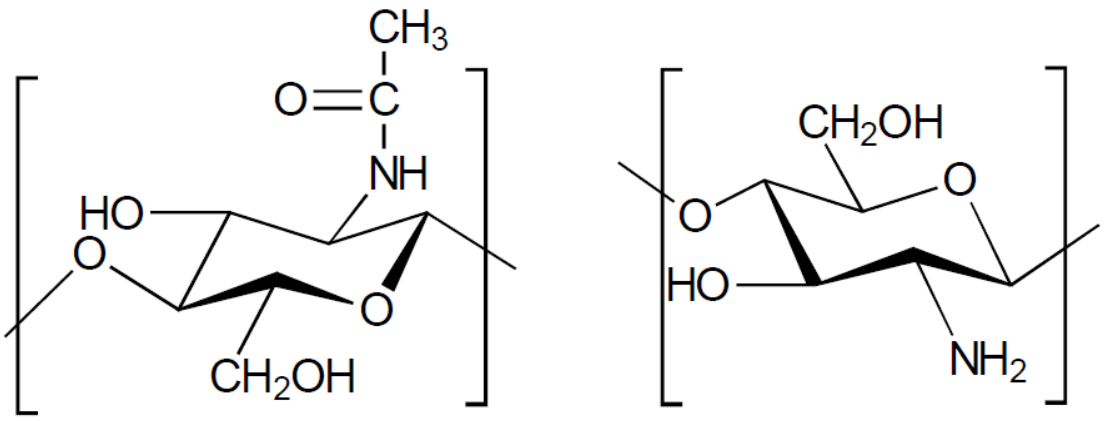


Figure 4-9 Chemical structure of chitosan monomeric unit (Acetylated residue on the left and deacetylated residue on the right).

In the IR spectrum of CALG microparticles, the strong peaks of at 2918 and 2873 cm^{-1} were recorded which are associated with the presence of chitosan. A narrow peak of the asymmetrical stretching of carboxylate group at 1601 cm^{-1} indicates less vibration from carboxylate groups in CALG compared to ALG microspheres. Moreover, disappearance of amide I vibration in chitosan at 1652 cm^{-1} and formation of a shoulder at 1651 cm^{-1} could be due to the interaction between carboxylate groups of alginate associated with chitosan. In addition, the absorption band at 1575 cm^{-1} in chitosan shifted and formed a shoulder at 1455 cm^{-1}, the stretching vibration of –OH and $–NH_2$ at 3360 cm^{-1} shifted to 3350 cm^{-1} and became broad after the reaction of chitosan and alginate in CALG microspheres (Li et al., 2008).

Table 4.3 Summary of major FTIR peaks and assignment of corresponding functional groups.

	Wavenumber (cm^{-1})	Functional group or contributor biomolecule
Alginate powder	1607	Asymmetric stretching vibration of carboxylate groups
	1412	Symmetric stretching vibration of carboxylate groups
	1031	C-O-C stretching in saccharide structure
Chitosan powder	3100-3500	Amine and hydroxyl groups
	2918	C-H stretching
	2873	-OH stretching
	1652	Stretching of Carbonyl group in the secondary amide (amide I band)
	1575	Bending vibrations of the N-H (N-acetylated residues, amide II band)
	1418	N-H stretching of the amide and ether bonds
	1375	N-H stretching (amide III band)
Calcium carbonate	1796	C=O bonds
	1394	C-O bonds
	871	C-O bonds
	712	Ca-O bonds

4.3.5 Bacterial release in seawater

To assess the protective characteristics of chitosan-coated and uncoated microparticles against the release of encapsulated bacteria in seawater, bacterial release studies were conducted in a simulated set up. *Exiguobacterium* sp. was selected for this experiment because it can be recognised easily under microscope and having high survivability during staining procedure. The cumulative number of bacteria released from the microparticles were calculated which are shown in Figure 4-10. The reported bacterial count may include the released bacteria from the microparticles, and the daughter bacterial cells produced by already released bacteria. However, the release test tubes were erected still and tightly capped during the experiment to limit the presence of oxygen. Furthermore, it was assumed that lack of fresh nutrients in the release media would limit and postpone the bacterial multiplication during the experiment. For

uncoated microparticles, bacterial release commenced at 5 hrs post incubation which slowly increased until 24 hrs followed by a fast release. No release was observed for coated microparticles over the first 6 hrs of incubation in seawater. The overall release of bacteria from chitosan-coated microparticles was significantly lower (P<0.05) than that of the uncoated microparticles. The encapsulated bacteria could still be detected within coated microparticles under fluorescent microscope after 48 hrs of incubation in seawater Figure 4-11. Previous studies have also reported a significantly lower release of encapsulated bacteria from chitosan-coated microparticles compared to uncoated ones (Iyer, Phillips, & Kailasapathy, 2005). In aquaculture, it is desired that delivery systems protect their bioactive contents when placed in sea water. Any bioactive release at this stage and before animals consume the microparticles can result in bioactives leach into sea water and potential environmental risks.

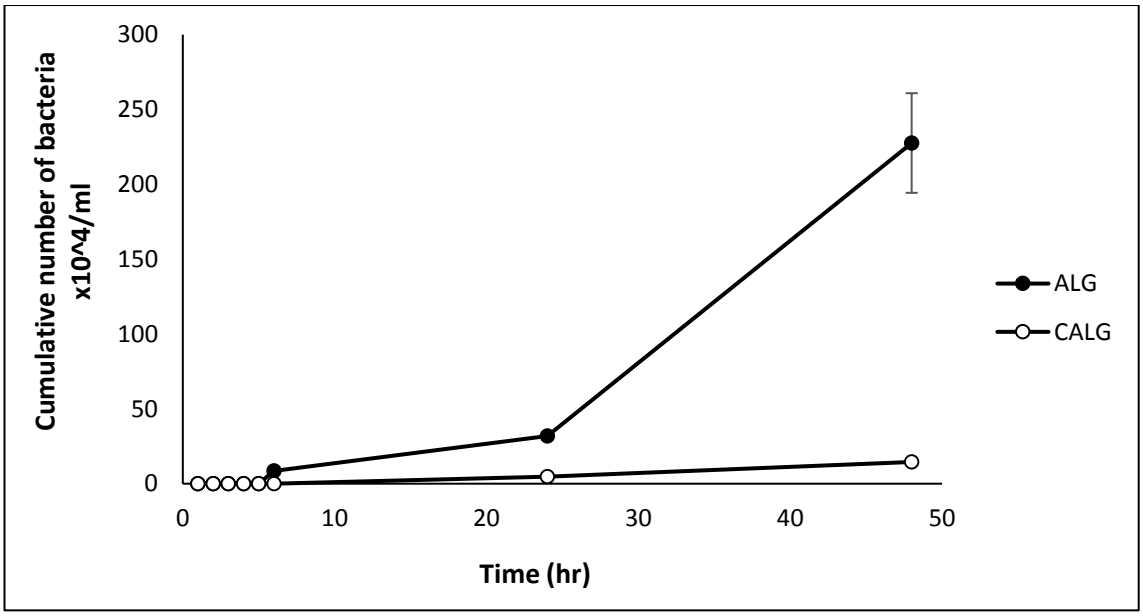


Figure 4-10 Release of encapsulated probiotic bacteria in seawater (n=3; mean ± SD).

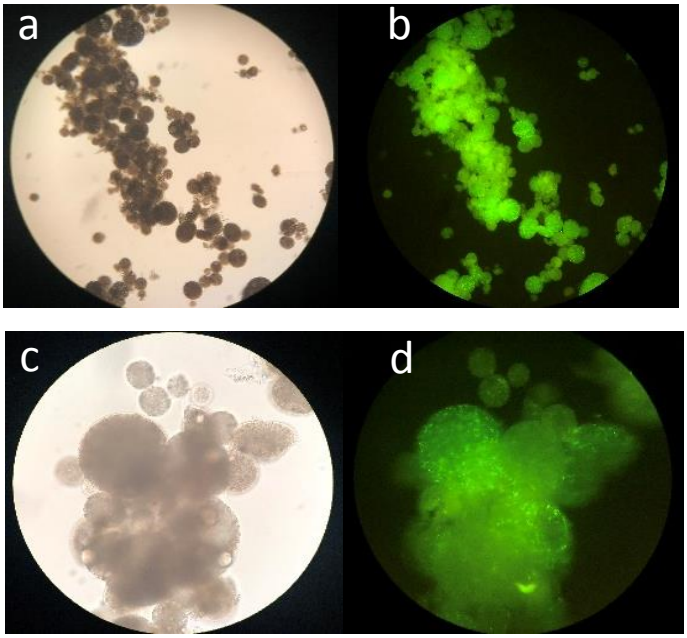


Figure 4-11 Images of chitosan-coated microparticles after 48 hrs incubation in seawater. (a,c) white light and (b,d) fluorescence images with (a,b) 100× and (c,d) 400× magnifications.

4.3.6 Tracking microparticles within the GIT of abalone

Tracking studies were carried out to explore the fate of microparticles within the abalone GIT. The *Exiguobacterium* cells were stained with SYTO9 (green) and PI (red) which are both nucleic acid stains. However, they differ in ability to penetrate live and dead cell membranes. Live cells with intact cell membranes are stained green whereas dead cells and cells with damaged membranes are stained red. The bacteria were successfully stained as illustrated in Figure 4-12. A small proportion of bacteria were stained red which were probably damaged during the staining process. However, for the purpose of this experiment, the microscopic observation was continued using green fluorescein filter as delivering live bacteria to animals was of top of importance.

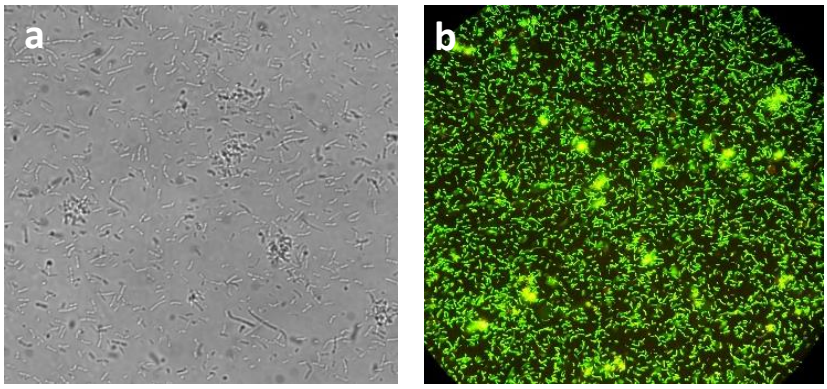


Figure 4-12 Micrograph of *Exiguobacterium* sp. Under (a) white light and (b) fluorescence filters (x400).

The stained bacteria were then encapsulated in chitosan-coated microparticles (Figure 4-13). The produced microparticles were immobilised in alginate beads to ensure they can sink to the bottom of the tank and do not float as abalone are bottom feeders. Bacterial staining, feeding, cryo-sectioning and microscopy were carried out in the dark to prevent quenching of the fluorescent dyes.

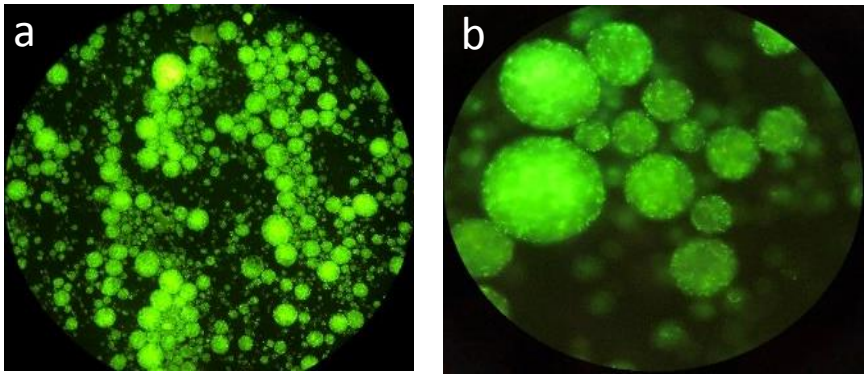


Figure 4-13 Microparticles containing stained bacteria under a fluorescence filter with (a) 100 and (b) 400 magnifications.

In abalone, food particles are grasped with foot muscle and graszed with radula. The food particles are broken down by mechanical forces exerted by radula and conveyed to buccal region. The food particles are mixed with mucus and carried down the oesophagus area by the strong ciliary movements and subsequently are transferred to

crop. The pH of the crop is reported to be around 5.2 in which food particles are stacked and mixed with strong digestive fluid and gradually enter to the stomach. Extracellular digestive enzymes are secreted into the stomach which are moved along with the food into the intestine (Figure 4-14). Intestine in abalone is long and consists of several sections with an average pH of 6.3. Food digestion and absorption continues in intestine due to the presence of enzymes such as amylase (McLean, 1970; Harris et al., 1998). A very limited information is available on the feed incubation, digestion and passage time for different segments of abalone digestive system where, signs of digestion have been observed from several minutes up to seven days after feeding (McLean, 1970; Harris et al., 1998).

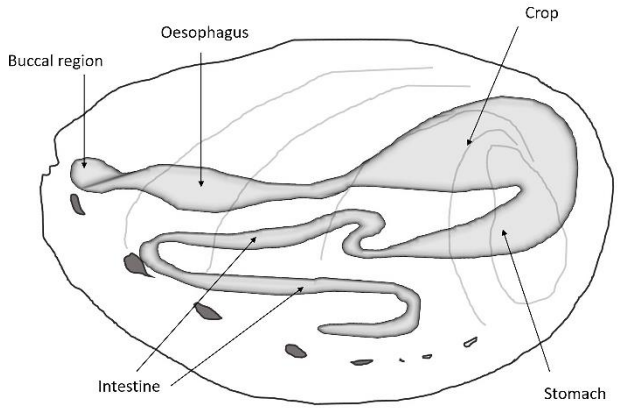


Figure 4-14 Schematic diagram of digestive tract of abalone.

Microscopic observations revealed the presence of microparticles in the entire length of abalone GIT 12 hrs post feeding showing successful delivery. A substantial load of microparticles was found in alimentary canal including buccal region, esophagus, post-esophagus regions, crop and stomach in specimens collected 12 hrs post feeding. The presence of microparticles in buccal region at this time point implies continuous consumption of microparticles which is common in abalone as they are slow-feeders (Wee, Maguire, & Hindrum, 1991).

A change in physical features such as size and shape of some of the eaten microparticles was observed in all specimens which could be due to the effect of radula, teeth-like structure in gastropods, and the effect of strong enzymatic digestion within the abalone GIT over the time (McLean, 1970).

At time points 12 and 24 hrs post-feeding, the amount of microparticles was highest in buccal pouches, esophagus regions, crop and stomach. Lesser amount of intact microparticles were found at the final sections of intestine. In addition, weaker fluorescein signals were observed toward the end of abalone GIT (Figure 4-15). Higher amount of microparticle residues with weak fluorescent signals were found toward the latter part of GIT at time point 48 and 72 hrs post feeding which could suggest a successful delivery of probiotics into the intestine of abalone. The chitosan coating should dissolve in acidic pH of stomach exposing alginate microspheres to enzymatic digestion and increased swelling. Bacteria that are released into the intestine may start to propagate and gradually lose their fluorescent signals.

From 12 to 72 hrs post feeding, the amount of microparticles inside the stomach was reduced which could be due to the unavailability of the food and gradual evacuation of intact and partially digested microparticles from the GIT. Weaker fluorescein signals from microparticles over time and towards abalone intestine could potentially indicate digestion of microparticles and release of bacteria from microparticles. However, it has to be mentioned that a small fraction of microparticles appeared in the animal faeces after 72 hrs post feeding which may be due to partial digestion and/or over feeding. However, the excreted microparticles showed holes and pores in their structure under SEM (Figure 4-16). This indicates that the bacteria were possibly released from the partially digested or undigested microparticles.

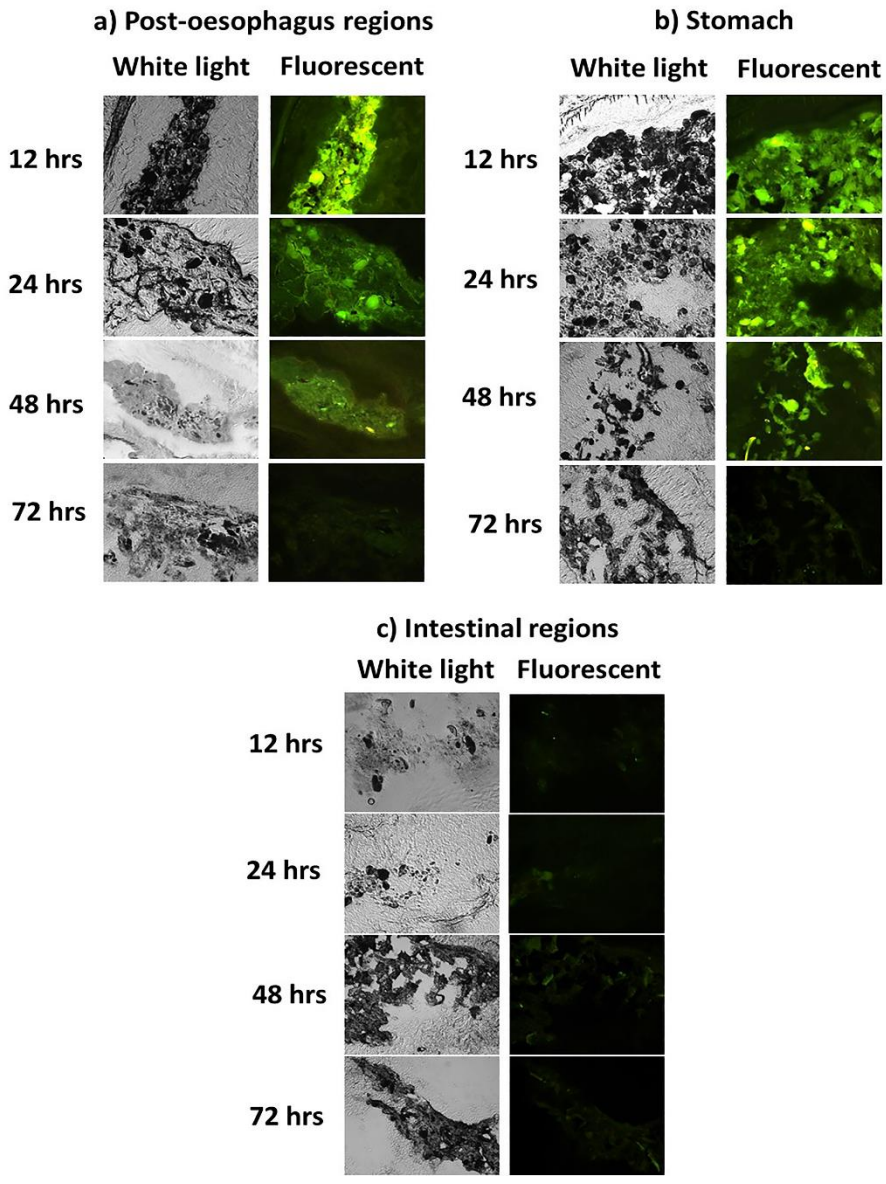


Figure 4-15 Fluorescence microscopy of abalone GIT at 12, 24, 48 and 72 hrs after feeding with SYTO9-labelled bacteria. Consumed microparticles were tracked mainly in three regions of abalone GIT including (a) oesophagus, (b) stomach and (c) intestine at each time point. Normal white and fluorescent images were captured from gut sections.

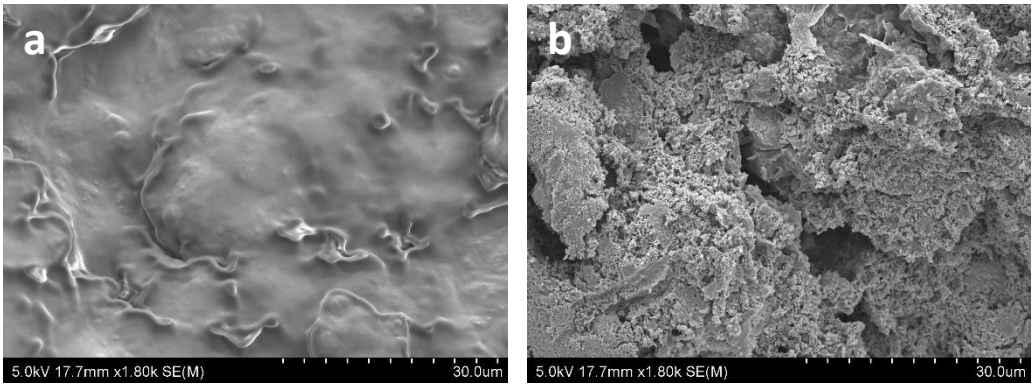


Figure 4-16 SEM images of (a) intact chitosan-coated microparticles and (b) excreted microparticles from animal's GIT.

4.3.7 Bacterial release in simulated gastric and intestinal fluid of abalone

The release of encapsulated bacteria was monitored over 48 hrs in simulated gastric medium (SGM) and simulated intestinal medium (SIM). The results suggest that the encapsulated bacteria start to be released in SGM and SIM two hrs post incubation. This could be due to acidic pH which dissolves the thin coating layer of chitosan. The release was slowly increased in both media; however, the number of released bacteria were always higher in SIM. The cell count of SIM was sharply increased by one log at 4 hrs differing from SGM by two logs. A significant difference ($P < 0.05$) was observed comparing the total bacterial release of SGM and SIM (Figure 4-17). The number of released bacteria increased dramatically in SIM (1.6×10^8/ml) as opposed to SGM (7.5×10^5/ml) after 48 hrs. This could be due to the stability of Ca-alginate core in acidic pH. While in SIM, the microparticles swelled as a result of increasing pH within intestine. Furthermore, the presence of strong enzyme activities such as carbohydrases (Garcia-Esquivel & Felbeck, 2006) could be another contributing factor to the digestion of microparticles and release of bacteria. The fluorescence images of released bacteria are shown in Figure 4-18.

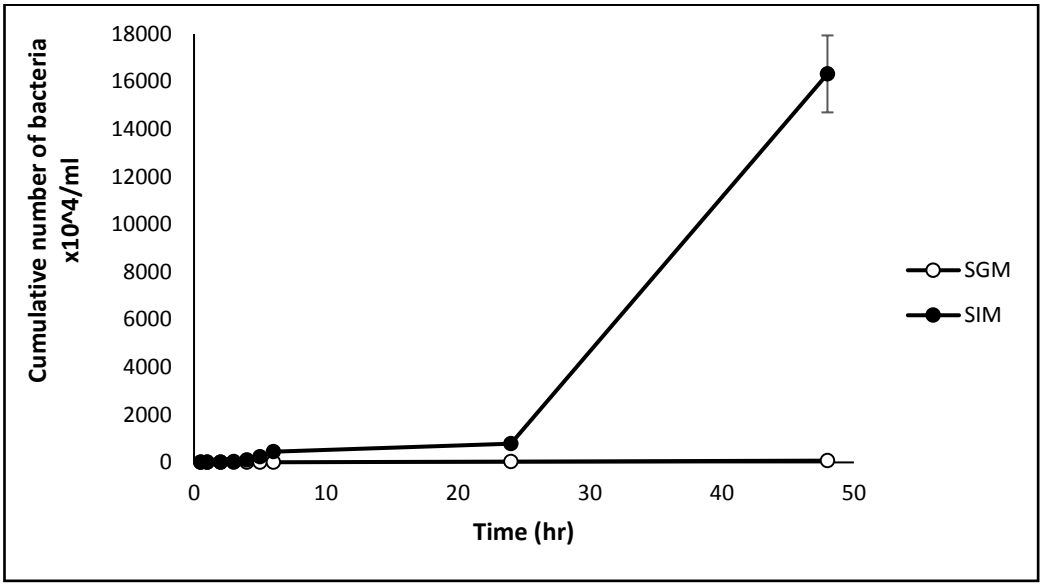


Figure 4-17 The release of encapsulated bacteria over time in SGM and SIM of abalone (n=3; mean ± SD).

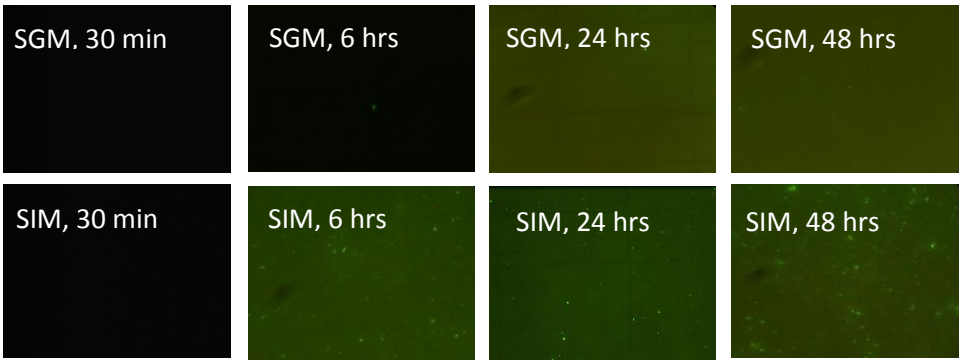


Figure 4-18 Fluorescent images of released bacteria in SGM and SIM of abalone.

4.3.8 Feeding trial

The bacterial load in the gastrointestinal tract of abalone fed with either commercial feed (control) or encapsulated probiotic were quantified two days after probiotic administration. The average amount of *Enterococcus* sp. was six logs higher in probiotic-fed (3.3×10^8 CFU/ml) than the control group (5×10^2 CFU/ml). This indicates the successful delivery of encapsulated probiotics into the gastrointestinal tract of abalone.

4.4 Overall discussion

Oral administration is one of the most practical ways to deliver probiotics to abalone. Although some researchers could successfully improve the growth and immunity of abalone by delivering probiotics through culture water or feed (Macey & Coyne, 2005; Doeschate & Coyne, 2008; Silva-Aciares et al., 2011; Hadi et al., 2014), dispersion of probiotics in water can lead to environmental and health issues while the uptake of sufficient dosages of live bacteria by animals is not guaranteed. The aim of this chapter was to formulate and evaluate the efficacy of chitosan-coated alginate microparticle as a carrier for probiotic administration to black-footed abalone.

The produced microparticles were composed of alginate and chitosan as the core and shell materials respectively. Alginate is one the most commonly used polymers for encapsulation due to simplicity of its formulation and compatibility with cells (Krasaekoopt et al., 2003). Several studies have reported the use of alginate microspheres for delivery of probiotics to fish (Rodrigues et al., 2006; Ghosh et al., 2016). Successful delivery, improvement in viability of probiotics and enhanced survival of probiotic-fed fish have been achieved using alginate microspheres. In addition, alginate can enhance palatability of microparticles as it is derived from seaweed which is the natural food for abalone. However, the porous structure has been reported for alginate microspheres produced by internal gelation which can accelerate the bacterial release from the microparticles via diffusion (Quong, Neufeld, Skjak, & Poncelet, 1998). Utilising chitosan as a coating layer around alginate microspheres could act as a physical barrier reducing the bacterial release into seawater.

Emulsification is a widely used technique that can be used for microencapsulation of probiotics (Shima, Matsuo, Yamashita, & Adachi, 2009; Song, Yu, Gao, Liu, & Ma, 2013; Rodriguez-Huezo et al., 2014). Alginate microspheres can be formulated using either external or internal gelation techniques. In external gelation, an external source of calcium is used during encapsulation. An emulsion is formed by dispersing polysaccharides with oil phase. A solution of $CaCl_2$ is then added to the stirred emulsion for gelation to occur. A high chance of clumping non-solidified materials was reported

as the addition of cross-linking solution to the emulsion disrupts the emulsion system (Poncelet et al., 1999). In internal gelation, an internal calcium source is used in form of adding powder to polysaccharide solution. The mixture is then added to an oil continuous phase to form an emulsion. Calcium ions will then be released by reducing pH of the system using a weak acid (Poncelet et al., 1995; Quong et al., 1998; Song et al., 2013).

In this study, an internal gelation emulsification method was used to produce microparticles. It has been reported that using this method a narrow size distribution, uniform and homogenous gels and minimal leakage during formation and storage can be obtained compared to external gelation (Quong et al., 1998; Song et al., 2013). During encapsulation process, alginate and probiotics were mixed homogenously. An insoluble salt of $CaCO_3$ was added as a source of calcium ions for cross-linking of alginate polymer chains. The mixture of alginate, probiotic and $CaCO_3$ was used as the dispersion phase during emulsification. Sunflower oil was selected as continuous phase and added to the mixture to generate a water in oil (W/O) emulsion. Sunflower oil was selected based on the safety, content of saturated fatty acid, stability of emulsion reported in literature (Garti & Remon, 1984) and market price. Span 80 was used as emulsifier to stabilise the newly formed alginate droplets within the continuous phase. This emulsifier has a low hydrophilic-lipophilic balance (HLB) value which is suitable for producing W/O emulsion. After an emulsion was formed, acetic acid was added to release calcium ions from $CaCO_3$ salt for cross-linking with alginate monomers (Poncelet et al., 1995). The developed microparticles were then collected and washed thoroughly to remove surface oil and to expose alginate surface for interaction with chitosan as coating layer. The pH of chitosan solution was increased before coating as low pH could negatively impact bacterial viability. Chitosan is a polycationic polymer which can easily form a network with anionic molecules such as alginate through ionic interactions (Berger et al., 2004). The chitosan-coated microparticles were collected by centrifugation and washed with distilled water to remove excess chitosan. After washing with distilled water, particles had a zeta potential value close to zero (Table 4.1) that caused agglomeration of CALG

microparticles. Therefore, a second washing step was carried out using $CaCl_2$ solution to increase surface charge of the particles and to avoid agglomeration of chitosan-coated microparticles.

The microparticles produced by internal gelation were in the micrometer range of 10 to 1000 µm with an average diameter of 113 µm. The mean diameter of CALG microparticles were within the particle size range edible by abalone (Campbell, 1965). The spherical shape of obtained microparticles could be due to the high degree of cross-linking (Antonio et al., 2005). The sphericity of obtained CALG particles are desirable as it can improve the mechanical and chemical stability of microspheres as opposed to particles with irregular or non-spherical shape (Al-Hajry et al., 1999). Furthermore, this can provide a better control over the release of encapsulates and allow generating reproducible results (Lee, Ravindra, & Chan, 2013).

The surface structure analysis of the microparticles using SEM imaging revealed a relatively spherical, but slightly uneven granulated surface structure for ALG microspheres (Figure 4-5a). In contrast, a smoother surface was observed for CALG microparticles (Figure 4-16a) which can show that chitosan coating was indeed present and modified the overall surface of the microparticles. Previous studies also reported a smoother surface structure in chitosan-coated particles as compared to uncoated particles (Pasparakis & Bouropoulos, 2006; Mokarram et al., 2009).

High encapsulation efficiency of 75 to 85 % was achieved indicating the minimal effect of encapsulation process on the viability of the probiotic bacteria. An encapsulation efficiency of about 37.9% was reported by Cai et al. (2014) encapsulating *Lactobacillus acidophilus* using emulsion/internal gelation (Cai et al., 2014). Another study reported 93.4% encapsulation efficiency for *Bacillus subtilis* using a mixture of alginate and gelatine during emulsification/internal gelation (Tu et al., 2015). These variations may be due to the sensitivity of the different probiotic strains. Furthermore, the solvent used to release the encapsulated bacteria from microspheres varies which is an influential

factor on probiotic viability as different bacterial strains are variously susceptible to environmental conditions.

FTIR spectra showed strong absorption bands at 1607 cm^{-1} and 1412 cm^{-1} that correspond to asymmetric and symmetric stretching vibration of carboxylate groups (Ribeiro et al., 2005; Li et al., 2008). The carboxylate vibration could be shifted by the presence of $CaCO_3$ from 1394 cm^{-1} to 1409 cm^{-1} in ALG microspheres. The presence of chitosan coating deviates the carboxylate bands of ALG spectrum due to the interaction with $–NH^{3+}$ groups of chitosan.

The main aim of adding an extra layer of chitosan around alginate microspheres was to provide a protective layer against bacterial release into the seawater. The release profile of encapsulated bacteria from chitosan-coated microparticles in seawater indicates that the addition of an extra layer of chitosan around the microparticles can delay the onset of bacterial release and reduce the total release of bacteria in seawater. Lack of bacterial release in the first six hours can undoubtedly minimise the chance of environmental contamination during probiotic administration. In addition, it maximises the number of deliverable probiotics to abalone via the microcapsule delivery system.

In all the feeding experiments, microparticles were immobilised in an alginate bead to prevent them from floating in seawater. This facilitates accessibility of microparticles to abalone. Microparticles float in water due to their small size and density. Having them imbedded in bigger alginate beads facilitates their settlement in the bottom of the tank and as abalone are bottom feeders, this maximised feed consumption rates. The increased bacterial load proved the successful delivery of bacteria to the GIT of animal using microparticles.

Tracking of encapsulated stained probiotics in the GIT of abalone was essential to study the fate of microparticles and visualise the successful delivery of probiotics into the intestine of abalone. Presence of intact microparticles with strong fluorescent signals indicated the resistance of microparticles to degradation at low pH and enzymatic content of crop and stomach sections. Therefore, chitosan-coated microparticles

imbedded in alginate beads were not only able to protect their content in seawater but also against degradation in abalone gastric region. This is because chitosan coating remains intact in sea water and starts to dissolve slowly in acidic pH of stomach and by the time microparticles reach the intestine, the coating may disappear which can facilitate bacterial release. To back this hypothesis, bacterial release was investigated in *ex-vivo* simulated gastric and intestinal media. Only a small number of bacteria was released from microparticles into simulated gastric medium which was ideal and in agreement with other studies (Joosten et al., 1997; Fundueanu et al., 1999; Romalde et al., 2004; Rodrigues et al., 2006). However, in the intestinal medium, a faster bacterial release rate was observed which is probably due to the swelling of alginate microspheres at slightly higher pH values. Similar results have been reported where alginate microspheres swelled when put in media with pH values higher than pH 5 which subsequently led to the release of encapsulated bioactives (Joosten et al., 1997). The stability of chitosan-coated alginate microparticles in slightly acidic environments (pH of 4-5) followed by a very high bacterial release rates at higher pH was also reported by Cook et al. (2011) which confirms a pH- dependent release of encapsulated bacteria from chitosan-coated alginate microparticles (Cook et al., 2011). This can also explain the weak fluorescent signals obtained in tracking studies from the particles in intestinal regions as more fluorescent bacteria are released and start to propagate in the presence of nutrients within the GIT of animal resulting in the loss of fluorescent signal. By increasing the bacterial counts in the intestinal region of abalone, this microencapsulated delivery system can increase beneficial effects of probiotics as relatively long intestine in bivalves provides more surface for probiotics to colonise. More favourable pH of intestinal sections for the three probiotics can also help in their propagation and implementation of their activities that they were selected for. The occurrence of bacterial release and the presence of highly porous and damaged microparticles in animal faeces indicate that the encapsulated bacteria can diffuse from the microparticles as microparticles start to swell and get digested by digestive enzymes.

Therefore, it can be speculated that the encapsulated bacteria can be released from CALG microparticles via diffusion and erosion. Diffusion can be facilitated via the porous structure of alginate particles. This release mechanism has been previously reported for alginate microspheres produced by internal gelation (Quong et al., 1998). Erosion can be considered as the second possible mechanism for bacterial release from the microparticles. Erosion of microparticles can occur with radula's scraping function, pH-dependent dissolution of microparticles in gastrointestinal tract of abalone and enzymatic degradation of microparticles (Figure 4-19).

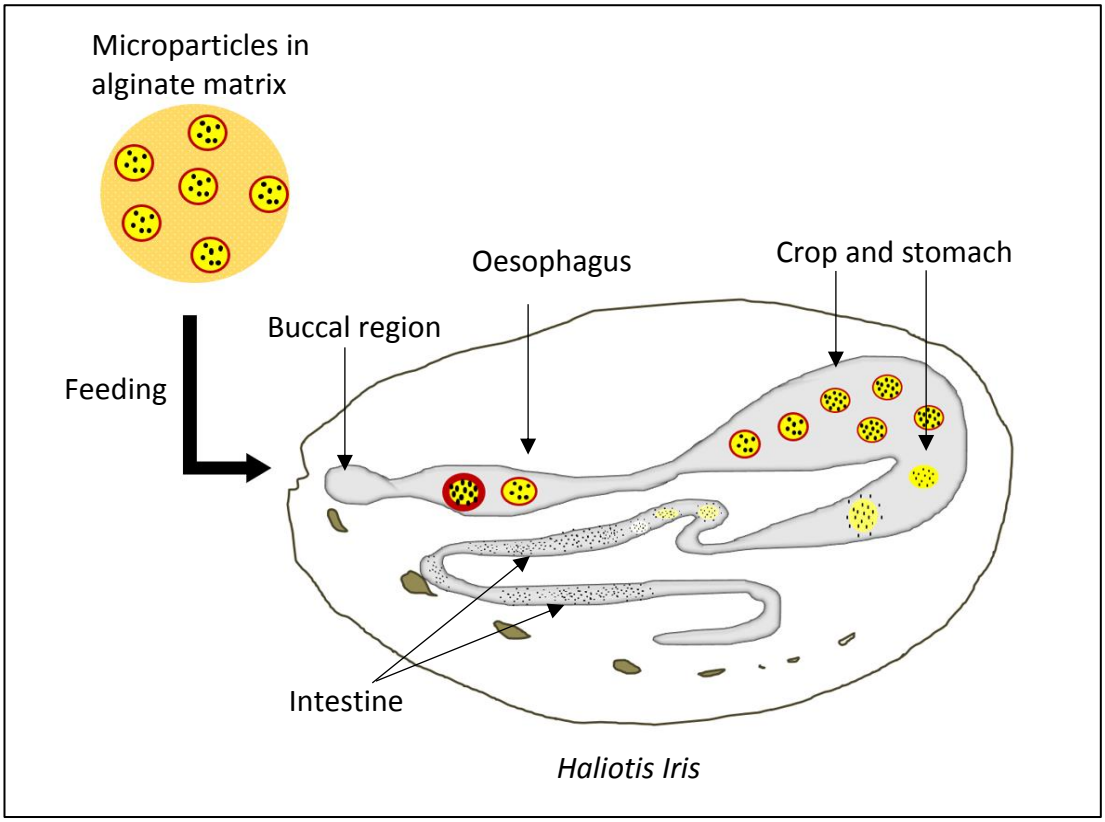


Figure 4-19 Schematic diagram of the controlled release of encapsulated bacteria. The alginate matrix may be broken down by radula's scraping function and the immobilised microparticles are released from the alginate matrix into the buccal region and oesophagus of abalone. The chitosan coating of the microparticles is then gradually dissolved in acidic pH of crop and stomach and encapsulated bacteria are partially released by diffusing from the particles. The

alginate cores of microparticles are swelled and dissolved in basic pH of intestine leading to a complete release of the encapsulated bacteria into the intestine of abalone.

This study proves that encapsulation of probiotics in chitosan-coated alginate microparticles can be potentially applied for targeted delivery of beneficial bacteria to black-footed abalone. This could address some of the concerns associated with traditional methods of probiotic administration such as risk of environmental contamination, loss of bacterial viability, lack of control over the dosage and site of release of administered probiotics.

4.5 Conclusion

In this study, an emulsion technique was used to produce microspheres for delivery of live probiotics to black-footed abalone. Spherical chitosan-coated alginate microparticles were palatable and could efficiently protect probiotics in seawater. The developed microspheres showed high encapsulation efficiency for live probiotic bacteria and were able to specifically deliver them to the abalone's intestine. This study is the first report to demonstrate that the encapsulation of probiotics in chitosan-coated alginate microparticles can be potentially applied for targeted delivery of beneficial bacteria to abalone. This approach could address some of the concerns associated with traditional methods of probiotic administration, such as risk of environmental contamination, loss of bacterial viability, lack of control over the dosage and site of release.

Chapter 5

Encapsulation of probiotic and characterisation of chitosan-coated alginate beads for oral administration to abalone

"When scientific literature says something isn't possible, you just have to create possibilities that don't exist."

Robert Langer

Abstract

The potential benefits of probiotics for growth of black-footed abalone (*Haliotis iris*) have been highlighted in previous studies. However, traditional methods of delivering probiotics to aquaculture species are inefficient due to possible environmental contamination and bacterial loss during delivery. The objective of this chapter is to develop an efficient delivery system based on chitosan-coated alginate beads (CCALG) for improved delivery of three probiotic bacteria (*Exiguobacterium sp., Enterococcus sp*. and *Vibrio sp*.) previously shown to enhance growth in this species. An extrusion technique was utilised to produce CCALG beads, which were then fed to juvenile abalone in controlled experiments.

A stepwise approach was used to optimise the final formulation of CCALG beads with desired morphology, gel stability, sinking time and release profile. Alginate beads (ALG) were obtained using a final concentration of 1.5% w/v sodium alginate with a viscosity of 0.41 ± 0.06 pa.s for the bead formulation. Sterilising (121˚C, 15 min) the alginate solution before bead formation reduced the viscosity of sodium alginate. Therefore, the characteristics of beads produced from heat treated sodium alginate (AtALG), were notably influenced by the sterilisation process. Lower sinking time (167 ± 12 sec) and matrix erosion (3.1% ± 0.3) after 72 hrs incubation in seawater were achieved for ALG beads compared to those of AtALG beads (254 ± 15 sec, 8.6% ± 0.1).

ALG beads were then coated with 0.8% w/v of chitosan for 20 min (CCALG-0.8-20). CCALG-0.8-20 beads were found to be more stable in seawater due to a lower percentage of matrix erosion (0.9% ± 0.2) after 72 hrs incubation in seawater compared to ALG beads (3.1% ± 0.3). In addition, a considerably lower release of encapsulated probiotics in seawater was achieved using CCALG-0.8-20 beads compared to ALG beads. An observational study revealed high palatability of CCALG beads for abalone. Probiotic-fed abalone had significantly higher probiotic bacterial loads ($1.3 \times 10^8\ CFU/ml$) than the control animals ($6 \times 10^3\ CFU/ml$) ($P < 0.05$) . The tracking experiment resulted in successful delivery of fluorescent-labelled encapsulated bacteria to the

gastrointestinal tract (GIT) of probiotic-fed abalone. The findings of this research indicate that CCALG beads are efficient in delivering probiotics to abalone.

5.1 Introduction

Advanced aquaculture practices are required for complete removal of synthetic agents and development of green strategies to achieve a sustainable production. The advent of probiotics in aquaculture research reflects targeted efforts by researchers around the globe to achieve this aim. The approval for the wide use of probiotics in food production from the Food and Agriculture Organisation (FAO) has paved the way and drawn more attention to the use of these beneficial organisms instead of chemotherapeutics in aquaculture (FAO/WHO, 2002). A great number of studies have also shown the beneficial effects of probiotics on aquatic animals (Sahu et al., 2008; Talukder Shefat, 2018; Zhao et al., 2018). Most probiotics applications for farmed animals are through water treatment or as feed additives (Alessandro & Chen, 2009). These conventional delivery methods can introduce environmental issues and increase production costs.

In conventional delivery methods, safety issues may arise due to the release of live microorganism into farm effluent water which may increase the risk of environmental contamination. This issue requires attention as one probiotic species may be beneficial for one aquatic species but pathogenic to others. Another concern around the delivery of probiotics is the dosage and viability of organisms since the initial dosages may be diluted in the culturing water before they reach the target animals. The loss in viability of probiotics during feed preparation and storage is another concern. Therefore, further research is required to develop economic and environmentally friendly methods for delivering probiotics to aquaculture settings.

Controlled and targeted drug delivery strategies used in pharmaceutics have been adopted by marine scientists to develop new delivery methods for nutrients, probiotics (Pirarat et al., 2006; Kumaree et al., 2014; Pinpimai et al., 2015; Madreseh et al., 2018; Xia et al., 2018) and immunostimulants in aquaculture (Adomako et al., 2012; Vidhya

Hindu et al., 2018). Biocompatible and biodegradable natural polymers have been used to develop confinement structures in the form of capsules and beads to maintain the viability of probiotics and to obtain a targeted delivery to a host (Zhang, Lin, & Zhong, 2015; Rodriguez et al., 2018).

Several materials and methods have been used to create confinement structures for delivery of bioactives to aquatic animals (Darmody et al., 2015; Pinpimai et al., 2015; Pirarat et al., 2015) among which alginate particles formed by dripping technique have been used frequently. Alginate is an excellent polymer to be used for cell encapsulation due to its non-toxic nature (Lee & Heo, 2000). Encapsulates can be immobilised easily in alginate matrix simply by extrusion into a hardening solution (Maurice et al., 2004; Romalde et al., 2004; Behera & Swain, 2014). However, the encapsulation process must be optimised to achieve a controlled release of encapsulated materials into a specific target site. In the present research, an extrusion technique was utilised to develop a formulation to deliver probiotics to black-footed abalone. A full characterisation of the developed carrier was performed to identify an optimised formulation with high stability in seawater, easy accessibility and palatability to deliver viable probiotics to abalone. Therefore, the specific objectives of this chapter are:

- to establish optimal conditions for preparation of chitosan-coated alginate beads,
- to characterise the optimised formulation according to morphological features, release profile in seawater and viability of encapsulated probiotics following encapsulation and different storage and temperature conditions, and
- to evaluate the palatability and efficacy of the developed beads to deliver encapsulated probiotics to gastrointestinal tract of abalone.

5.2 Materials and methods

5.2.1 Materials

Sodium alginate was purchased from AcrosOrganics (Beijing, China). Medium molecular weight chitosan was acquired from Sigma (St. Louis, USA). Calcium chloride was obtained from Ajax Finechem (New South Wales, Australia). LIVE/DEAD Bacterial Viability Kit (L7012) was purchased from BacLight (Oregon, USA). Fluorescence-labelled chitosan was purchased from Creative PEGWorks (Durham, USA). Gaspak^{TM} EZ anaerobic container system was obtained from Becton Dickinson (New Jersey, USA). All the other chemicals were of analytical grade. Juvenile black-footed abalone (25-35 mm shell length) were provided by Moana New Zealand (Ruakaka, New Zealand). Bacterial isolates were kindly provided by the Aquaculture and Biotechnology Research Group at Auckland University of Technology (Auckland, 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).

5.2.2 The optimisation process

This study aimed to develop and optimise CCALG bead formulation for encapsulating probiotics. Beads were intended to be used as oral delivery vehicles for probiotics to abalone. A step-by-step approach was applied to optimise the properties of CCALG beads. The core and shell of the beads were characterised in three main experimental phases as listed in Table 5.1. In phase I, alginate was considered as the core material to produce plain polymeric ALG beads. Here, the percentage of alginate was optimised considering viscosity of obtained formulations, morphology and sinking time of ALG beads in seawater. The concentration of alginate which produced spherical ALG beads with lowest settling time in seawater was selected as optimised and was further used in phase II of the optimisation process. In the second phase, AtALG beads were produced using autoclaved alginate solution. The purpose of using autoclaved alginate was to keep the encapsulated cells free from contamination as well as to reduce the chance of contamination of alginate stock solution during storage. The characteristics of both ALG and AtALG beads were compared to find the best formulation in terms of settling time

in seawater, morphology and percentage of matrix erosion. The optimised form of sodium alginate solution was selected and used in phase III of the optimisation process. In this phase, the optimised form and concentration of alginate were used to produce CCALG beads. The percentage of chitosan in the coating solution and the coating time were then studied to determine a single formulation with minimum bacterial release and matrix erosion in seawater.

Table 5.1 Optimisation of the beads: parameters and variables.

Phase of optimisation process	Parameters	Formula Code	Final concentration of Alginate in bead formulation (% w/v)	Concentration of chitosan in coating solution (% w/v)	Coating time (min)	Selected formulation
I	Concentration of sodium alginate	ALG-0.5	0.5	-	-	ALG-1.5
		ALG-1.0	1.0	-	-	
		ALG-1.5	1.5	-	-	
		ALG-2.0	2.0	-	-	
II	Form of sodium alginate solution	ALG	1.5	-	-	ALG
		AtALG	1.5	-	-	
III	1-Concentration of chitosan in coating solution 2- Coating time	CCALG-0.2-20	1.5	0.2	20	CCALG-0.8-20
		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	

5.2.3 Formulation of alginate beads

Figure 5-1 illustrates the diagrammatic flow of the encapsulation process used to produce alginate beads using extrusion. In order to facilitate the optimisation process and reduce experimental cost, blank media (marine broth containing yeast extract (1% w/v)) was used instead of probiotic cultures, unless otherwise stated.

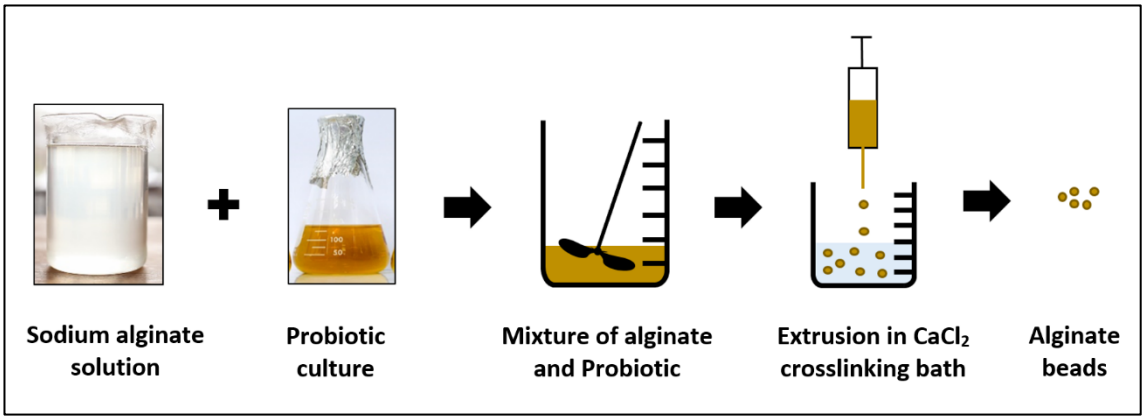


Figure 5-1 Flow diagram of the formulation of alginate beads containing probiotics.

5.2.3.1 Preparation of alginate beads (ALG)

Sodium alginate (SA) stock solutions (2% w/v and 2.5% w/v) were prepared by dissolving sodium alginate powder in distilled water (dH_2O) which was stirred overnight to obtain a fully dissolved alginate solution. The SA stock solutions were stored in a refrigerator (4˚C) until use.

Alginate beads (ALG) 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 fall from about 10 cm into a 100 ml $CaCl_2$ cross-linking bath (0.1 M). The produced particles were collected using a sieve after 30 min incubation in the agitated cross-linking bath (200 rpm). Alginate beads were then washed with dH_2O (100 ml) twice to remove unbound $CaCl_2$ 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 5.2).

Table 5.2 Composition of ALG beads.

Formula Code	SA (ml)	Media (ml)	dH_2O (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

5.2.3.2 Preparation of alginate beads (AtALG) with autoclaved alginate solution

A stock solution of autoclaved alginate was used to produce alginate beads (AtALG). The alginate stock solution was prepared as described in section 5.2.3.1. AtALG beads were prepared as previously described apart from the sodium alginate solution which was autoclaved at 121˚C for 15 min prior to formulation. The alginate final concentration of 1.5% w/v was used in order to produce AtALG beads. The morphology, sinking time and stability of these beads in seawater were compared with those of ALG beads.

5.2.3.3 Preparation of chitosan-coated alginate beads (CCALG)

Chitosan coating solutions with different concentrations of 0.2, 0.4 and 0.8% w/v were prepared by dissolving appropriate amounts of chitosan in lactic acid (1% v/v in dH_2O) with pH adjusted to 5.70 by gradual addition of NaOH (1M). The chitosan solution was stirred (200 rpm) until a clear solution 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 chitosan-coated alginate beads (CCALG) were then separated using a plastic grid and washed with dH_2O (100 ml) twice for further characterisation.

5.2.4 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 measurement was carried out using a rheometer fitted with cone NO. CCT-25 accepting 15 ml of sample.

Measurements were made at room temperature (26˚C) while the shear rate was increased from 50 to 400 s^{-1}. The duration time was 120 sec with a total of 100 measurement points for each sample.

5.2.5 Spectroscopic analysis

Fourier-transform infrared (FTIR) spectra of ALG and CALG were obtained using a spectrometer (Nicolet™ iS10, USA) equipped with a horizontal attenuated total reflectance (ATR) crystal. Pure powders of alginate, chitosan and $CaCO_3$ were used to obtain references. An average of 32 scans at 4 cm^{-1} resolution were recorded within the wave number range of 400-4000 cm^{-1}.

5.2.6 Size and morphology

The mean diameter of fifty beads was measured using a light microscope equipped with a scaled eyepiece and a stage micrometre 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 aluminium 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).

5.2.7 Sinking time in seawater

The sinking time of particles 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 travelled the column height was recorded as its sinking time. The measurements were repeated for at least ten individual particles.

5.2.8 Matrix erosion

Beads produced in section 5.2.3. containing *Exiguobacterium* sp., *Enterococcus* sp. and *Vibrio* sp. probiotics (7-9× 10^9 CFU/g) were used to study the stability in seawater. The beads were then placed in beakers (3.0 g/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 hrs 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 formula:

$$\text{Matrix erosion } (\%\ \text{w/w}) = \frac{(\text{W0} - \text{W})}{\text{W0}} \times 100$$

where W0 is the initial dry weight of the beads and W is the mass of dry beads after incubation in seawater.

5.2.9 Bacterial staining with fluorescent dye

A single colony of *Exiguobacterium* sp. was inoculated in marine broth (100 ml) containing yeast extract (1% w/v). The inoculated medium was incubated at room temperature for 18 hrs 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 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 µL from each pre-warmed fluorescence dyes were combined in a dark tube. 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.

5.2.10 Bacterial release study

Fluorescent-labelled bacteria were used to produce chitosan-coated and uncoated beads as described in section 5.2.3. *Exiguobacterium* sp. was used in this test because it

can be recognised 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 µm). The tubes were kept at 15˚C to mimic the temperature at which abalone were maintained. Aliquots of 40 µl were withdrawn from the release medium at various time intervals (1, 2, 3, 4, 5, 6, 24, 48 and 72 hrs). A volume of 10 µl 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.

5.2.11 Fluorescence microscopy of chitosan coating

The presence of chitosan coating around the alginate beads was examined using fluorescence-labelled chitosan (Creative PEGWorks, USA). Alginate particles were produced as described in section 5.2.3. The rhodamine-labelled chitosan solution was prepared by dissolving fluorescent-labelled chitosan powder (0.6 mg/ml) in normal chitosan solution (0.8% w/v, pH: 5.7). CCALG beads were then prepared by immersing ALG beads in rhodamine-labelled chitosan solution and stirring at 100 rpm for 20 min. The produced CCALG beads were then rinsed with dH_2O (100 ml) twice. The uncoated and chitosan-coated particles were observed using a fluorescence microscope (Olympus BX51TRF, Japan) with excitation/emission wavelength of 552/575 nm.

5.2.12 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 depolymerising 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 µl of each dilution on KAA and marine agar plates. Cultured KAA plates were incubated in anaerobic conditions for 48 hrs. Cultured marine agar plates were incubated at room temperature until the colony colours were fully developed. The percentage of bacterial survivability after encapsulation was calculated as follow (Martin et al., 2013):

$$Survivability\ (\%) = \frac{N}{N0} \times 100$$

Where N is the number of released viable bacteria from the beads and $N0$ is the number of viable bacteria added to the formulation before encapsulation.

5.2.13 Observational evaluation of acceptability and palatability of the developed beads

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 (8 pellets) to acclimatise 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, 8 commercial pellets and 8 encapsulated probiotic beads were placed in the tank simultaneously (Figure 5-2). Feeds were then distributed evenly within the tank. The number of uneaten feed pellets and CCALG beads were counted after 24 hrs of incubation. The experiment was performed in triplicate.

Figure 5-2 Set up for assessing feed palatability.

The eating behaviour of abalone was recorded using a digital single lens reflex camera (Figure 5-3). 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.

Figure 5-3 Camera placed under the aquarium containing abalone to record the feeding behaviour of the animals.

5.2.14 Tracking of encapsulated bacteria in the gastrointestinal tract of abalone

The efficiency of CCALG beads in delivering encapsulated probiotics to the GIT of abalone was investigated by labelling *Exiguobacterium* sp. with fluorophores (similar to section 5.2.9) and encapsulating them in chitosan-coated alginate beads. A total of 16 juvenile abalone (25-35 mm) were transferred into two different tanks with 10 L of filtered seawater maintained at 15˚C. All animals were starved for 24 hrs. CCALG beads (4.0 g) with encapsulated fluorescent labelled *Exiguobacterium* sp. were placed in one tank. Empty CCALG beads were used as a diet for the control animals. The uneaten beads were removed from each tank after 12 hrs. Two animals were collected from each tank at 12, 24, 48 and 72 hrs post feeding. Two animals collected at each time point were

dissected to remove the gastrointestinal tract. Tissue samples were frozen in a tissue freezing medium (Leica, UK) and Surgipath Frostbite rapid coolant (Leica, UK) and cut using a Leica CM1850 cryostat (UK). Thin sections (40 µm) from various layers were collected on a glass slide and kept in the dark at -25°C until microscopic observational could be made. Fluorescence images were obtained using an Olympus BX51 microscope equipped with a camera (Optronics, Japan) and MagniFire imaging software (DP12, Olympus, Japan).

5.2.15 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 hrs before the beginning of the experiment. Each tank contained 10 L of filtered seawater (50 µm) and was aerated with two air stones and seawater was checked regularly for dissolved oxygen (100%), pH (8-8.3), ammonia (0-0.25 ppm) and nitrite (0-0.25 ppm) concentrations.

Water temperature was maintained at 15˚C and the water was replaced with fresh seawater before removal of uneaten beads and placement of fresh beads every two 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 two 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 homogenised 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 homogenised solution. A volume of 100 µl from each dilution was cultured on KAA plate and incubated for 72 hrs at room temperature until the colours of colonies were fully developed. Differences between bacterial cell counts were identified using the Student's t-test analysis ($P < 0.05$).

5.2.16 Effect of storage condition on the viability of encapsulated bacteria

The viability of encapsulated bacteria in optimised 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 following protocols described in section 5.2.3. The produced beads were then stored at 4˚C for 96 hrs. The number of bacteria were enumerated every two 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 µl) on KAA and marine agar plates. The number of bacteria was recorded after 72 hrs of incubation at room temperature when the colour of the colonies was fully developed. The experiment was performed in triplicate.

The second part of the storage study focused on the viability of encapsulated probiotics after freeze drying (lyophilisation). Overnight cultures of the three probiotic bacteria were prepared as explained in section 3.2.2. 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 section 5.2.3.3. The produced beads (4.0 g) were incubated in a freezer (-20˚C) for 12 hrs and subsequently lyophilised for 24 hrs (-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 µl from all dilutions was plated on marine agar and KAA plates. The cultured petri dishes were incubated for 72 hrs at room temperature until the colour 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.

5.2.17 Statistical analysis

Statistical analysis of matrix erosion and bacterial release parameters were conducted with Two Way ANOVA (Coating concentration and coating time as fixed factors). Data from release tests were subjected to One Way ANOVA with a SPSS computer program

(version 20) with the significance value set to 0.05. For multiple comparison Tukey's post-hoc test was performed with a *P* value set to 0.05.

5.3 Results and discussion

5.3.1 Phase I: effect of concentration of sodium alginate on the characteristics of the beads

5.3.1.1 Viscosity

While the pH of all alginate formulations remained similar (7.05 ± 0.02), an increase in viscosity of alginate formulations was observed as alginate concentration increased (Table 5.3). The apparent viscosity of alginate formulations was gradually decreased with increasing shear rate (Figure 5-4). This fluid behaviour is a characteristic of non-Newtonian shear-thinning fluids which can be seen in polymeric systems (Belalia & Djelali, 2014). The extent of changes in the viscosity of the tested formulations was related to different concentration 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 offers higher resistance against deformation or flow by demonstrating higher viscosity. These interactions then gradually disappeared at higher shear rates which facilitate flow.

Table 5.3 Viscosity of alginate solutions used in the formulation of beads (n=100 ± SD).

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

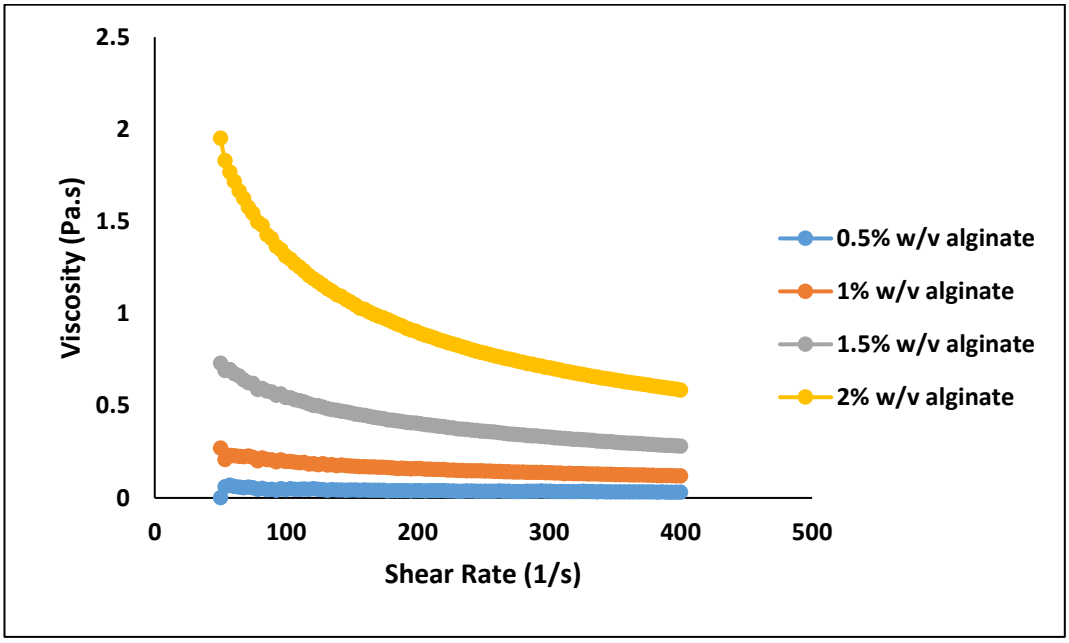


Figure 5-4 Plot of viscosity versus shear rates. Conditions: Average of 100 measurements. Time: 120 sec, Shear rate= 50-400, Temp: 26˚C.

5.3.1.2 Morphology of beads

The diameter of fifty individual particles was measured and the results are presented as mean diameter ± SD in Table 5.4. The size of alginate particles was increased by increasing alginate concentration.

Table 5.4 Measured characteristics of ALG beads (n= 50 ± SD for size, n= 10 ± SD for sinking time).

Formula code	Shape	Average size (mm)	Sinking time in seawater (sec)
ALG-0.5	Irregular	3.0 ± 0.1 × 2.7 ± 0.1	473 ± 12
ALG-1.0	Spherical	3.2 ± 0.0	280 ± 18
ALG-1.5	Spherical	4.1 ± 0.1	157 ± 37
ALG-2.0	Pear	6.7 ± 0.3 × 4.6 ± 0.2	83 ± 14

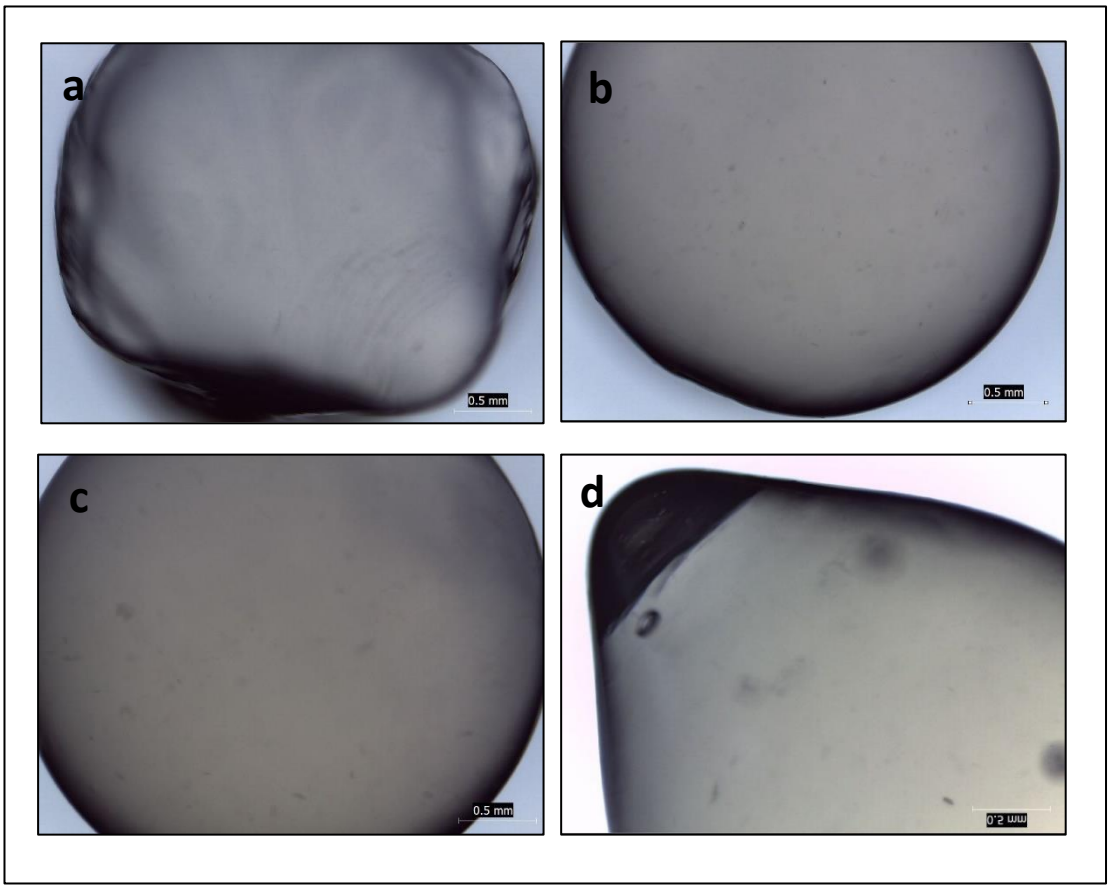


Figure 5-5 Morphology of alginate beads with (a) 0.5, (b) 1, (c) 1.5 and (d) 2% w/v alginate under a light microscope (x40).

The morphologies of alginate beads are illustrated in Figure 5-5. 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 encapsulates. This can happen due to the presence of areas with different thicknesses in non-spherical particles which can generate a unique degradation and release profile due to changes in their shape over time in the release media (Velings & Mestdagh, 1995).

5.3.1.3 Sinking time in seawater

The sinking time of alginate beads was measured as the time that a particle moved a specific distance through seawater. 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 (Velings & Mestdagh, 1995). As shown in Table 5.4, 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 (Del Gaudio et al., 2005).

In order to improve the sinking rate, ALG beads could be soaked in seawater for 5 min. The results showed that the pre-soaking 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 isosmotic with seawater. Therefore, they exhibited significantly higher sinking rate compared to ALG bead with no soaking step in seawater (Figure 5-6).

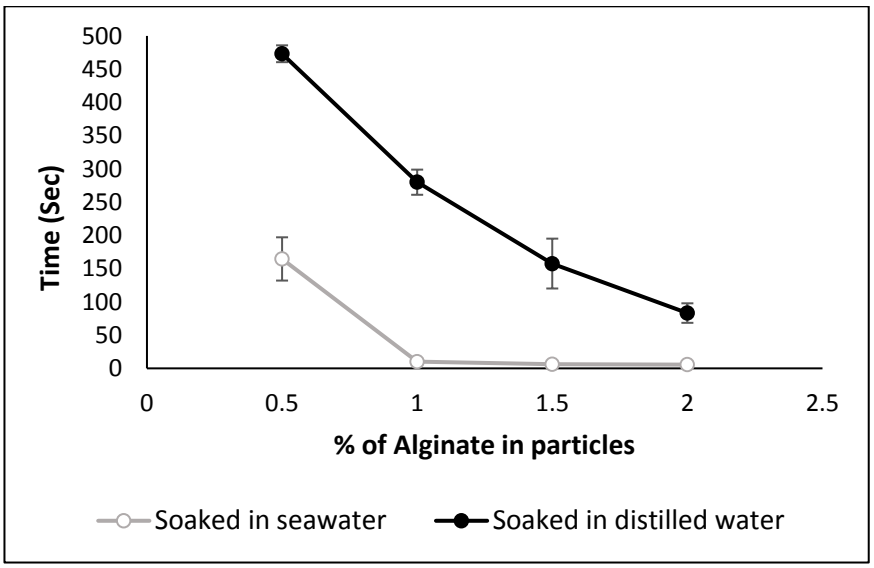


Figure 5-6 Sinking time of alginate beads in seawater (n=15; mean ± SD).

5.3.1.4 Phase I conclusion

The concentration and viscosity of the alginate solution are influential factors that affect the final size and shape of beads (Lee et al., 2013). At very low alginate concentrations, alginate droplets may deform when coming in contact with the surface of the cross-linking solution (Seifert & Phillips, 1997; Meiser et al., 2009). This could explain why at 0.5% w/v alginate concentration, the beads were non-spherical. Similarly, beads containing 2% w/v alginate (ALG-2.0 beads), which had the highest viscosity among the tested formulations, were non-spherical (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 (Leo, Mcloughlin, & Malone, 1990; Meiser et al., 2009). ALG-1.5 beads also had a lower sinking time in seawater which is ideal for delivering probiotics to bottom feeder abalone. Therefore, 1.5% w/v sodium alginate was selected as the optimised concentration to be used in the next two phases of the optimisation process.

5.3.2 Phase II: Effect of autoclaving on physico-chemical properties of alginate beads

It may be required to autoclave alginate solutions before formulation to avoid contamination. Therefore, in order to investigate the effect of autoclaving on alginate solution and its corresponding beads, AtALG beads with 1.5% w/v alginate were produced using autoclaved alginate.

5.3.2.1 Changes in the viscosity of alginate, sinking time and matrix erosion of beads

The viscosities of AtALG and ALG formulations were 0.08 ± 0.00 and 0.41 ± 0.06 Pa.s, respectively. The viscosity of the alginate solution was significantly reduced after the heat treatment at 121˚C. This can be due to the partial loss of glycosidic bonds within the polysaccharide chains resulting in a reduction in the degree of polymerisation. Bead size was also influenced by the viscosity of alginate.

A notable increase in sinking time of AtALG beads was observed in seawater compared to the ALG beads (Table 5.5). This may be explained by cross-linking of shorter polymer chains in autoclaved alginate which may lead to the formation of pores within the AtALG bead matrix. This may lead to a greater level of porosity in AtALG beads compared to ALG beads (Leo et al., 1990). Due to increased porosity, AtALG beads will float on the surface of seawater for a longer time before sinking.

The matrix erosion is an important factor which can affect the release of encapsulated materials. The matrix disintegration of alginate beads was examined over 3 days in seawater as the long-term stability of particles in seawater is critical for aquaculture application. The matrix erosion in ALG beads was only 3% w/w as opposed to 8% w/w in AtALG beads (Table 5.5).

Table 5.5 Characteristics of ALG and AtAlG beads (n= 50 ± SD for size, n= 10 ± SD for sinking time and n= 3 ± SD for matrix erosion).

Sample	Morphology	Size (mm)	Sinking time (s)	Matrix erosion (% w/w)
ALG	Spherical	4.1 ± 0.1	167 ± 12	3.19 ± 0.3
AtALG	Spherical	4.3 ± 0.1	254 ± 15	8.60 ± 0.1

5.3.2.2 Spectral analysis

To investigate the effect of autoclaving on the chemical properties of alginate beads, FTIR spectra of crude alginate, AtALG and ALG beads were collected (Figure 5-7). Comparison of spectra revealed no notable difference and beads showed bands at 1593 cm^{-1}, 1406 cm^{-1} and 1026 cm^{-1} corresponding to asymmetric $–COO^-$, symmetric $–COO^-$ and asymmetric C-O-C stretching vibrations, respectively. A slight shift in $–COO^-$ vibration at 1406 cm^{-1} was observed in both ALG (1422 cm^{-1}) and AtALG (1422 cm^{-1}) beads due to the interaction of carboxylate groups with Ca^{2+} ions during cross-linking. Other than that, the spectra of both beads were very similar.

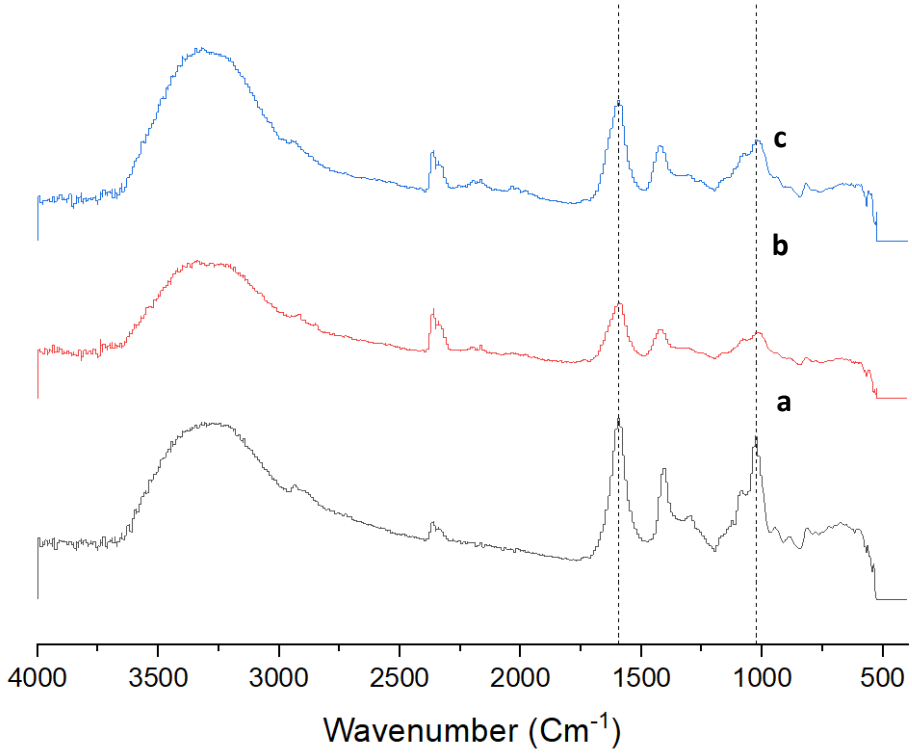


Figure 5-7 Infrared spectrum of (a) crude sodium alginate, (b) ALG beads and (c) AtALG beads.

5.3.2.3 Elemental analysis

EDS analysis was performed to investigate possible differences in percentage and distribution of elements between the two samples. As summarised in Table 5.6, the amount of C and O atoms are approximately the same. However, some differences were observed in the amount of Ca and Na atoms. This maybe be due to higher degree of cross-linking in ALG beads. The lack of Na atoms in ALG beads shows that all sodium atoms are replaced by Ca during cross-linking (Figure 5-8) (Fernández-Hervás et al., 1998).

Table 5.6 percentage of atomic composition of ALG and AtALG beads using EDS.

Atom (%)	C	O	Na	Ca
ALG	33.85	49.44	-	7.47
AtALG	32.78	50.20	2.30	5.49

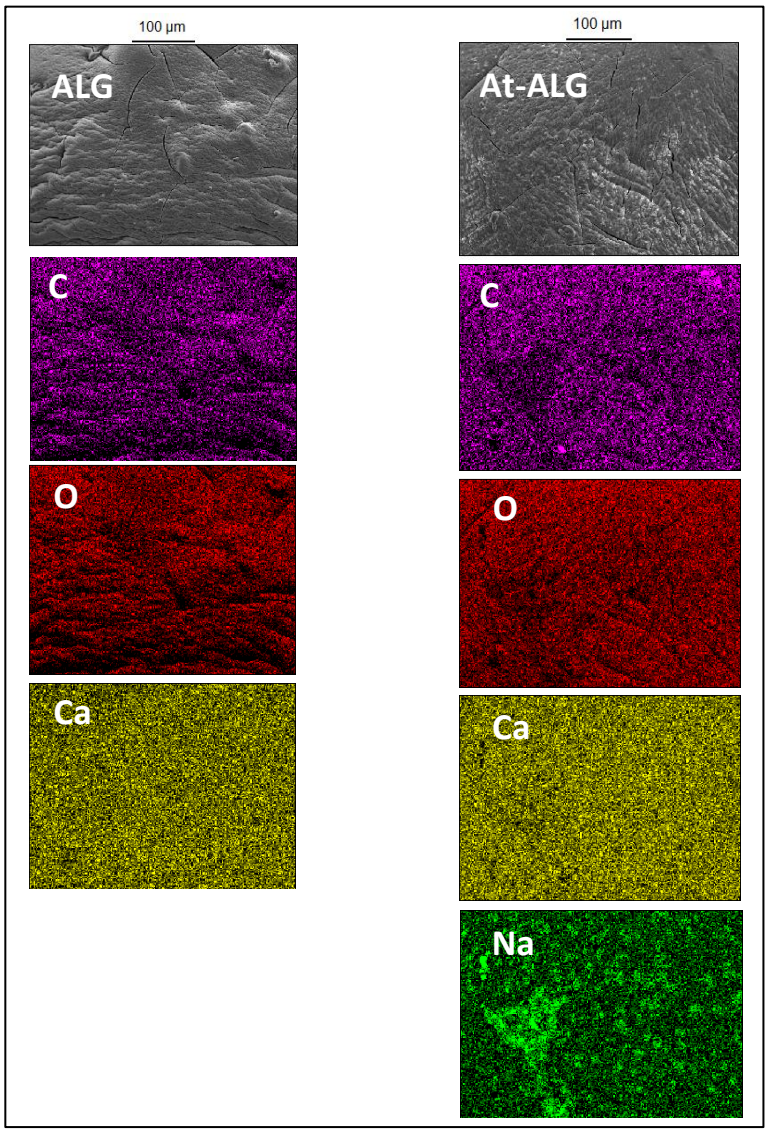


Figure 5-8 EDS mapping of AtALG and ALG beads.

5.3.2.4 Phase II conclusion

Sterilising alginate solution through standard autoclaving procedure can depolymerise alginate which can modify the hydrogel structure and increase sinking time and percentage of erosion in seawater. Higher percentages of matrix erosion in AtALG beads as well as longer sinking time can accelerate the release of encapsulated bacteria from the particles into the seawater which is not favourable. It is also reported that autoclaving alginate can decrease gel stability resulting in poor bead formation (Leo et al., 1990). Based on this, ALG beads were selected for the next phase of optimisation.

5.3.3 Phase III: Optimisation of chitosan coating

Chitosan coating is proposed to increase the stability of beads in seawater. Therefore, in phase III optimisation, 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.

5.3.3.1 Matrix erosion

The percentage matrix erosion for CCALG beads prepared using different concentration of chitosan and coating times is illustrated in Figure 5-9. A slight reduction (not significant, $P > 0.05$) in erosion was observed when the concentration of chitosan increased. The lowest and highest erosion was obtained from CCALG-0.8-20 with 0.9% ± 0.1 and CCALG-0.2-60 with 2.2% ± 0.3 respectively over 3 days of incubation in seawater. There was also no significant difference in matrix erosion with increasing coating time.

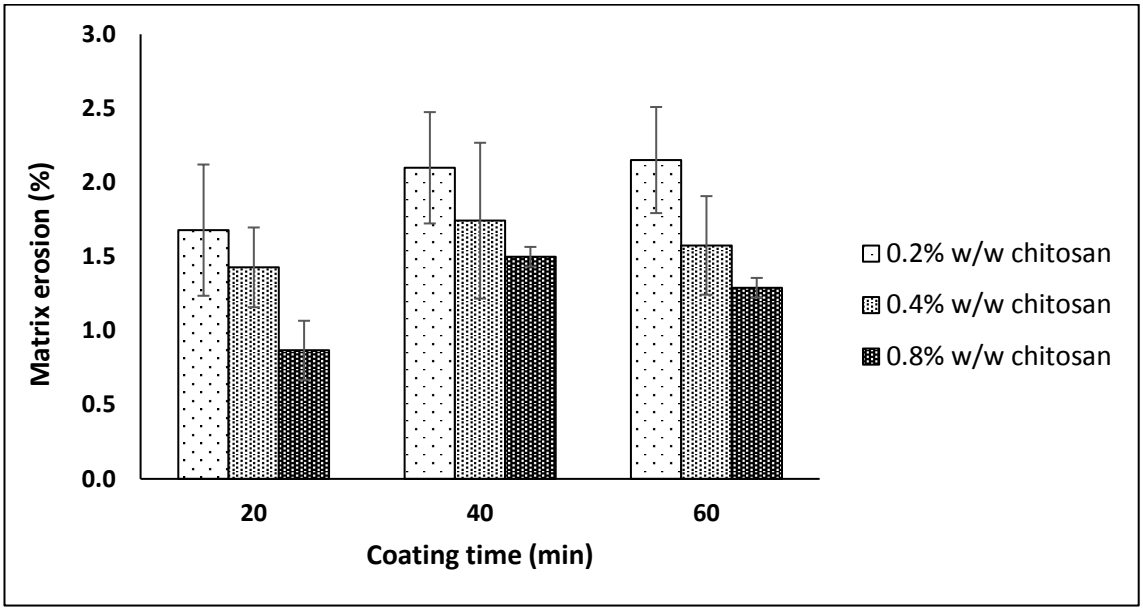


Figure 5-9 Percentage of matrix erosion of chitosan-coated alginate (CCALG) beads with different coating time (20, 40 and 60 min) and chitosan concentration (0.2, 0.4 and 0.8 w/v) 3 days post incubation in seawater (n=3; mean ± SD).

5.3.3.2 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 ($P < 0.05$). However, there was no significant difference between CCALG-0.4-20 and CCALG-0.8-20 ($P > 0.05$) Figure 5-10.

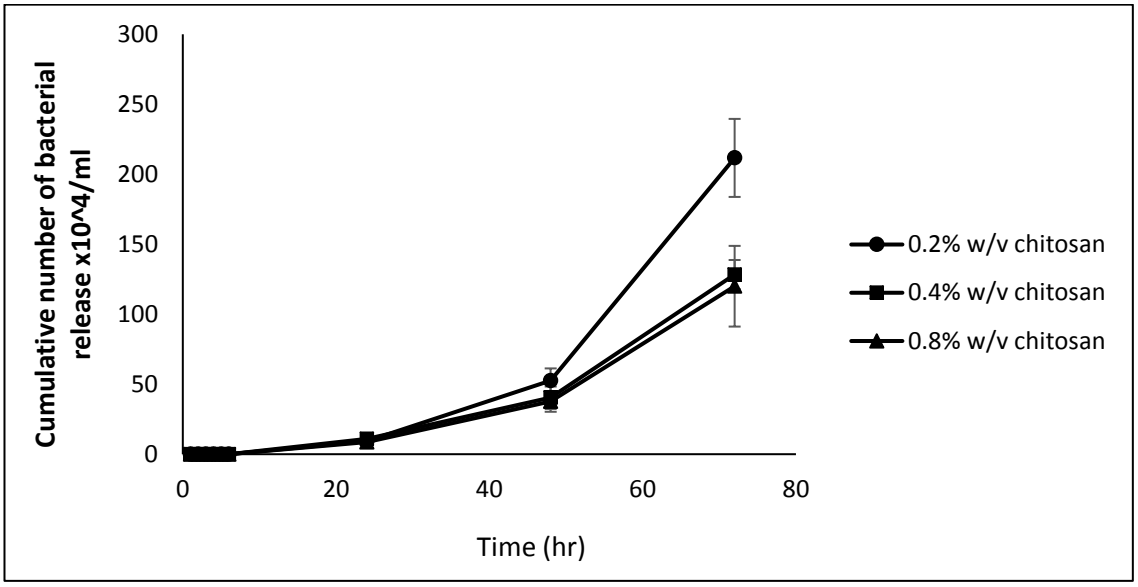


Figure 5-10 Cumulative release curves of *Exiguobacterium* sp. from CCALG coated with different concentration of chitosan (0.2, 0.4 and 0.8 w/v). Release media was seawater (n=3; mean ± SD).

5.3.3.3 Phase III conclusion

The aim of the third phase of optimisation was to attain an optimised 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 characterisation due to having a lower bacterial release in seawater and matrix erosion.

5.3.4 Characterisation of the optimised formulation (CCALG-0.8-20)

5.3.4.1 Microscopy of CCALG beads

Fluorescent microscopic images of ALG and CCALG beads are shown in Figure 5-11. Fluorescent-tagged chitosan was used for coating the beads in this experiment. No fluorescent signal was detected for uncoated ALG beads under the fluorescent filter while a strong red fluorescent signal confirmed the presence of chitosan coating around CCALG beads.

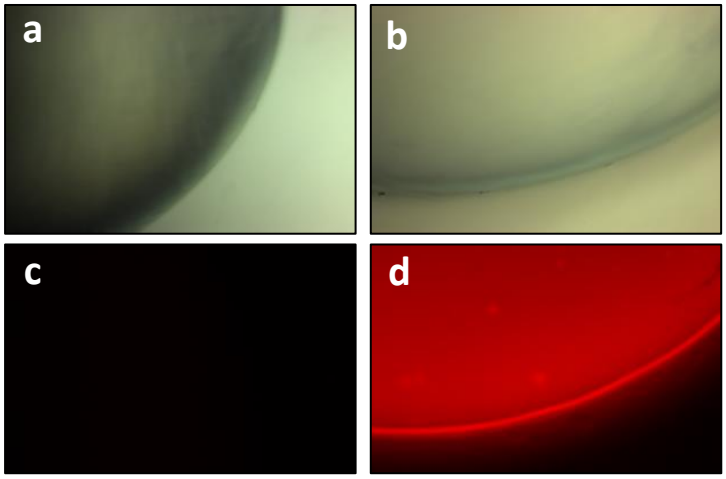
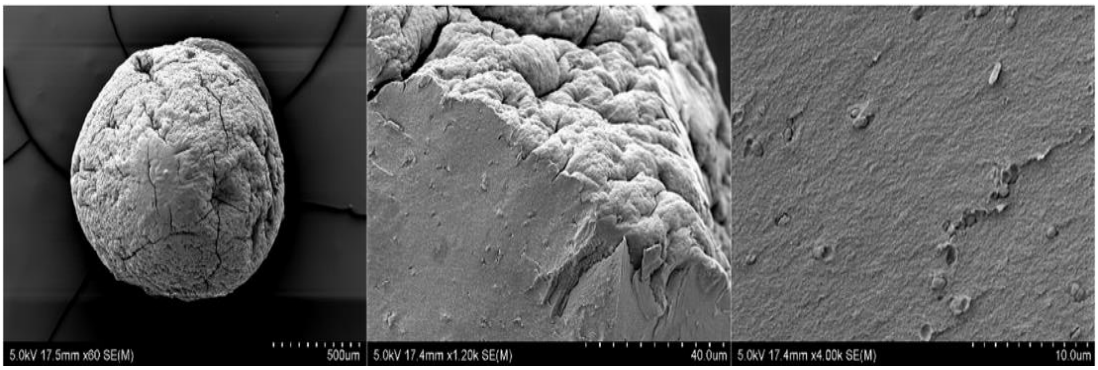


Figure 5-11 Light microscopic images of (a) ALG and (b) CCALG beads and fluorescent images of (c) ALG and (d) CCALG beads (x100) confirming the presence of chitosan in coated beads. Excitation/emission wavelength 552/575.

Scanning electron micrographs of air dried CCALG beads are illustrated in Figure 5-12. CCALG beads retained their 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.



a b c

Figure 5-12 SEM micrographs of (a) an air-dried CCALG bead, (b) cross-section and surface microstructure and (c) the encapsulated probiotics within the bead matrix.

While examining CCALG 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 5-13. The higher amount of carbon and oxygen is related to the presence of carboxyl groups in alginate chemical structure (Figure 5-14). In addition, the presence of sodium is due to the use of sodium alginate in the formulation which is remained after cross-linking. A significantly 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 previous study by Pasparakis & Bouropoulos (2006).

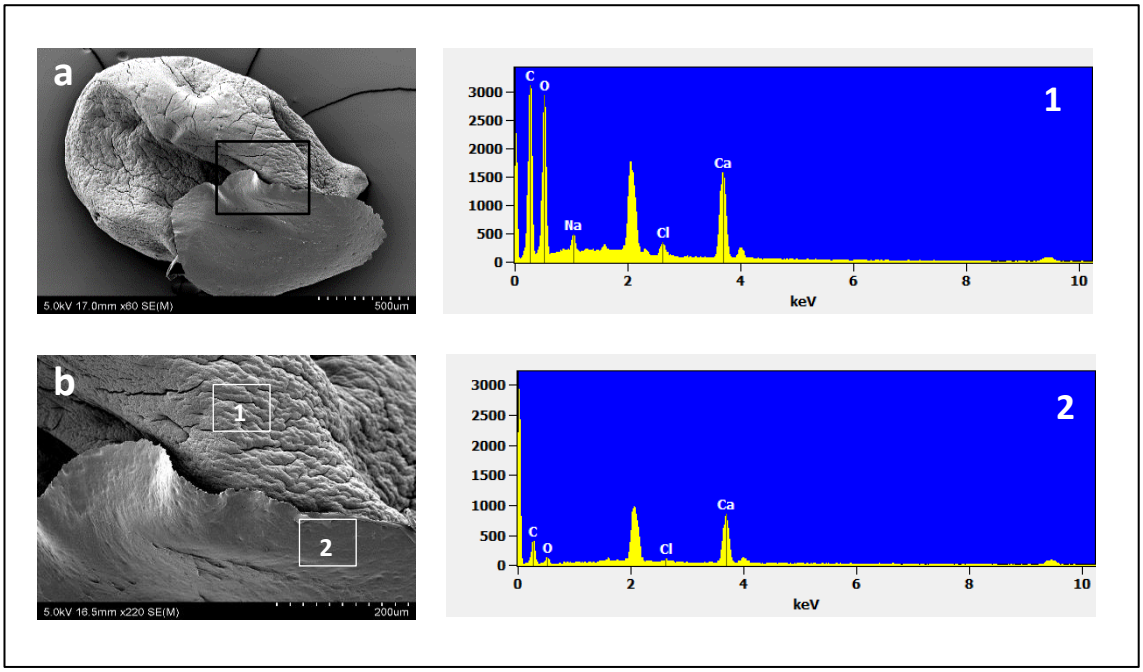


Figure 5-13 SEM images of (a, b) a CCALG bead and the corresponding EDS graphs of (1) alginate matrix and (2) chitosan coating designated as area 1 and 2.

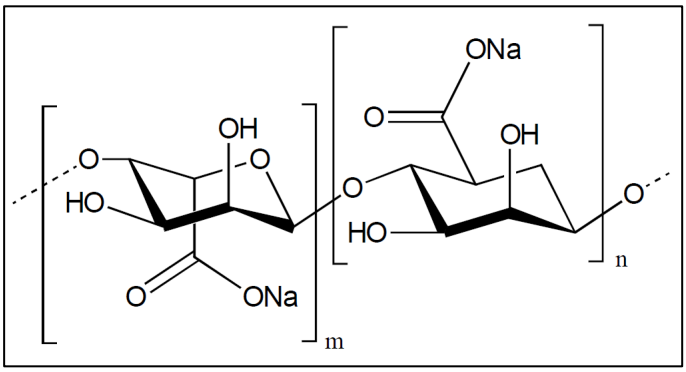


Figure 5-14 Chemical structure of sodium alginate.

5.3.4.2 Effect of the encapsulation process on the viability of bacteria

Viability of the three probiotic bacteria encapsulated in CCALG beads are shown in Table 5.7. The results suggest that only *Vibrio sp*. exhibited a low encapsulation efficiency of less than 2%. This could be due to the sensitivity of *Vibrio sp*. to chemical and mechanical stressors involved in the encapsulation process which are either detrimental to *Vibrio* sp. or makes this bacterium non-culturable.

Table 5.7 Percentage of viable probiotics released from chitosan-coated alginate beads (n=3; mean ± SD).

Bacteria	Entrapped viable cells (%)
Enterococcus sp.	81.2 ± 0.1
Exiguobacterium sp.	55.9 ± 5.8
Vibrio sp.	1.6 ± 0.1

5.3.4.3 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 CCALG beads are shown in Figure 5-15. The encapsulated probiotics were released from ALG after 5 hrs of incubation in seawater while the release from CCALG beads started 24 hrs post incubation. A higher level of bacterial release was obtained for ALG throughout release period. The difference in the bacterial release from the two formulations after 72 hrs can be clearly

seen in Figure 5-16. The number of released bacteria was 1.1×10^8 ml^{-1} and 1.2×10^6 ml^{-1} for ALG and CCALG beads after 72 hrs of incubation, respectively. This indicates that the addition of chitosan coating provides a barrier to reduce the release of the bacteria into the seawater. Figure 5-17 shows the presence of encapsulated bacteria within the matrix of CCALG after 72 hrs of incubation in seawater which indicates the efficiency of CCALG beads in retaining encapsulated bacteria. The number of viable bacteria retained within CCALG beads after 72 hrs of incubation in seawater was further determined using viable plate count method. The results showed that approximately 48 ± 0.5% of the initial encapsulated bacteria remained viable within the matrix of CCALG even after 72 hrs of incubation in seawater.

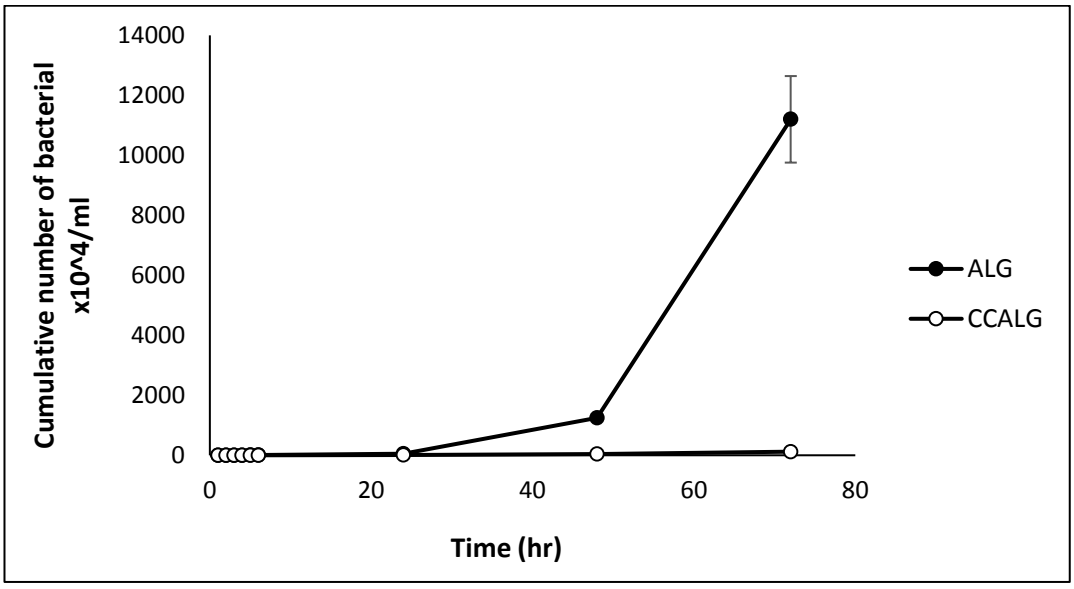


Figure 5-15 Cumulative release curves of encapsulated *Exiguobacterium* sp. from ALG and CCALG beads. The coating media was 0.8% w/v chitosan. The release medium was seawater (n=3; mean ± SD).

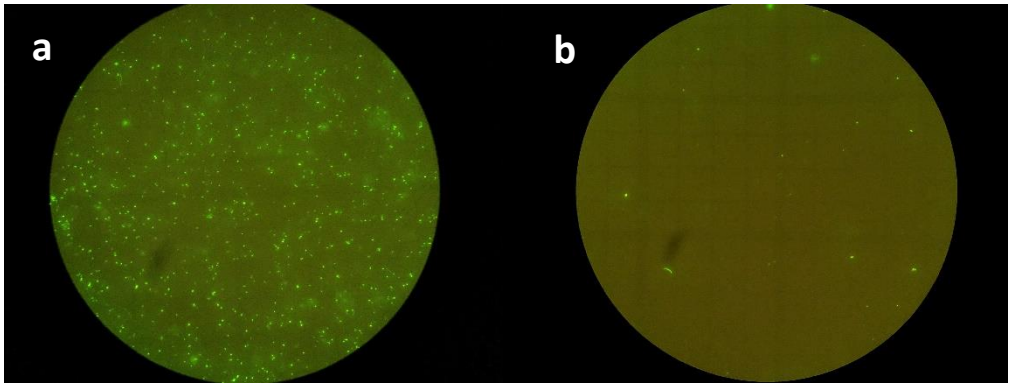


Figure 5-16 Bacterial release from (a) ALG and (b) CCALG beads at 72 hrs post-incubation in seawater under fluorescence microscope (magnification x100).

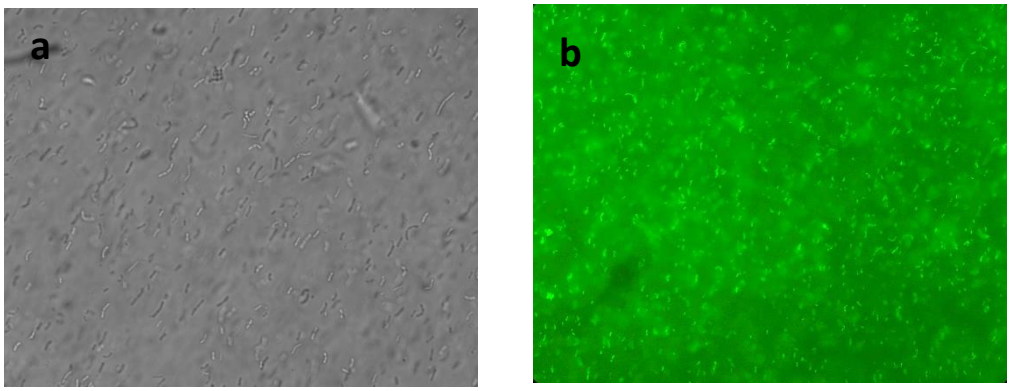


Figure 5-17 Cross-section of CCALG beads after 72 hrs incubation in seawater under (a) normal white light and (b) fluorescent filter (magnification x400).

5.3.4.4 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 hrs 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 behaviour 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 5-18).

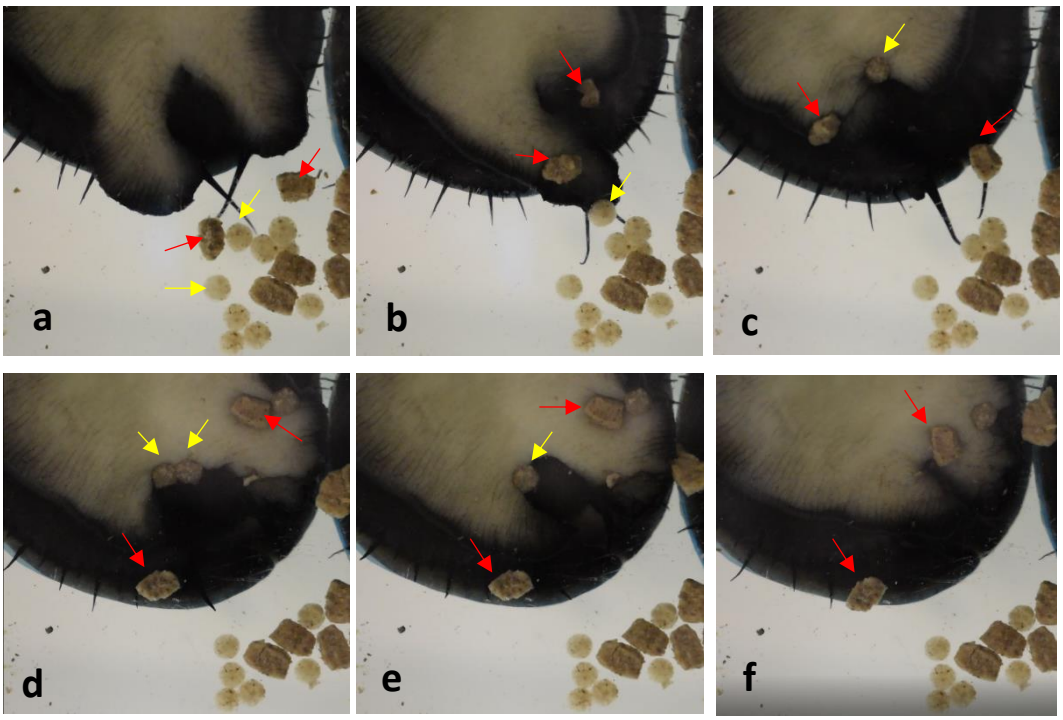


Figure 5-18 A series of captured images showing abalone eating behaviour for the two particular feeds. (a) Sensing food including both probiotics encapsulated CCALG beads (indicated by yellow arrows) and commercial feed (indicated by red arrows). (b) 2 commercial feeds and 1 CCALG bead being grabbed 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.

5.3.4.5 Tracking of fluorescent-labelled bacteria encapsulated in CCALG beads within the GIT of abalone

In this study, animals were given beads containing fluorescent-labelled bacteria and at specific time intervals, animals were cryosectioned to track the encapsulated bacteria within the animals' GIT. Fluorescent-labelled bacteria could be readily identified within the digestive tract of probiotic-fed abalone as small green dots. No intact beads were found within the GIT of abalone. This shows that beads were broken down during intake due to mechanical forces generated by radula (teeth-like structure). Fluorescence images showed that a high concentration of labelled probiotic bacteria was delivered to the buccal region at 12 hrs post feeding (Figure 5-19). Furthermore, the presence of labelled bacteria could be detected throughout the alimentary canal at the same time

point. A lower bacterial concentration was found in the stomach and intestine, which could be due to propagation of bacteria after being released into the gut thereby utilising nutrients and therefore, reflecting loss of fluorescent signals. Moreover, the consumed materials could still en route to the posterior sections of the GIT after only 12 hrs. No labelled bacteria were found within the GIT of abalone at 24, 48 and 72 hrs post feeding. This could be explained by the destruction of the beads by the radula that resulted in a quick release of encapsulated bacteria within the gut and subsequent propagation and loss of fluorescent signal.

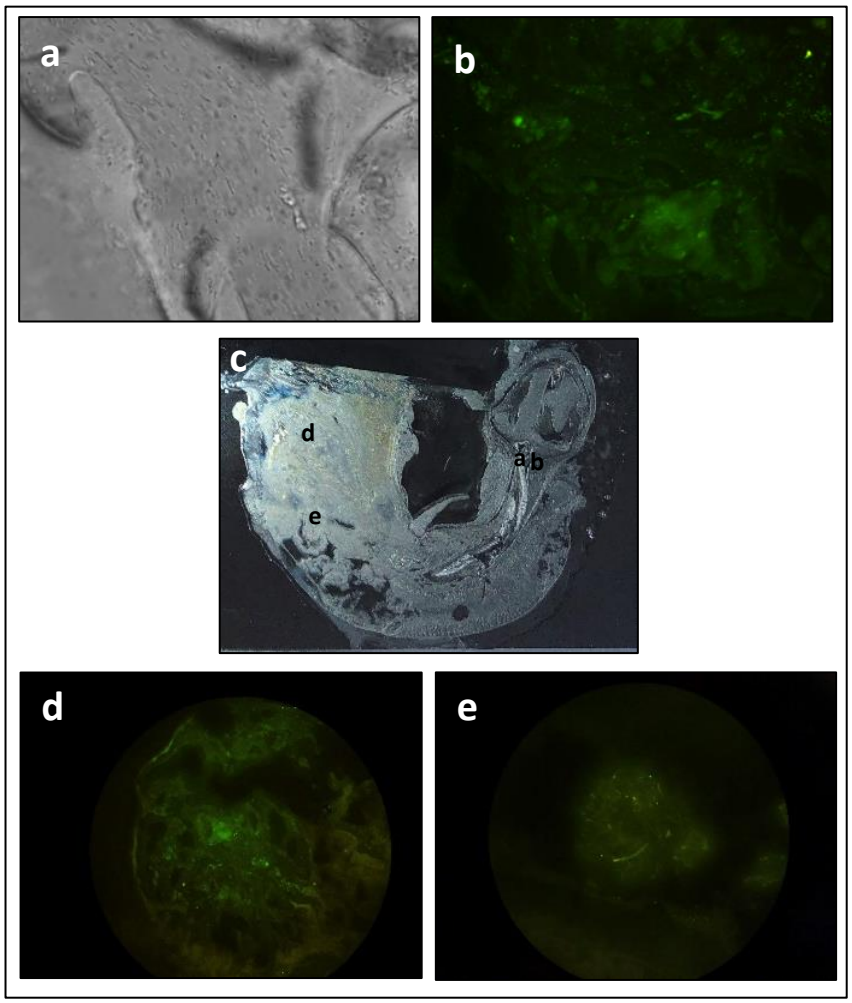


Figure 5-19 Tracking of encapsulated probiotics within the GIT of abalone at 12 hrs post feeding. The presence of labelled bacteria is demonstrated in (a) white light (x400) and (b) fluorescent images in buccal region, (c) stomach and (d) intestine of abalone. Image (c) presents an example of a microsection from the GIT and approximate location of the captured images.

5.3.4.6 Feeding trial

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 (1.3×10^8 CFU) than the control group (6×10^3 CFU). This indicates that the developed CCALG beads could efficiently deliver viable probiotic bacteria into the GIT of abalone.

5.3.4.7 Effect of storage conditions on the viability of encapsulated bacteria

Table 5.8 summarises 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 hrs. A slight increase was then observed in the number of viable bacteria when the storage time further increased from 48 to 96 hrs. Perhaps, the live bacteria can adapt and slowly propagate within beads' matrices.

The counts of bacteria after 96 hrs 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 5.8 Effect of refrigeration on survivability of encapsulated bacteria (n=3; mean ± SD).

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

Table 5.9 shows the average bacterial count post-lyophilisation. The results indicate that the number of viable bacteria reduced 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 lyophilisation. 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 lyophilisation.

Table 5.9 Average bacterial viable count after freeze drying of encapsulated probiotics (n=3; mean ± SD).

Bacteria	Initial viable count (CFU/g)	Viable count after freeze drying (CFU/g)
Exiguobacterium sp.	$(119 \pm 16.2) \times 10^7$	$(44 \pm 0.049) \times 10^5$
Enterococcus sp.	$(94 \pm 4.94) \times 10^9$	$(30 \pm 6.36) \times 10^4$
Vibrio sp.	$(281 \pm 8.48) \times 10^8$	$(33 \pm 7.07) \times 10^4$

5.4 Overall discussion

In this chapter, a simple extrusion technique was used to prepare alginate beads. A mixture of sodium alginate with probiotics was extruded dropwise into a solution containing calcium ions ($CaCl_2$ solution). $CaCl_2$ is a preferred cross-linker for alginate as it is cheap, non-toxic, readily available and importantly, a cross-linking process can occur at neutral pH which in turn can maintain the viability of probiotics (Foroughi, Mirabedini, & Warren, 2018). A cross-linked layer is formed immediately around spherical droplets as they enter the hardening solution (Krasaekoopt et al., 2003). The cross-linking reaction can then proceed from the surface toward interior regions of the beads as Ca^{2+} ions continuously diffuse into the beads (Velings & Mestdagh, 1995).

Alginate beads with distinct shapes and sizes were produced using different concentration of alginate. A higher viscosity was observed as the alginate concentration increased in bead formulations. The shape of alginate beads was influenced by the viscosity of alginate solution. Beads deformation was observed at low (0.04 pa.s) and high (0.93 pa.s) viscosities. 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 (Seifert & Phillips, 1997; Lee et al., 2013). 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 non-spherical droplets at the dripping tip, retaining the shape during dripping distance and finally entering the gelling bath (Lee et al., 2013). Furthermore, it should be considered that high viscosity can complicate the encapsulation process due to handling and process difficulties. Some desirable features

such as improved mechanical and chemical stability, better aesthetic qualities, well-defined size and shape, higher reproducibility and controlled release profiles have been suggested for spherical particles as opposed to particles with non-spherical and irregular shapes (Velings & Mestdagh, 1995; Al-Hajry et al., 1999; Woo et al., 2007; Lee et al., 2013).

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. Furthermore, ALG-1.5 yielded more spherical beads compared to other formulations. 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 pre-soaking step can reduce the osmotic pressure gradient between ALG beads and seawater and facilitate sinking within a few seconds.

In order to minimise the possibility of contamination, it may be necessary to autoclave the alginate solution. It was found that, heat treatment of the alginate solution causes a remarkable decline in the degree of polymerisation of alginate. This has been previously reported by other studies (Leo et al., 1990; Al-Hajry et al., 1999). The depolymerisation of alginate resulted in a significant drop in the viscosity of alginate solutions. Therefore, autoclaving alginate solution can result in higher level of matrix erosion obtained, lower mechanical stability and greater porosity in the produced beads (Leo et al., 1990). These could lead to a faster degradation and release of encapsulated materials from alginate beads, longer sinking time due to higher porosity and lower density. Therefore, it was decided that preparation of the beads from non-autoclaved alginate solution should produce beads with improved qualities for delivery of probiotics to abalone. In order to retain the characteristics of alginate beads in the process of sterilisation, it may be possible to increase the initial concentration of alginate to

counteract the reduction in the degree of polymerisation after sterilisation. Alternative sterilisation techniques can also be explored. However, similarly to autoclaving, methods such as γ-radiation and ethylene oxide treatment have also been reported to change the degree of polymerisation of alginate solution (Leo et al., 1990). In this study, ALG beads produced from non-autoclaved alginate solution were selected for further evaluation due to lower percentage of erosion and smaller sinking time in seawater.

Uncoated ALG beads showed a very fast release of probiotics which is mostly due to the porous structure of alginate particles (Gouin, 2004; Abbaszadeh et al., 2014). However, when the beads were coated with chitosan, the release of probiotics could be successfully controlled (Shu & Zhu, 2002; Ribeiro et al., 2005; Cook et al., 2011). Chitosan, a cationic polymer derived from the shell of crustaceans, can form a strong complex with alginate. In this study chitosan was used to create a polyelectrolyte complex with alginate to reduce the bacterial release and dissolution of beads in seawater (Gombotz & Wee, 1998; Shu & Zhu, 2002; Abbaszadeh et al., 2014). Chitosan coating also delayed the release of probiotics from CCALG bead into seawater where the percentage of matrix erosion in seawater was significantly suppressed, similar to reports elsewhere (Murata et al., 1993). Matrix erosion 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 (Shu & Zhu, 2002; Sahasathian, Praphairaksit, & Muangsin, 2010). On the other hand, coating duration had minimal effect on matrix erosion of the beads (Cook et al., 2011). Therefore, CCALG beads with 0.8% w/v chitosan-coated for 20 (CCALG-0.8-20) was selected as the optimised formulation for delivery of probiotics to abalone.

These beads demonstrated ideal morphological properties such as spherical shape and smooth surface. The release study showed no bacterial release within the first 6 hrs of incubating CCALG beads in seawater which is ideal in terms of minimising the potential environmental impact. This timeline is satisfactory as these beads had a high consumption rate within the first 24 hrs of being placed in abalone culture tank further

minimising the risk of environmental contamination. The developed beads were also able to deliver encapsulated probiotics to the GIT of abalone as shown in tracking experiments. The mechanism of probiotic release from the beads within the gut of abalone can be explained via physical destruction of beads by the radula 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. In order 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 (Chávarri, Marañón & Villarán, 2012). 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* species was less than 2% which could be due to higher sensitivity to calcium in the gelling bath or stirring during encapsulation (Rosas-Ledesma et al., 2012). 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 (Camelin et al., 1993). A wide range of survival levels have been reported using the same method of encapsulation (Albertini et al., 2010; Rosas-Ledesma et al., 2012) and other encapsulation techniques (Annan, Borza, & Hansen, 2008; Martin et al., 2013).

The survivability of the encapsulated probiotics during refrigerated storage at 4˚C showed only one log reduction in viability of encapsulated probiotics after 96 hrs 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 (Macey & Coyne, 2005; Doeschate & Coyne, 2008; Hadi et al., 2014).

Lyophilisation as an alternative storage option for encapsulated probiotics showed a significant 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 (Rybka & Kailasapathy, 1995; Capela, Hay, & Shah, 2006). It needs to be noted that other additives such as skim milk or sugars can be utilised as cryoprotectants to reduce the chance of cell damage during freeze drying (Pinpimai et al., 2015).

5.5 Conclusion

In conclusion, the developed chitosan-coated alginate beads could be used as an efficient probiotic delivery system for abalone. Considering different stages of delivery process, a high level of viable bacteria can be obtained during encapsulation using CCALG beads. A very low bacterial release in seawater can maintain the quality of culture water when beads are placed in abalone culture tanks and high consumption rates ensure delivery and minimise environmental impact. The physical rupture of the beads during intake by radula can initiate bacterial release followed by dissolution and diffusion of encapsulated bacteria into abalone's GIT in the first 12 hrs after feeding. A high concentration of probiotics (10^8 CFU/ml) was found within the GIT of abalone which indicates successful delivery of probiotics.

Chapter 6

Effect of encapsulated feed and probiotics on the growth and immunity of *Haliotis iris*

"Never promise more than you can perform." -- Publilius Syrus

Abstract

This chapter investigates the efficiency of feed encapsulation and probiotics delivery on the growth, immunity and feed digestibility in juvenile black-footed abalone (*Haliotis iris*). Juvenile abalone were fed with four different dietary treatments, including conventional feed pellets (diet 1), conventional feed pellets sprayed with probiotics (diet 2), combination of conventional feed and chitosan-coated alginate (CCALG) beads containing probiotics (diet 3) and CCALG beads encapsulating probiotics and feed powder (diet 4).

Abalone fed with diet 4 performed significantly better than the other groups, resulting in a significantly higher growth rate both in shell length (50.06 ± 5.91 µm/day) and weight (25.55 ± 2.55 mg/day). Furthermore, the feed conversion rate (FCR) for abalone fed with diet 4 (1.04 ± 0.06) was significantly lower than those for diet 1 (3.39 ± 0.07), diet 2 (2.71 ± 0.14) and diet 3 (3.21 ± 0.36). Compositional analysis of abalone faecal matter revealed the lowest percentage of protein content in abalone reared with diet 4 compared to those provided with the other diets, which indicates enhanced feed digestibility and utilisation. Analysis of haemolymph samples with flowcytometry revealed a significantly lower level of oxidative stress associated with diet 4 (13.24 ± 4.2%) compared to that of diet 1 (26.8 ± 3.8%). Metabolomics analyses of foot muscle samples from abalone showed a total of 227 detected metabolites, of which 184 could be reliably identified. A total of 31 differently regulated metabolites across the four treatments were revealed with a characteristic enhancement of free amino acids in the samples obtained from abalone fed with diet 4. These results suggest greater incorporation of proteinaceous amino acids from the diet and/or differences in amino acid turnover in foot tissue (endogenous biosynthesis vs use). The combined results from the growth parameters, proximate analysis, flow cytometry and metabolomics indicate that CCALG beads containing probiotics and nutrients (diet 4) can efficiently improve the growth and immunity of juvenile abalone. Moreover, encapsulated

probiotics-feed can markedly reduce feed cost by significantly improving FCR and consumption rate.

6.1 Introduction

The New Zealand abalone industry is considered as an important economic sector with a total revenue of around NZ$4 million in 2018, mostly from fisheries (Seafood New Zealand, 2019). In addition, abalone farming is rapidly growing to address market demands. However, the long growth period of black-footed abalone has imposed significant restrictions on the development of the industry. Commercially produced abalone usually takes around 4-5 years to grow to market size (80-100 mm). This cultivation time presents a major challenge to the industry, reducing profitability and increasing risk of disease outbreaks (Wang et al., 2004; Hooper et al., 2007; Sawabe et al., 2007). Therefore, improving the growth rate and general health of these animals is crucial for the growth and sustainability of this important aquaculture sector.

The application of probiotics in aquaculture is an eco-friendly strategy that can enhance growth rates and immunity of aquatic animals. Application of probiotics, either as feed supplements or in the culturing water have been shown to improve the resistance of marine animals against pathogens, ameliorate unfavourable environmental and nutritional conditions and enhance the growth rate of animals (De et al., 2014). This approach has been used to increase the growth rate and health of several species of abalone, including *Haliotis midae* in South Africa (Macey & Coyne, 2005; Doeschate & Coyne, 2008), *H. rufescens* in Chile (Silva-Aciares et al., 2011; 2013), *H. iris* in New Zealand (Hadi et al., 2014; Grandiosa et al., 2018), *H. asinine* in Indonesia (Faturrahman, F., Rohyani, I., & Sukiman, 2015), *H. rubra x H. laevigata, a* hybrid abalone in Australia (Amin et al., 2017), and *H. discus hannai* (Gao et al., 2018) and *H. diversicolor* in China (Zhao et al., 2018).

Delivery of live probiotics has been mostly through administration in water or as feed supplements. This may lead to an uncontrolled release and instability of probiotics during processing and storage (Anal & Singh, 2007). Additionally, traditional ways of

delivering probiotics may result in an insufficient dosage of probitic bacteria which can hamper their beneficial effects. In recent years, a number of studies have shown that, by encapsulating probiotics, one can maintain viability, control the release and deliver a sufficient dose of probiotics to a specific site within the animal's body (Wu et al., 2011; Rosas-Ledesma et al., 2012; Ghosh et al., 2016; Dezfooli et al., 2018). In aquaculture, encapsulation has also been used to preserve nutrients, minimise nutrient leakage and control the release of nutrients in target areas (Anas, Philip & Singh, 2008; Zhu, & Zhang, 2012; Jiménez-fernández et al., 2014). Therefore, this could be a viable approach to minimise environmental impact of probiotics.

In the past few years, encapsulation techniques have been investigated for delivery of nutrients to abalone. Zhu et al. (2002) used microencapsulation for delivery of thiamine-HCL to *H. discus*. A significantly lower leaching of the encapsulated vitamin into seawater was reported compared to non-encapsulated ones (Zhu, Mai, & Wu, 2002; Miao et al., 2013). Similarly, Shipton et al. (2002) used microencapsulation to avoid leaching of encapsulated amino acids into seawater prior to ingestion by *H. midae* (Shipton, Britz, & Walker, 2002). Wu et al. (2011) improved the activity of immunoglobulin Y in the digestive tract of *H.diversicolor* using microencapsulation (Wu, Wang, Chan, & Li, 2011). Together, the reviewed studies indicating the potential of encapsulation for developing more efficient delivery methods of bioactives to abalone. However, the use of encapsulation to deliver probiotics to abalone is quite new and has not been previously investigated.

The aim of this study is to explore the effect of the probiotics encapsulated in chitosan-coated alginate (CCALG) beads on growth and health of juvenile abalone (*H. iris).* Specifically, four different diets including a basal diet of conventional feed (diet 1), a probiotic supplemented feed (diet 2), an encapsulated probiotic diet (diet 3) and an encapsulated feed-probiotics (diet 4) were used and assessed in this study. The efficiency of the encapsulated bioactives to improve the growth performance, immunity, feed conversion rate and metabolomics profile of *H. iris* was compared to non-probiotic and non-encapsulated diets. The specific objectives of this chapter are:

1. to identify growth variations of *H. iris* reared with different diets,
1. to study the feed wastage and feed conversion rate of *H.iris* fed with different diets,
2. to investigate the effect of different diets on immune parameter of *H. iris* and
3. to perform a metabolomics analysis of foot muscle tissue of abalone fed with different diets.

6.2 Materials and methods

6.2.1 Materials

Medium molecular weight chitosan, sodium hydroxide, pyridine, methyl chloroformate (MCF), L-alanine-2, 3, 3, 3-*d*4 and GC glass vials were purchased from Sigma Aldrich (St. Louis, USA). Sodium alginate was purchased from AcrosOrganics (Beijing, China). Sodium bicarbonate, calcium chloride and anhydrous sodium sulphate were purchased from Ajax Finechem (NSW, Australia). Chloroform, methanol, sodium bicarbonate and a standard amino acid mix were obtained from Merck (Darmstadt, Germany). Kimble silanised borosilicate glass tubes (12 x 75 mm) were purchased from Thermofisher (Auckland, NZ). Anhydrous sodium sulphate was purchased from BDH Chemicals (Poole, UK). A Gaspak^{TM} EZ anaerobic container system was obtained from Becton Dickinson (New Jersey, USA). All other chemicals were of analytical grades. Juvenile black-footed abalone (average shell length of 25-35 mm) were provided by Moana New Zealand (Ruakaka, Northern New Zealand). Bacterial isolates were kindly provided by the Aquaculture Biotechnology Research Group at Auckland University of Technology (Auckland, New Zealand). Seawater was collected from Okahu Bay, Auckland. All bacterial growth media were purchased from Difco (New Jersey, USA). Cell Count and Viability Assay Kits and Muse Oxidative Stress Kit were purchased from Merck Millipore (Massachusetts, USA). Commercial abalone feed from Marifeed (Hermanus, South Africa) was used in this study.

6.2.2 Rearing tanks and water system

Seawater was stored in a 2000 L tank continuously being recirculated to maintain water quality using membrane filtration (50 µm) and UV sterilisation before use. Furthermore, the water was cooled and maintained at 15˚C. Animal holding tanks were aerated vigorously and seawater was checked regularly for dissolved oxygen (100%), pH (8-8.3), ammonia (0-0.25 ppm) and nitrite (0-0.25 ppm) concentrations.

6.2.3 Probiotic cultivation

A probiotic cocktail consisting of three species of bacteria was used in this study. The probiotic cocktail was cultivated as described in Section 3.2.2, Chapter 3.

6.2.4 Feeding trial

6.2.4.1 Experimental animals

About 180 juvenile abalone (*H. iris*) with an average shell length of 34.7 ± 2.8 mm and wet weight of 4.0 ± 1.1 g were transported from Moana New Zealand to the Auckland University of technology (AUT), Auckland in cold and wet conditions. Once at the AUT lab, the animals were acclimatised for one month, fed with commercial pellets, labelled with numbered water-proof tags and measured for initial shell length and wet weight. Animals were randomly distributed into 12 tanks with 10 L of seawater one week prior to the feeding trial (Figure 6-1). An additional stock containing 40 juvenile abalone was maintained under similar conditions to replace test animals in the case of mortalities during the experiment.

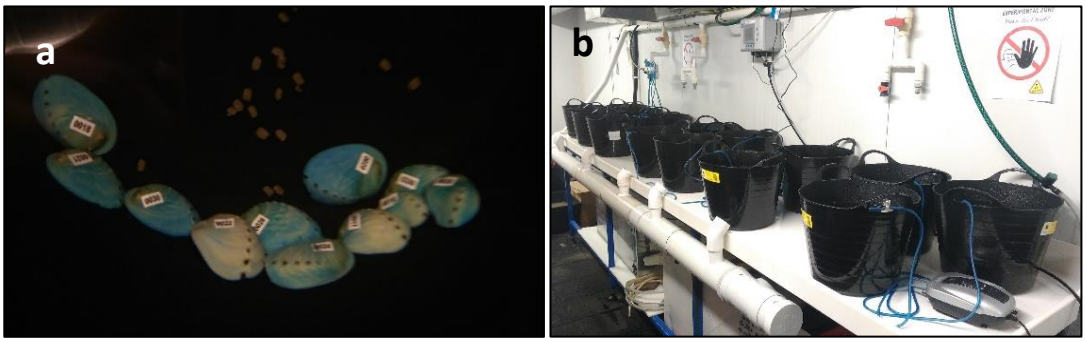


Figure 6-1 (a) Juvenile abalone labelled with numbered water-proof tags and (b) set-up of experimental tanks in the lab.

6.2.4.2 Experimental diet

Three tanks with a total of 45 abalone each were set up for each experimental diet. Four different diets were examined, including conventional feed (diet 1), probiotic-supplemented feed (diet 2), encapsulated probiotics (diet 3) and encapsulated probiotic-feed (diet 4) (Table 6.1). Diets containing probiotics were prepared every four days. A bacterial suspension containing the three probiotic bacteria was prepared by inoculating marine broth with bacterial colonies from cultured agar plates. *Exiguobacterium* sp. were inoculated in 140 ml of marine broth containing 1% w/v yeast extract. *Vibrio* sp. were transferred into 280 ml of marine broth supplemented with 1% w/v yeast extract. The inoculated media were incubated in a shaker (100 rpm) at room temperature for 16 hrs. A volume equal to 140 ml of marine broth with 1% w/v yeast extract was inoculated with *Enterococcus* sp. colonies. The media were incubated in an anaerobic jar for 16 hrs. All the three overnight bacterial cultures were transferred into sterile centrifuge tubes. A volume equal to 45 ml of each *Exiguobacterium* sp. and *Enterococcus* sp. cultures and 90 ml of *Vibrio* sp. culture was mixed. A total of 180 ml of the three bacterial species were utilised for preparation of each probiotic-containing diet sufficient for two days feeding. Cultures were centrifuged at 4000 rpm, for 15 min at 4˚C and the pellets were resuspended in 3 ml of fresh marine broth which was used in the diet preparations. The total number of bacteria added to the probiotics containing diets was approximately 1.3×10^{11} CFU/ml.

In all experimental diets, the conventional feed was used as the source of base nutrients for either the feed pellets in diet 1, 2 and 3, or the encapsulated feed powder in diet 4. An identical daily feeding ratio of 0.88% of average body weight of all animals in each tank was maintained throughout the study. The amount of feed presented to animals across all diets were kept the same except for diet 4 which was lower due to the high viscosity of the final formulation after addition of feed powder.

Table 6.1 The composition of experimental diets.

Diet	Diet composition	Conventional feed (g)	Dose of probiotics (CFU)	Delivery method	
				Probiotics	Feed
1	conventional feed	1.056	-	-	pellets
2	probiotic-supplemented feed	1.056	3.9×10^{11}	sprayed on feed	pellets
3	encapsulated probiotic	1.056	3.9×10^{11}	encapsulated	pellets
4	encapsulated probiotic-feed	0.792	3.9×10^{11}	encapsulated	encapsulated

Diet 1: Conventional feed

The conventional feed specifically designed for pāua was manufactured by Marifeed (Hermanus, South Africa). It was obtained from Moana New Zealand and kept in the lab (15-17˚C) until use (Figure 6-2).

Figure 6-2 Conventional feed pellets used as diet 1 during the feeding trial.

Diet 2: Probiotic-supplemented feed

Feed pellets were weighed and placed in a sterilised tube under a laminar flow hood. Bacterial pellets were retrieved from bacterial cultures and resuspended in 3 ml of fresh marine broth. The mixture was sprayed over the feed pellets and air dried under laminar flow for 2-4 hrs (Figure 6-3).

Figure 6-3 Probiotic-supplemented feed used as diet 2 in the feeding trial.

Diet 3: Encapsulated probiotic

To ensure that animals would receive enough nutrients during the feeding trial, the encapsulated probiotics were added as a supplement to the conventional feed (Figure 6-4). Each tank received a total amount of 4 g of encapsulated probiotics with 1.056 g (dry weight) of conventional feed every two days.

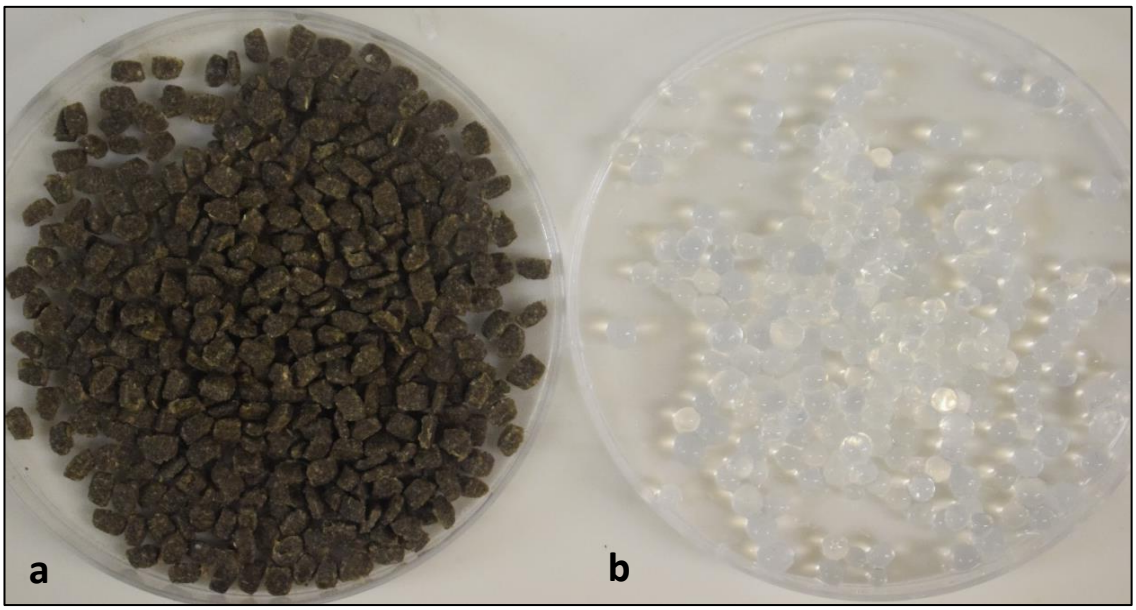


Figure 6-4 Diet 3 containing both (a) conventional feed as the source of nutrients and (b) encapsulated probiotics as a supplement.

The encapsulated probiotics were produced every four days. The three probiotics (*Exiguobacterium* sp., *Vibrio* sp. and *Enterococcus* sp.) were cultured in marine broth containing 1% w/v yeast extract and collected as described in Section 3.2.2, Chapter 3. The collected bacterial cells were resuspended in 3 ml of fresh marine broth and mixed with 22.5 ml alginate from a 2% w/v sodium alginate stock solution. The total volume of the formulation was set to 30 ml by addition of dH_2O. The homogenised mixture was then extruded through a syringe with an aperture size of 2.22 mm into a calcium chloride bath (0.1M). The produced alginate beads containing probiotics were collected using a sieve after 30 min stirring at 100 rpm (Figure 6-5). A coating solution with the final

concentration of 0.8% w/v chitosan was prepared by dissolution of 0.8 g of medium molecular weight chitosan in 1% v/v lactic acid. The pH of the coating solution was then adjusted to pH 5.70 by adding sodium hydroxide (1 M). The alginate beads were then coated with chitosan for 20 min. The chitosan-coated beads were collected and washed with dH_2O (100 ml) twice. Half of the produced encapsulated probiotics beads were used on the day of preparation and the second half was stored at 4˚C to be used within the next 48 hrs.

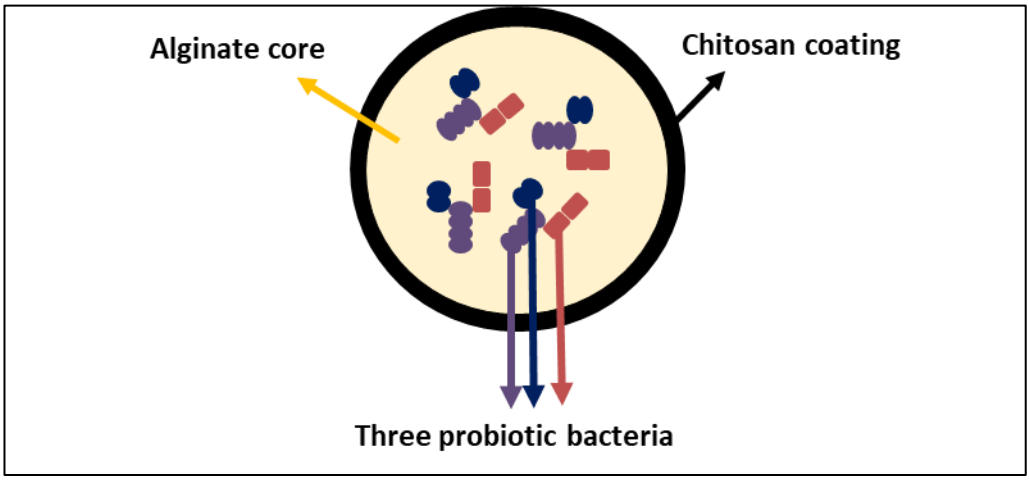


Figure 6-5 Diagrammatic illustration of an encapsulated probiotic bead.

Diet 4: Encapsulated probiotic-feed

Encapsulated probiotics were prepared as described above with the addition of 4.752 g of powdered conventional pellets (Figure 6-6).

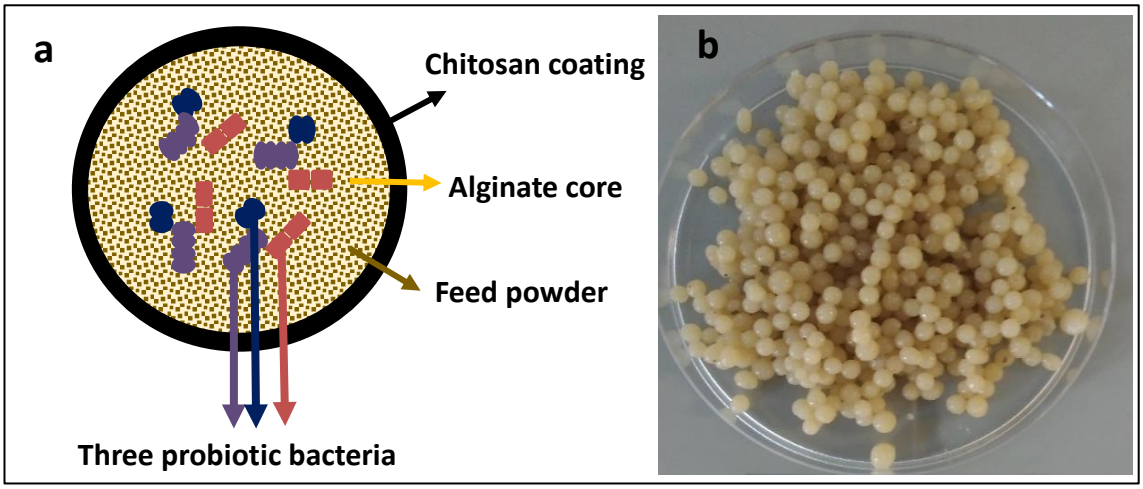


Figure 6-6 (a) Schematic illustration and (b) picture of produced encapsulated probiotic-feed used in the feeding trial.

6.2.4.3 Feeding trial

The duration of the feeding trial was 57 days (July to August). Three tanks each containing 15 abalone were assigned for each diet (Figure 6-7). Each tank was aerated using a separate air pump to avoid cross contamination during the feeding trial and the water was replaced every two days. Uneaten food and faecal matter were collected at the same time using 800 and 125 µm sieves, respectively.

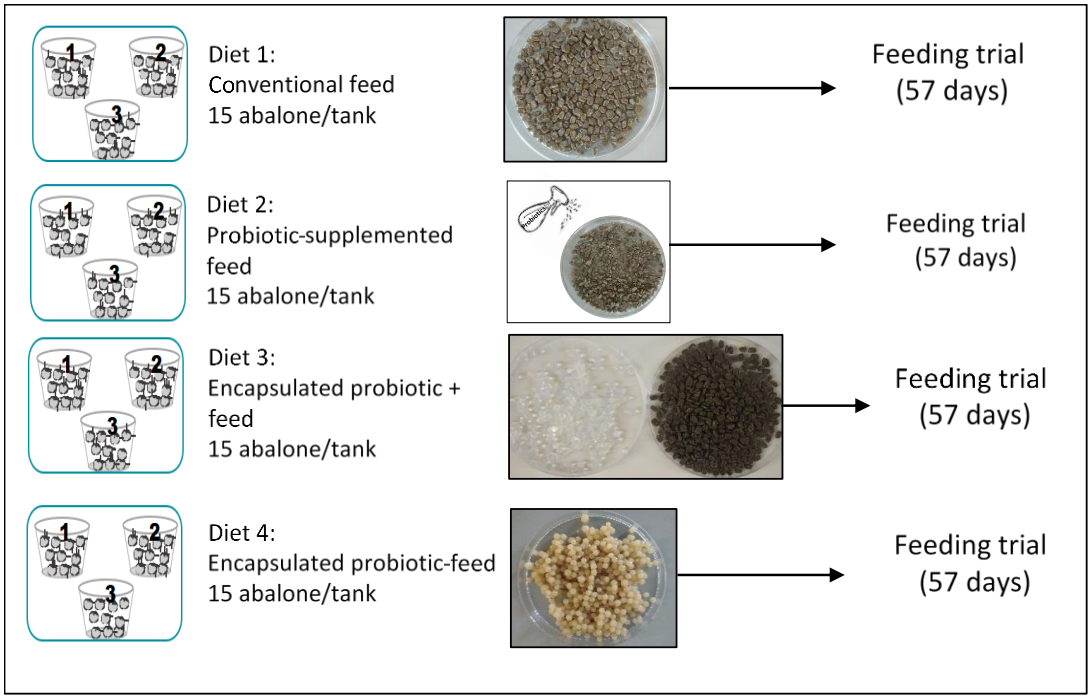


Figure 6-7 Schematic representation of four different diets used in the feeding trial.

6.2.5 Bacterial load

A probiotic dose of approximately 3.9×10^{11} CFU was added to each probiotics-containing diet. The concentration of probiotics bacteria in the digestive tract of abalone was quantified at the end of the feeding trial using similar procedures described in section 5.2.15, Chapter 5.

6.2.6 Growth parameters

The longest axis of shell length for each abalone was measured using a Fuller digital calliper (Quebec, Canada) (Figure 6-8) at the beginning and end of the experiment. Shell growth was calculated using the below formula:

$$Daily\ shell\ growth = \frac{(Final\ shell\ length - Initial\ shell\ length)}{57}$$

The wet weight of each abalone was measured using a Mettler Toledo balance (PB602-S, USA) at the beginning and end of the experiment. Before each measurement abalone were blot dried using a tissue paper. The weight gain of animals was calculated as:

$$Daily\ weight\ gain = \frac{(Final\ weight - Initial\ weight)}{57}$$

Figure 6-8 Measurement of abalone shell length.

6.2.7 Feed wastage and conversion rate

Uneaten feed from each tank was collected and dried in an oven at 35˚C for 48 hrs and accurately weighed. The percentage of feed wastage and feed conversion rate (FCR) was calculated using the below formula:

$$Feed\ wastage\ (\%) = \frac{Amount\ of\ uneaten\ feed\ (g)}{Amount\ of\ given\ feed\ (g)} \times 100$$

$$FCR = \frac{Amount\ of\ given\ feed\ (g)}{Weight\ gain\ (g)}$$

6.2.8 Biochemical analysis

6.2.8.1 Protein analysis

Total protein was estimated using the Kjeldahl method which is used to determine protein concentration in organic samples which calculates the overall protein concentration derived from the amount of nitrogen in the sample (AOAC, 2010). The amount of total protein in each sample was calculated using the below formula:

$$Total\ protein\ \% = \frac{6.25 \times 14.01 \times (ml\ titrant - ml\ blank)}{sample\ weight\ (g)} \times \frac{0.1M\ HCl}{1000} \times 100$$

The value of 6.25 is the standard Kjeldahl factor that is generally used to convert nitrogen content into protein content (FAO, 2003). The atomic weight of nitrogen is 14.01.

6.2.8.2 Lipid analysis

Samples of collected faecal matter and conventional feed were used to extract the lipid content using a method described by Bligh & Dyer (1959). A weight of 0.5 g of each sample was transferred into a 15 ml centrifuge tube. Samples were rehydrated by adding 2 ml of dH_2O. An aliquot of 3 ml of methanol: chloroform (2:1) was added to obtain a homogenised suspension of sample. Chloroform (1 ml) was added to the mixture, mixed for and centrifuged at 2000 rpm for 10 min. The chloroform layer containing extracted lipid was transferred into a clean glass tube and was exposed to nitrogen gas to eliminate chloroform. The extracted lipid remained as a yellow layer at the bottom of the tube and the lipid content was calculated using the following formula:

$$Lipid\ content\ (\%) = \frac{Extracted\ lipid\ (g)}{Initial\ weight\ of\ sample\ (g)} \times 100$$

6.2.8.3 Carbohydrate analysis

The percentage of carbohydrate content was calculated by summing percentages of protein, lipid, moisture and ash subtracted from 100 (Porter & Earl, 1990).

$$Carbohydrate\ content\ (\%) = 100 - [protein\ (\%) + lipid\ (\%) + moisture\ (\%) + ash\ (\%)]$$

6.2.8.4 Moisture analysis

The moisture content was calculated based on the weight differences of each sample before and after drying at 100˚C for 3 hrs using the following formula (De Knegt & Brink, 1998; McClements, 2003):

$$Moisture\ content\ (\%) = \frac{M1 - M2}{M1} \times 100$$

Where, M1 is the initial weight of sample and M2 is the weight after drying.

6.2.8.5 Ash analysis

Samples were placed in a porcelain crucible and then placed in a furnace at 520˚C for 3 hrs. The amount of ash was weighed, and the ash content was calculated using the below formula:

$$Ash\ content\ (\%) = \frac{M1 - M2}{M1} \times 100$$

Where, M1 is the weight of the dried sample before burning and M2 is the weight of the ash obtained after burning.

6.2.9 Haemolymph analysis

Haemolymph samples from 9 animals from each experimental treatment (3 animals per tank) were assessed using a MUSE® Cell Analyser (EMD Millipore, Hayward, CA, USA). Haemocyte counts, viability and reactive oxygen species (ROS) were measured by extracting haemolymph from the tested animals at the beginning and at the end of the feeding trial. The haemolymph was extracted using a pre-cooled 1 ml syringe and needle (27 G) from the anterior arterial sinus of abalone (Figure 6-9). The extracted

haemolymph was transferred into a pre-cooled centrifuge tube and immediately mixed with MUSE reagents.

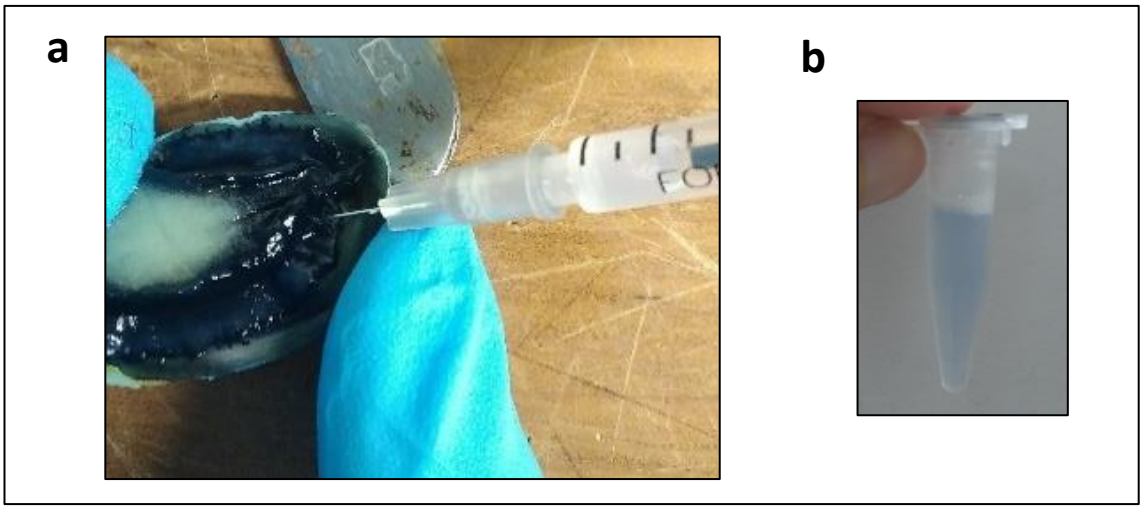


Figure 6-9 (a) Extracting haemolymph from a juvenile abalone using a syringe and needle and (b) abalone haemolymph in a vial.

6.2.9.1 Haemocyte cell count and viability

The total number of haemocyte cells and percentage of viable and dead cells were estimated via flow cytometry on the Muse® Cell Analyser using a viability assay kit following the manufacturer's instruction (Merck Millipore, 2013). A volume equal to 20 µl of extracted haemolymph was transferred into a pre-cold dark 1.5 ml centrifuge tube. The Muse Cell Count and Viability Assay reagent (380 µl) was then added and mixed with the haemolymph. The tubes were incubated at room temperature for 5 min. The mixture was then briefly vortexed for 5 seconds and analysed.

6.2.9.2 Quantification of ROS

The amount of ROS as superoxide radicals in haemocytes was estimated using the Muse Oxidative Stress Kit following the manufacturer's instructions (Merck Millipore, 2013). The data output was set as the relative percentages of cells with (ROS+ cells) and without (ROS- cells) intracellular ROS. A solution of ROS intermediate reagent was prepared by mixing 2 µl of ROS reagent in 198 µl of 1x assay buffer. The ROS intermediate reagent

was further diluted 1:80 with 1x assay buffer to prepare Muse® oxidative stress working solution.

For the purpose of adjusting the ROS threshold, a negative and positive control were used to adjust and verify settings, respectively (Figure 6-10). A fresh sample of extracted haemolymph was used as a sample with minimum ROS+ cells (negative control). Haemolymph (20 µl) was mixed with 380 µl of ROS working regent, vortexed and incubated for 30 min at room temperature. The sample was vortexed briefly and then analysed using the MUSE® Cell Analyser immediately. The threshold was adjusted to remove cell debris from the cell population. A positive control was then used to confirm the accuracy of the setting by preparing a solution of titanium oxide (10,000 µM) as a ROS inducer (Zhu, Zhou, & Cai, 2011). A sample of positive control was prepared by mixing 20 µl of titanium oxide (10,000 µM) with 20 µl of extracted haemolymph. Samples were vortexed briefly and incubated for 5 min at RT. The ROS working reagent (380 µl) was added and incubated at room temperature for another 210 min before analysis. The adjustments were then saved to be used for examining the haemolymph samples from each dietary treatment. ROS measurements were performed immediately after haemolymph extraction from animals in each dietary treatment group. The samples were analysed after 30 min incubation at RT.

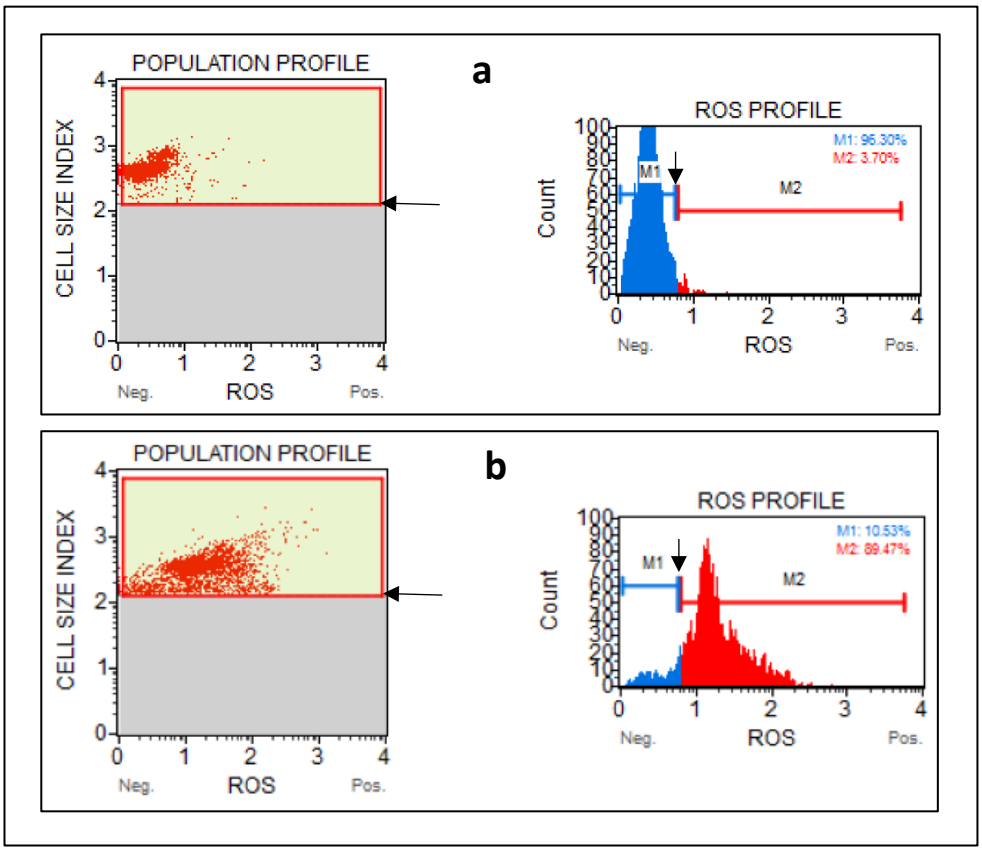


Figure 6-10 Settings of ROS threshold indicated by black arrows in a MUSE® Cell Analyser. Adjustment of setting prior to ROS measurements using (a) negative and (b) positive control by moving the thresholds up and down to exclude cellular debris from studied cell populations.

6.2.10 Metabolomics study on abalone tissue

6.2.10.1 Sample preparation

Four animals from each experimental tank (12 per treatment) were randomly selected to collect tissues for metabolomics analysis at the end of the feeding trial. Animals were then euthanised and dissected to obtain a piece of foot and adductor muscle tissue with an average weight of 200 mg per animal. Each dissected tissue was placed in a cryovial and snap frozen in liquid nitrogen. The frozen tissues were lyophilised for 24 hrs using a freeze dryer (-80˚C, 0.001 mbar) and then ground with a mortar and pestle.

6.2.10.2 Extraction of metabolites

Extraction of metabolites was performed using the cold-methanol water method (Villas-Bôas et al., 2011; Nguyen et al., 2018). Tubes containing powdered samples (5 mg) were kept on ice to which was added 500 µl of cold methanol (50% v/v) and 20 µl of an internal standard (10 mM L-alanine-2,3,3,3-*d*4). Samples were mixed well, freeze-thawed, mixed and centrifuged 4˚C at 1000 rpm for 10 min to collect extracted metabolites. Extraction was repeated on recovered pellets with 800 µl of cold methanol (80% v/v), twice freeze-thawed, pooled with the extracted metabolites in previous step and dried in a SpeedVac Concentrator with a Refrigerated Vapor trap (Savant™ SC250EXP, Thermo Scientific) for 4 hrs. Dried samples were stored at -80˚C until the derivatisation.

6.2.10.3 Derivatisation of metabolites

Derivatisation of metabolites was performed using methyl chloroformate (MCF) (Villas-Bôas et al., 2011). Standard samples were prepared by adding 20 µl of a standard amino acid mix solution (20 mM) into a fresh centrifuge. A volume equal to 400 µl sodium hydroxide (1M) was added to each individual lyophilised sample. The mixture was then transferred into glass tubes (Kimble™ silanized borosilicate 12×75 mm) containing 334 µl of methanol and 68 µl of pyridine. Then, 40 µl of MCF reagent were added and mixed. Another 40 µl of MCF reagent were added to the tubes and mixed. Then, 400 µl of chloroform were added to the tubes and shaken well for 10 sec to separate the MCF derivatives from the reactive mixture. This was followed by addition of 800 µl of sodium bicarbonate solution (50 mM) and centrifugation at 2500 rpm for 6 min. The upper aqueous phase was removed using a glass Pasteur pipette followed by addition of a small amount of anhydrous sodium sulphate to remove the remaining water. Samples were then analysed using gas chromatography (GC).

6.2.10.4 GC-MS analysis

MCF derivatives from each tissue sample, blank and standard were analysed using GCMS (GC7890, Agilent Technologies, USA) coupled with a quadrupole mass spectrometer MSD 5975 (Agilent Technologies, USA). A capillary column with stationary phase of 86% dimethylpolysiloxane and 14% cyanopropylphenyl was used, the volume of injection

was 1 µl, helium flow rate 1 ml min^{-1}, injection temperature 260˚C, oven temperature initially at 45˚C then increased gradually to 240˚C over 15 min and maintained for a further 11.5 min before being elevated to 280˚C for 2 min (Smart et al., 2010). The temperature of the quadrupole was set at 200˚C. A washing step was performed using pure chloroform before and after each injection to clean the column.

6.2.10.5 Data processing

Raw chromatographic data were obtained from GC-MS and deconvolution of data was carried out using the Automated Mass Spectral Deconvolution and Identification System (AMDIS v2.66) software (National Institute of Standards and Technology, USA, http://www.amdis.net/). Metabolites were identified based on their retention time and peak height using Chemstation software (Agilent Technologies). A customised R xxms-based script (Aggio, Villas−Bôas & Ruggiero, 2011) was used to compare the list of metabolites against an in-house library of MCF derivatised commercial standards and the National Institute of Standards and Technology (NIST) mass spectral library. An excel file with the list of metabolites and their peak height was obtained from data processing. The list of metabolites was additionally checked for repetitive data and contaminants (i.e. derivatisation artefacts and non-biologically derived compounds). The peak intensities were further normalised based on the exact biomass of individual samples and the peak intensity of internal standard (*d*4-alanine).

6.2.11 Statistical analysis

The data of bacterial load, growth rate, immunological parameters and biochemical analysis were analysed using One Way ANOVA (SPSS, version 20), Tukey's post-hoc test with a *P* value set to 0.05. Statistical analyses for metabolomics data were performed using the free web-based Metaboanalyst 2.0 developed for analysis of high-throughput metabolomics studies (Xia et al., 2015). Multivariate data analysis including unsupervised principal component analysis (PCA) was used to determine classification of samples.

6.3 Results and discussion

6.3.1 Bacterial load in the digestive tract

Results from the bacterial count in the digestive tract of abalone (Table 6.2) indicate that the number of probiotic bacteria were higher in probiotics-fed abalone compared to animals fed the conventional feed (diet 1) (Figure 6-11 and Figure 6-12). The higher probiotic bacterial load in the digestive tract of abalone fed with probiotic containing diets indicates the successful delivery of probiotics to abalone using both encapsulated and non-encapsulated probiotic diets (diets 2, 3 and 4). Despite using the same bacterial concentration for preparation of all probiotic-containing diets, the bacterial load in animals fed the encapsulated probiotics diets (diet 3 and 4) was lower than the other probiotics-containing diet (diet 2), which could have been due to a partial loss of bacterial viability during encapsulation. Additionally, the bacterial load of abalone fed with diet 3 was slightly lower ($1.2 \times 10^8\ CFU$) than that in diet 4 ($1.8 \times 10^8\ CFU$). However, no significant difference ($P > 0.05$) was found between the two. The measured viable bacterial densities in all probiotic-fed animals were between $10^7\ to\ 10^{10}$ CFU, which is within the optimum range required for probiotic function (Macey & Coyne, 2005; Hadi et al., 2014; Grandiosa et al., 2018).

Table 6.2 Viable count of bacterial population within the digestive tract of abalone.

Diet	Average number of bacteria in GIT of abalone (CFU)
Conventional feed (Diet 1)	$(4.8 \pm 0.9) \times 10^6$
Probiotic-supplemented feed (Diet 2)	$(1.2 \pm 0.2) \times 10^9$
Encapsulated probiotic (Diet 3)	$(1.2 \pm 0.3) \times 10^8$
Encapsulated probiotic-feed (Diet 4)	$(1.9 \pm 0.6) \times 10^8$

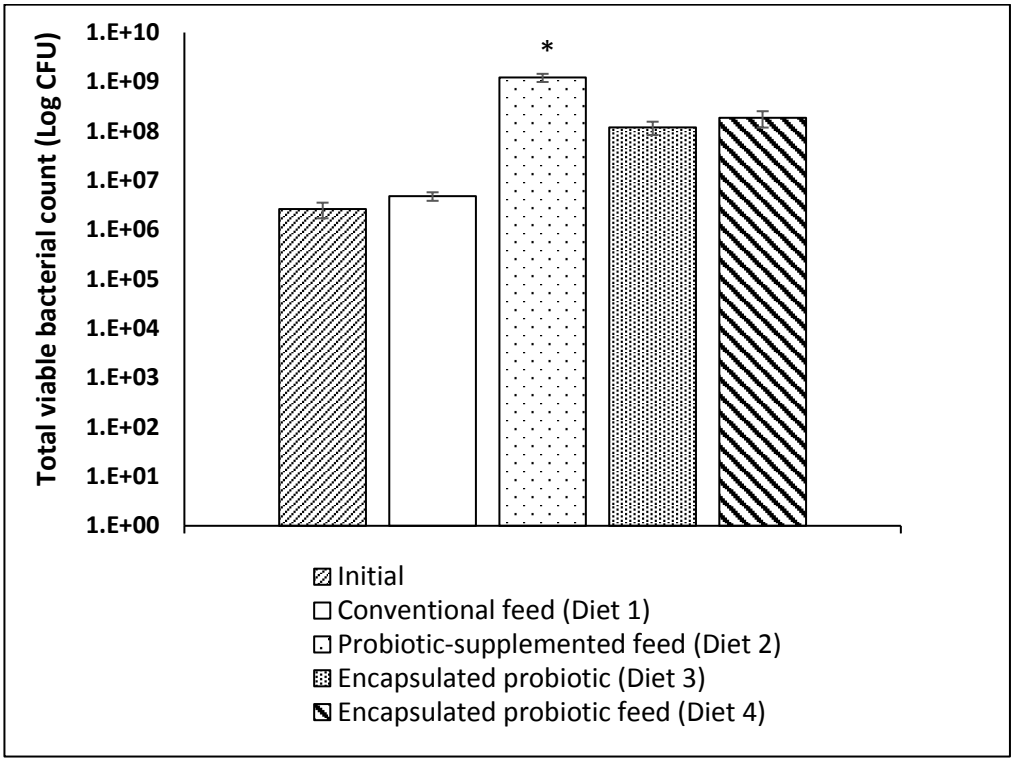


Figure 6-11 Total number of probiotic bacteria present in the digestive tract of abalone before and at the end of the feeding trial. Initial counts of bacteria present the number of bacteria in the digestive tract of abalone before the commencement of feeding trial. The data represents the mean (n=3) ± standard error (SE). Asterisk indicates statistical significance ($P < 0.05$) between different diets and non-probiotics fed (diet 1) animals (n=3; mean ± SE).

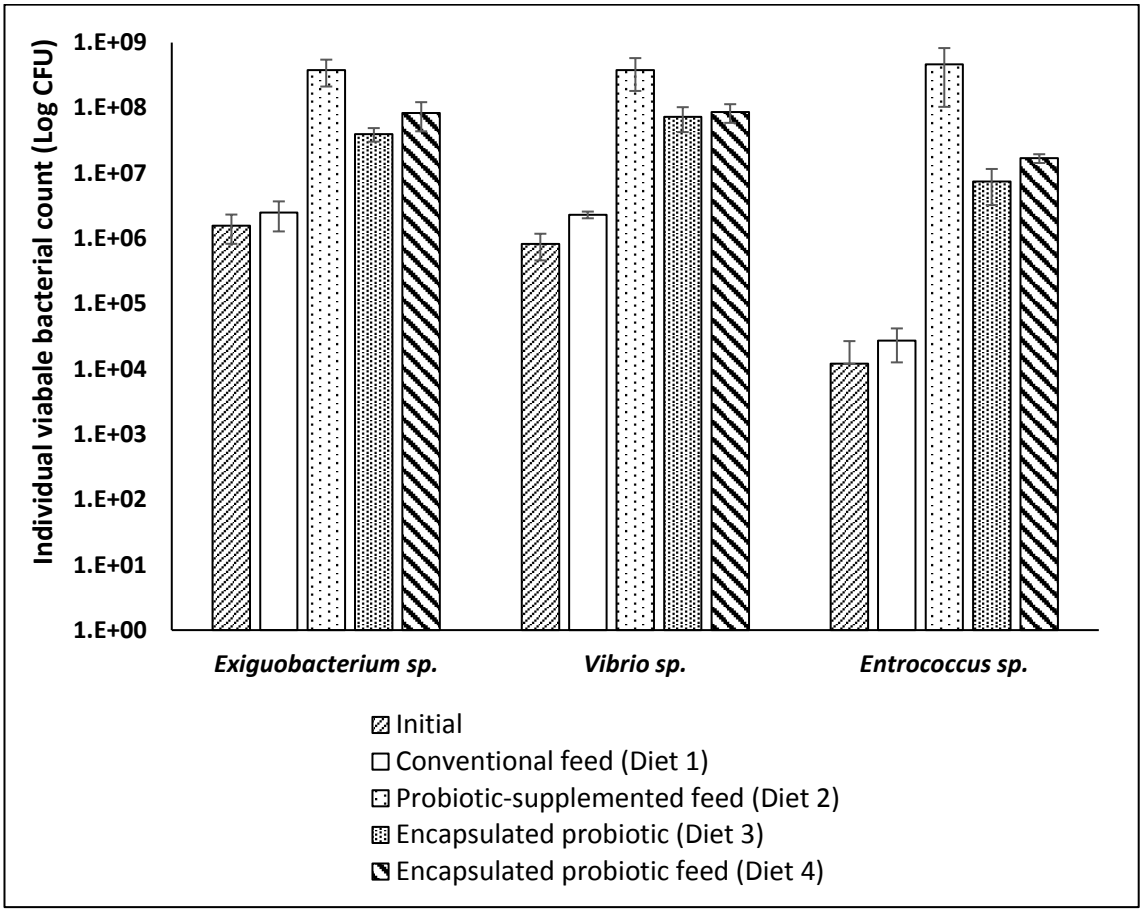


Figure 6-12 The number of viable *Exiguobacterium* sp., *Vibrio* sp. and *Enterococcus* sp. cells in abalone treated with different diets. Initial counts of bacteria represent the number of bacteria in the digestive tract of abalone before the commencement of the feeding trial (n=3; mean ± SE).

6.3.2 Mortality and growth rate

There were no mortalities throughout the period of the feeding trial. A general increase in the shell length and weight of abalone was observed in all four groups after 57 days. The amount of daily shell length gain and weight gain for abalone fed with different diets are summarised in Table 6.3. Greater shell lengths and weight gains were obtained for probiotics-fed animals compared to those provided with conventional feed (Figure 6-13). The mean ± SE increase in shell length for animals on diets 1, 2, 3 and 4 was 4.83 ± 1.29, 8.40 ± 0.94, 5.21 ± 0.80 and 8.26 ± 1.17%, respectively. Shell length gains for

animals on diets 2 and 4 were significantly higher ($P < 0.05$) compared to those fed the conventional feed (diet 1).

Animals on diet 4 showed the highest increase in wet weight after 57 days. The percentage of weight gain was 33.42 ± 3.4% which was significantly higher than those fed diet 1 (25.22 ± 1.60%), diet 2 (27.71 ± 1.51%) and diet 3 (24.81 ± 1.94%) (Figure 6-14). This was obtained despite the fact that 25% less nutrients were administered to abalone with diet 4 compared to the other three dietary regimes. The value of daily weight gain of *H. iris* treated with the basal diet was lower (17.25 ± 1.76 mg/day) than that (25.55 ± 2.55 mg/day) achieved by improving the delivery of nutrients to abalone using encapsulation. The daily weight gain using the same probiotic strains was previously reported to be 7.12 ± 0.15 mg/day (Hadi et al., 2014) and approximately 18 mg/day (Grandiosa et al., 2018) which were lower than that obtained in the present study using encapsulated probiotic-feed (diet 4). Similarly, Macey et al. (2005) and Doeschate & Coyne (2008) reported an improved daily weight gain of 7.2 mg/day and 14 mg/day after 8 months feeding trial in probiotic-fed *H. midae* using different strains of probiotics which are not comparable to the results obtained using diet 4.

An improved weight gain was observed in abalone fed diet 2 compared to those fed diet 1 which could be due to the presence of probiotics. However, the difference in weight gain was not significant ($P < 0.05$) between the two diets. This result is in agreement with previous studies (Tuterangiwhiu, 2015; Grandiosa et al., 2018) and in contrast with the results obtained by Hadi et al. (2014). These variations in results could be due to the health status of animals and technical variations in feeding trials.

A similar growth performance was observed for abalone fed with diet 3 and diet 1. This could be due to the abalone preferring CCLAG beads to conventional feed pellets. This probably led to abalone receiving less nutrients when CCALG beads was provided as supplement during the feeding experiment. A significantly higher ($P < 0.05$) growth rate (weight gain and shell length increase) was obtained for abalone when both probiotics and feed were encapsulated within CCALG beads (diet 4).

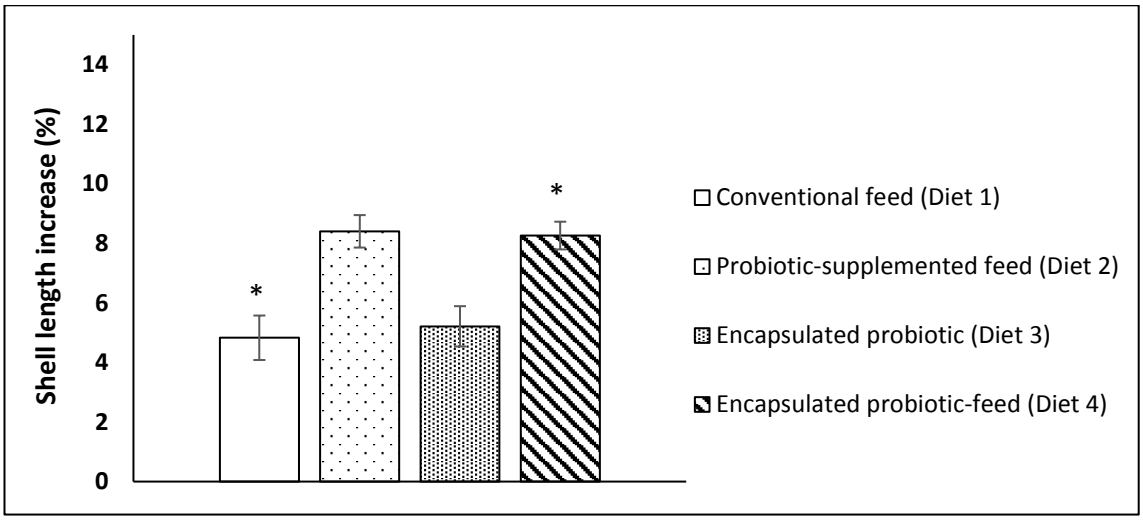


Figure 6-13 Shell length gains for abalone fed with different diets after 57 days of feeding trial. Symbols (*) above the bars indicate statistical significance between different diets and non-probiotics fed (diet 1) animals (one-way ANOVA, Tukey's post-hoc, $P < 0.05$) (n=3; mean ± SE).

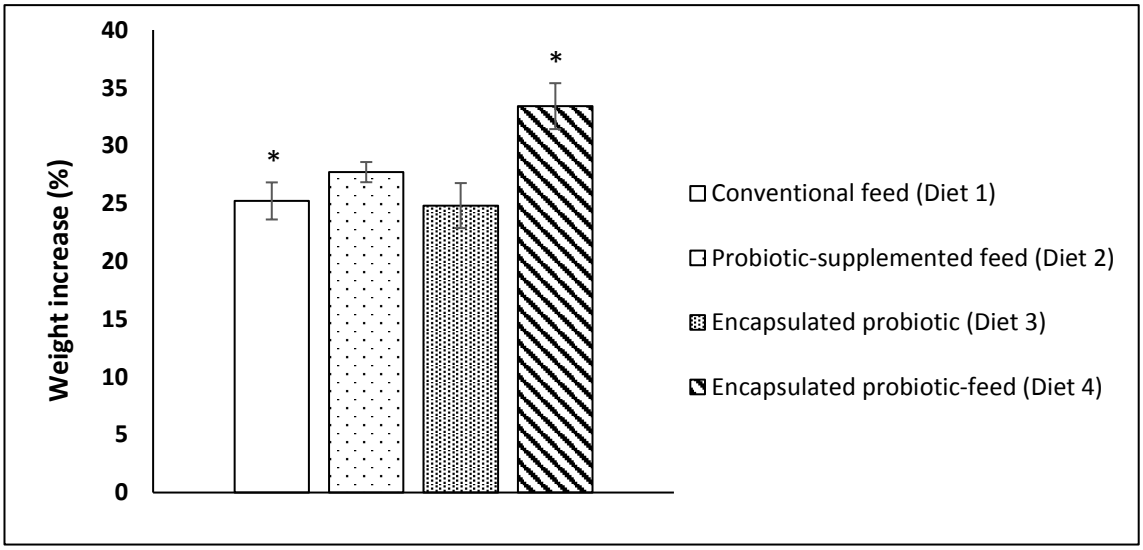


Figure 6-14 Percentage of weight gain by abalone after 57 days of the feeding trial. Asterisks indicate statistically significant differences between diets and control (diet 1) (one-way ANOVA, Tukey's post-hoc, $P < 0.05$) (n=3; mean ± SE).

Table 6.3 Increase in shell length and weight of abalone fed with different experimental diets (n=3; mean ± SE).

Diet	Shell length gain/57 days (mm)	Shell length/day (μm)	Weight gain/57 days (g)	Weight gain/day (mg)
Conventional feed (Diet 1)	1.65 ± 0.42	29.08 ± 7.49	0.98 ± 0.10	17.25 ± 1.76
Probiotic-supplemented feed (Diet 2)	2.82 ± 0.30	49.49 ± 6.69	1.04 ± 0.09	18.26 ± 1.75
Encapsulated probiotic (diet 3)	1.81 ± 0.29	31.76 ± 5.23	1.00 ± 0.14	17.65 ± 2.49
Encapsulated probiotic-feed (Diet 4)	2.85 ± 0.33	50.06 ± 5.91	1.45 ± 0.14	25.55 ± 2.55

6.3.3 Feed wastage and FCR

The percentages of feed wastage were approximately the same across all treatments except for diet 4. A significantly low ($P < 0.05$) percentage of feed wastage of 18 ± 2.1 was achieved by encapsulating feed in CCALG beads (diet 4) (Figure 6-15). The amount of feed wastage was relatively similar for the other diets (1, 2 and 3) ($P > 0.05$). This indicates that encapsulating the feed can potentially reduce the feed cost while sustaining the nutritional requirements of abalone.

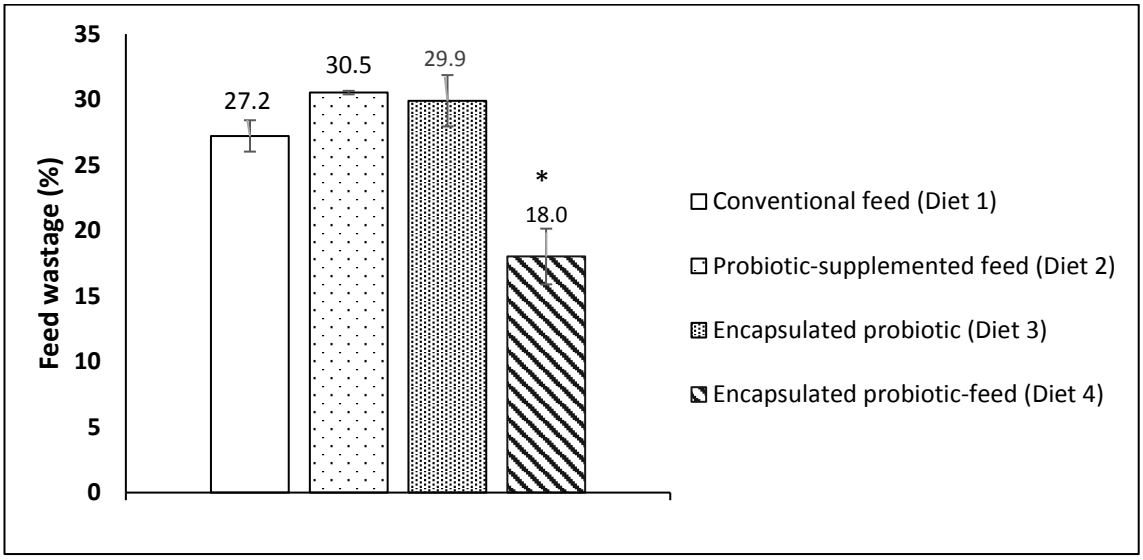


Figure 6-15 Feed wastage for each dietary treatment (n=3; mean ± SE). Asterisk indicates statistically significant differences between diets and control (diet 1) (one-way ANOVA, Tukey's post-hoc, $P < 0.05$) (n=3; mean ± SE).

The FCR did not vary significantly among diet 1 (3.39 ± 0.07), diet 2 (2.71 ± 0.14) and diet 3 (3.21 ± 0.36). However, the FCR value for abalone fed with diet 4 (1.04 ± 0.06) was significantly lower ($P < 0.05$) than for the other treatments (Figure 6-16).

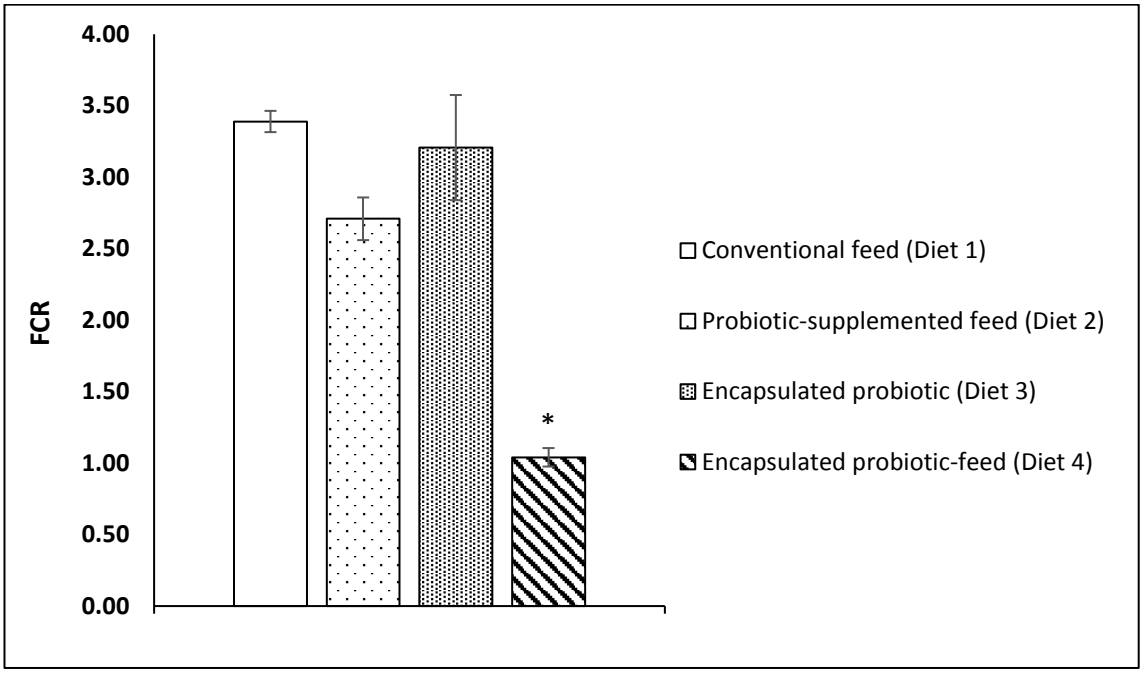


Figure 6-16 FCR values for juvenile abalone reared with different experimental diets (n=3; mean ± SE). Asterisk indicates statistically significant differences (one-way ANOVA, Tukey's post-hoc, $P < 0.05$) (n=3; mean ± SE).

6.3.4 Compositional analysis of feed and faecal matter

Compositional analysis of faecal matters showed differences in crude protein content between all tested groups. Lower amounts of crude protein were found in faecal matter from diet 2, 3 and 4 compared to conventional feed (diet 1) (Figure 6-17). However, the lower protein content of feed used as diet 4 compared to other three diets must be considered when interpreting these results.

The extent of protein digestion in abalone is very important in the weight gain of animals as dietary protein is mainly used for growth rather than as a source of energy (Taylor, 1997). Lesser amount of protein remained undigested in the faeces of probiotic-fed abalone compared to faeces collected from abalone fed with the basal diet which is in agreement with those reported by Macey & Coyne (2005) and Hadi et al. (2014). This could be due to the presence of probiotics which can improve the feed digestibility and utilisation. No significant difference was observed in lipid and carbohydrate contents in the faeces from animals in different treatments. Relatively similar results were obtained

for the moisture content in the faeces of animals treated with different diets (Table 6.4). Ash content in faeces of animals fed with diet 4 was significantly higher than other groups. This may be attributed to higher consumption of Ca-alginate beads which may deposit higher amount of minerals in the faecal matters.

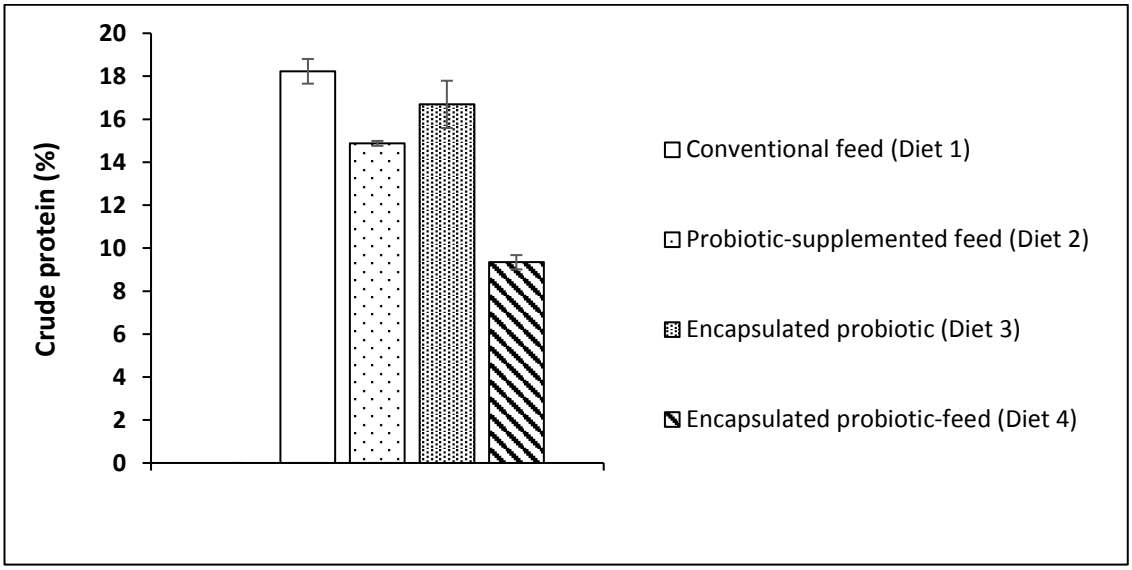


Figure 6-17 Protein content in abalone faeces fed with different dietary regimes.

Table 6.4 Compositional percentage of faecal matter (FM) and conventional feed (CF) (n=3; mean ± SD).

Samples	Diet	Protein (%)	Lipid (%)	Carbohydrate (%)	Moisture (%)	Ash (%)
FM	1	18.22 ± 0.57	3.45 ± 0.50	58.63 ± 1.67	9.79 ± 0.34	9.88 ± 1.42
FM	2	14.87 ± 0.11	4.78 ± 1.63	61.50 ± 2.48	9.66 ± 0.41	9.16 ± 0.84
FM	3	16.69 ± 1.09	3.28 ± 0.33	61.40 ± 0.39	9.30 ± 0.41	9.31 ± 0.83
FM	4	12.42 ± 0.33	5.23 ± 2.67	57.17 ± 2.00	9.34 ± 0.23	15.82 ± 1.25
CF		33.13 ± 0.35	4.81 ± 0.74	45.56 ± 0.68	10.56 ± 0.32	5.92 ± 0.20

6.3.5 Haemolymph analysis

6.3.5.1 Estimation of oxidative stress in abalone

ROS measurements showed that the number of haemocytes exhibiting ROS was reduced in probiotics-fed animals compared to those provided with conventional feed (Figure 6-18). A significant reduction ($P < 0.05$) was observed in abalone fed with encapsulated probiotics-feed (13.5 ± 4.2%) compared to the control (26.8 ± 3.8%).

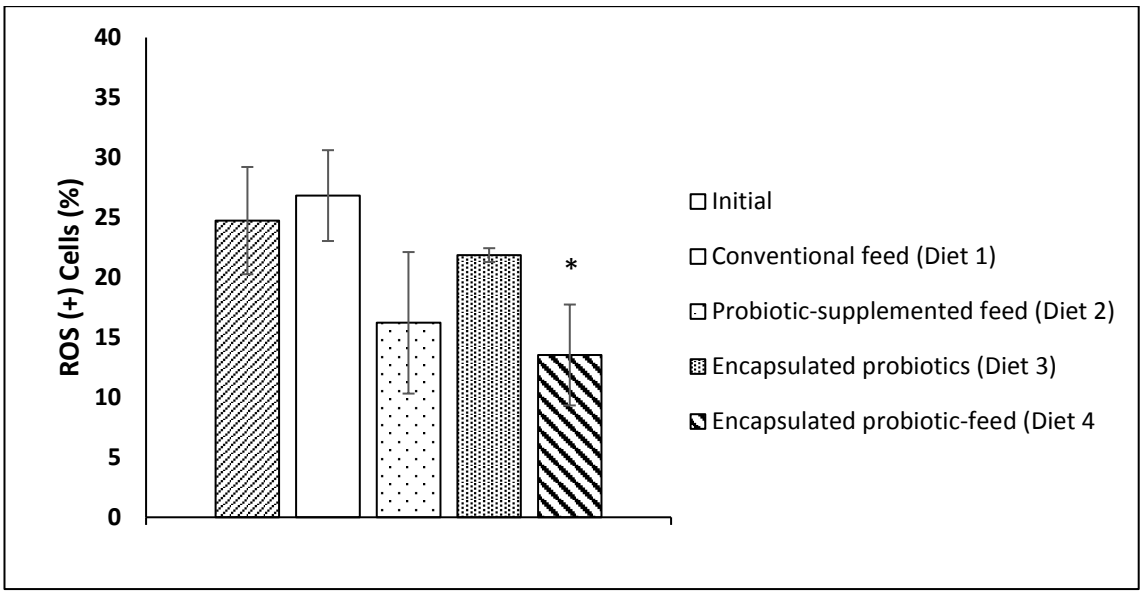


Figure 6-18 Percentages of haemocytes exhibiting ROS in abalone fed with different diets before and at the end of the feeding experiment. Asterisks indicate statistically significant differences (n=9; mean ± SE, one-way ANOVA, Tukey's post-hoc, $P < 0.05$).

6.3.5.2 Haemocyte cell counts and viability

The percentages of viable cells were not statistically different ($P > 0.05$) in animals fed with different diets. The percent viability remained high after 57 days of the feeding trial in probiotic-fed and conventional pellet-fed abalone (Figure 6-19). The number of haemocytes was lower in probiotics-fed animals. However, a statistical difference was observed only between animals treated with the conventional feed (diet 1) and probiotics-supplemented feed (diet 2) (Figure 6-20).

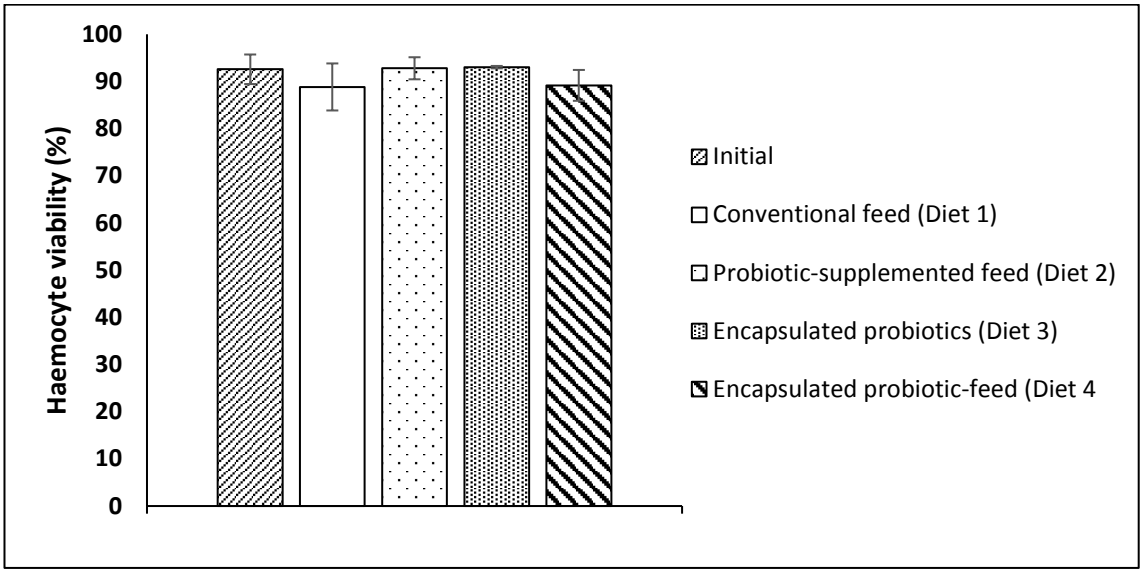


Figure 6-19 Haemocyte viability (%) of abalone fed with different diets before and at the end of feeding experiments (n=9; mean ± SE, one-way ANOVA, Tukey's post-hoc, $P < 0.05$).

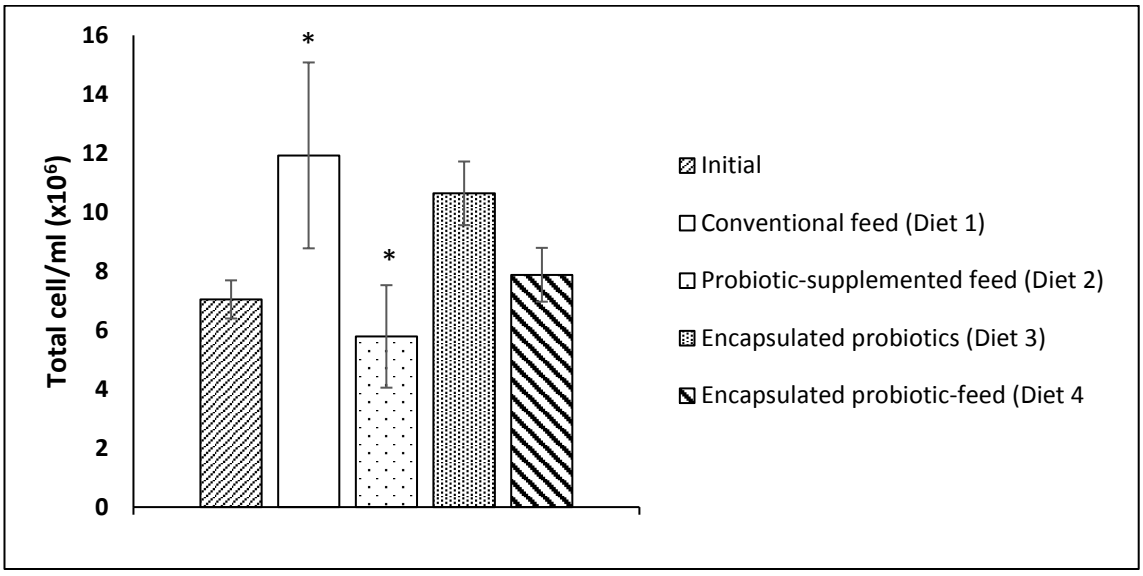


Figure 6-20 Total haemocyte cell counts of abalone fed with different diets before and at the end of feeding experiments. Asterisks indicate statistically significant differences between dietary diets (n=9; mean ± SE, one-way ANOVA, Tukey's post-hoc, $P < 0.05$).

6.3.6 Metabolomics study

GC-MS analysis was performed to identify extracted metabolites from abalone foot muscle. A total number of 227 metabolites were detected, of which 184 could be reliably identified. Levels of 31 metabolites (including 7 unknown metabolites) were significantly different among the feeding treatments (ANOVA; $P < 0.05$; FDR < 5%) which included amino acids and organic acids (Table 6.4). Unsupervised principal components analysis (PCA) was performed to visualise grouping of samples which resulted in four different groups in which samples from four different dietary treatments were clustered (Figure 6-21). A well-defined separation can be observed between diet 4 and the rest of the treatments, while less separation was observed among diet 1, 2 and 3.

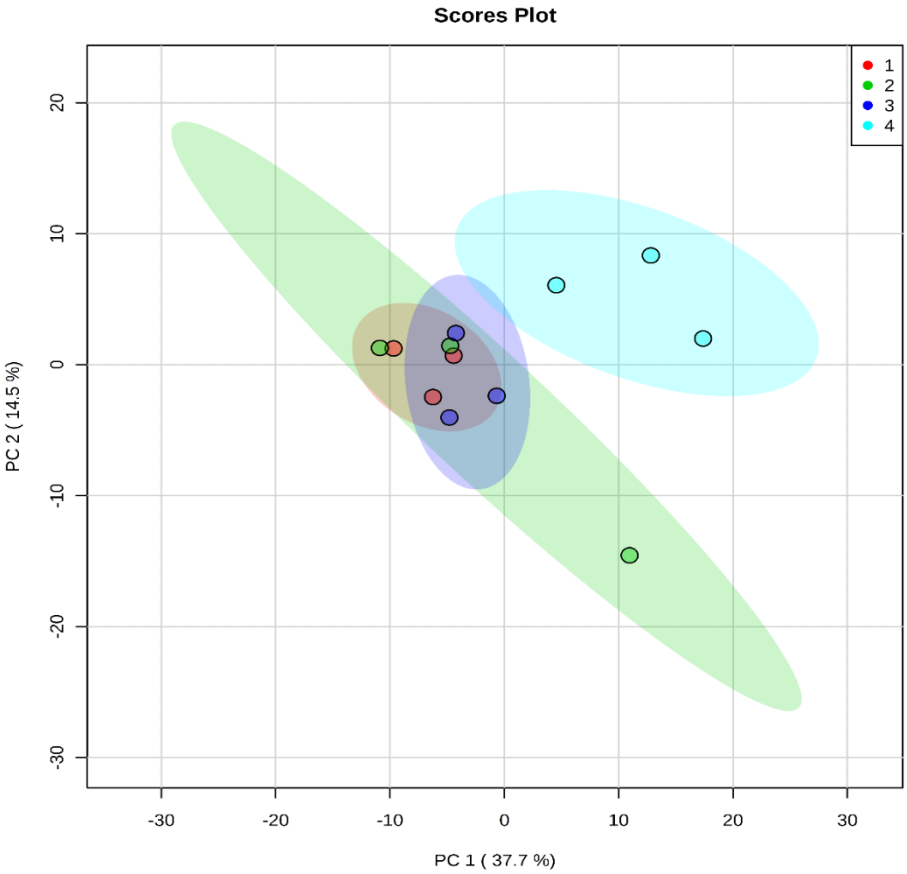


Figure 6-21 2D PCA score plot of abalone samples treated with four different diets based on all identified metabolites (n=227).

A heat map was developed to visualise potential differences among groups with different dietary treatments (Figure 6-22). Hierarchical cluster analysis (HCA) combined with the heat map shown the differentiation of only two main groups with one group having three subdivisions. Each group had a distinctive abundance of metabolites. The abundances of metabolites were generally higher in diet 4 compared to the other three diets except for an unknown compound which had the lowest abundance in diet 4.

Table 6.5 List of significant metabolites found in four groups of abalone reared for 57 days with different diets (One Way ANOVA, Tukey's post-hoc, $P < 0.05$, FDR < 0.05).

Metabolite name	*P* value	FDR	Nature of metabolite	Tukey's HSD (Post-hoc tests)
Isoleucine	0.0065	0.0477	Amino acid	4-1,4-2,4-3
Valine	0.0054	0.0435	Amino acid	4-1,4-2,4-3
Cysteine	0.0032	0.0353	Amino acid	4-1,4-2,4-3
Tyrosine	0.0056	0.0435	Amino acid	4-1,4-2,4-3
Tryptophan	0.0002	0.0075	Amino acid	4-1,4-2,4-3
Methionine	0.0002	0.0080	Amino acid	4-1,4-2,4-3
Glutamine	0.0003	0.0081	Amino acid	4-1,4-2,4-3
Creatinine	0.0065	0.0477	Amino acid	4-1,4-2,4-3
Threonine	0.0038	0.0371	Amino acid	4-1,4-2,4-3
Phenylalanine	0.0016	0.0240	Amino acid	4-1,3-2,4-3
Histidine	0.0008	0.0152	Amino acid	4-1,4-2,4-3
Oxalic acid	0.0012	0.0205	Organic acid	4-1,4-2,4-3
2-Aminobutyric acid	0.0013	0.0206	Amino acid	3-1,4-1,4-2
trans-Cinnamic acid	0.0017	0.0241	Organic acid	4-1,4-2,4-3
1,2,3,4-tetrahydro-8-hydroxy-4-oxo-, L-quinaldic acid	0.0028	0.0334	Organic acid	4-1,4-2,4-3
Pyroglutamic acid	0.0048	0.0420	Amino acid	4-1,4-2,4-3
1,5-Naphthyridine	0.0002	0.0075	Nitrogen containing compound	4-1,4-2,4-3
1-ethylbutyl-Benzene	0.0007	0.0146	Aromatic compound	4-1,4-2,4-3
4-Methoxyphenoxyphenylacetamide	0.0002	0.0075	Intermediate in phenylalanine metabolism	4-1,4-2,4-3
5-methyl-2-oxatricyclo6.5.0.04,8trideca-9, 11, 13-triene	0.0036	0.0371	polycyclic aromatic compounds	4-1,4-2,4-3
5,6-Dihydropyrazolo5, 1-aisoquinoline	0.0001	0.0075	Derivative of Tyrosine	4-1,4-2,4-3
5-3-Methyl-5-pentyl-2-furylpentanal	0.0001	0.0075	Fatty aldehyde	4-1,4-2,4-3
1-Aminocyclopropane-1-carboxylic acid	0.0046	0.0420	Amino acid	4-1,4-2,4-3

4-Hydroxyphenylacetic acid	0.0054	0.0435	Organic acid	4-1,4-2
Unknown 1	0.0000	0.0025		4-1,4-2,4-3
Unknown 2	0.0007	0.0146		4-1,3-2,4-3
Unknown 3	0.0007	0.0146		4-1,4-2,4-3
Unknown 4	0.0018	0.0242		4-1,3-2,4-3
Unknown 5	0.0025	0.0310		4-1,4-2,4-3
Unknown 6	0.0033	0.0353		4-1,4-2,4-3
Unknown 7	0.0043	0.0403		4-1,4-2,4-3

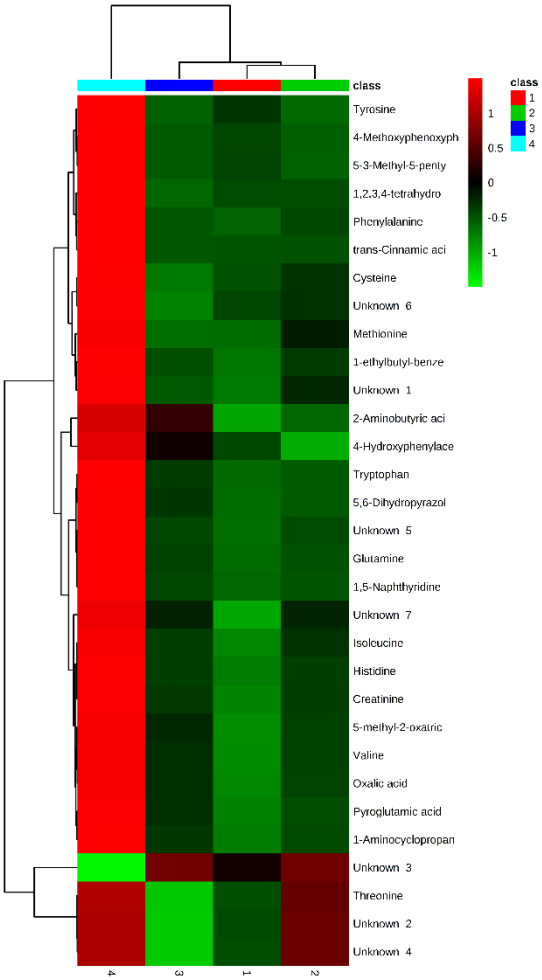


Figure 6-22 Heat map of all significantly different metabolites (n=31) found in abalone fed with four different experimental diets. Each column represents the average of 3 replicates. The abundance of each metabolite is illustrated with colour codes (red represents high abundance and green low abundance).

6.4 Overall discussion

This chapter was designed to assess the effect of an encapsulated diet on the growth rate and health of New Zealand black-footed abalone. As mentioned in the literature review, prior studies have reported the importance of encapsulating bioactives to improve the stability and reduce the leaching of bioactives for a better delivery to aquatic species (Zhu, Mai, & Wu, 2002; Miao et al., 2013). Therefore, it was hypothesised that by using encapsulated feed and probiotics, a controlled release of probiotics and nutrients may be achieved within the gastrointestinal tract (GIT) of abalone which could subsequently lead to a better growth performance and an improved health of the animals. The hypothesis was tested using four different diets to examine the response of juvenile abalone to each treatment over a 57-day feeding trial.

Conventional feed (Diet 1) served as basal diet (control) in this study. A conventional feed with probiotics sprayed on the feed's surface was used as diet 2. Diet 2 was included to investigate the effect of traditional method of probiotics delivery on abalone growth which has been the most common route of probiotics administration in previous studies (Macey & Coyne, 2005; Hadi et al., 2014). However, this diet may not be practically useful in an abalone farm due to the fact that it releases a large number of live bacteria in the water, raising environmental issues. Diets 3 and 4 were encapsulated containing only probiotic and a mixture of feed and probiotics, respectively. Several evidences support the hypothesis of this study including the enhanced growth rate, improved immunity due to lower level of ROS using encapsulated feed and probiotics. Likewise, a different and interesting metabolic profile with higher content of free amino acids was found when probiotics and nutrients were delivered to abalone in encapsulated form.

The high number of probiotic bacteria found within the GIT of black-footed abalone fed with diet 4 shows the efficiency of the developed beads in delivering probiotics to abalone. The highest level of improvement in both shell length and weight of abalone associated with diet 4 may be explained by a better delivery of probiotics and nutrients to the animals. The routes by which nutrients are transferred to aquatic animals are

critical to obtain satisfactory growth performance. Previous studies reported that encapsulating nutrients can retain nutrients in a capsule or bead and prevent nutrients leaching into the seawater (Lopez-Alvarado et al. 1994; Onal & Langdon 2000; Anas et al. 2008). Furthermore, encapsulation can improve the survival rate of probiotic bacteria significantly (Mandal et al. 2006; Morinigo & Vsanchez 2008; Rosas-Ledesma et al. 2012; Dong et al. 2013). Hence, it could be conceivably concluded that diet 4 is able to retain nutrients and viable probiotics within the beads structure until consumed by abalone.

A significantly low percent of feed wastage was obtained for diet 4 which could be due to the high palatability of alginate beads. Interestingly, the lowest FCR was obtained for diet 4. These results would therefore suggest that diet 4 can lead to higher abalone production in shorter time frames and surprisingly by using lesser amount of feed. In addition, lower feed wastage from diet 4 can result in the lower nutrification of culture water which can prevent the exposure of abalone to undesirable environmental factors such as pH stress, high load of organic materials and bacterial contamination.

A lower level of cells generating ROS was observed in probiotic-fed abalone with the lowest value obtained using diet 4. This could indicate lower levels of oxidative stress in probiotic-fed animals. Quantification of ROS has been frequently used to evaluate the level of oxidative stress in molluscs (Grandiosa et al., 2018; Nguyen et al., 2018). The presence of high amount of ROS generated in haemocytes was reported to be associated with oxidative stress as a result of environmental stressors such as hypoxia, undesirable pH of culture water, pathogens and elevated temperatures (Thorsten, 1999; Wang et al., 2009; Catarina et al., 2012). Moreover, excess ROS was reported to contribute to the degradation of essential biomolecules and harm cells and tissues (Ottaviani & Nappi, 2000). In agreement with this research, other studies also described application of probiotic dietary supplementation to enhance the antioxidant status, survival and immunity of marine animals (Chiu et al., 2007; Castex , Lemaire & Wabete, 2009) including *Haliotis* species (Macey & Coyne, 2005; Jiang et al., 2013; Hadi et al., 2014). This is probably due to the probiotic assistance in feed digestion which may lead to better nutrient absorption or competitive exclusion of pathogens (Turick & Tisa, 2002;

Zadeh et al., 2010). Although literatures support the finding of this study, the effect of nutrient encapsulation on ROS improvement obtained from diet 4 cannot be ruled out.

Investigation on haemocytes showed no significant differences between THC of probiotic-fed and control group. This agrees with another study by Macey at al. (2005), who reported no significant differences in the number of circulating haemocytes between probiotic-fed and control animals. The concentration of haemocyte within haemolymph is a critical factor in the defence mechanism of molluscs. Peraza-Gómez et al. (2009) reported that a high number of haemocytes is correlated with better resistance against pathogens. The high viability of haemocytes across all experimental diets are also indicative of lack of internal or external stress factors in all tested animals across different experimental diets. However, these results were in contrast to Roffi et al. (2018) who reported a lower level of cell viability in control animals compared to probiotic-fed (Grandiosa et al., 2018). These variations might be due to general biological variations between different batches of animals and the differences in maintenance conditions.

A major difference in metabolite profiling of abalone foot muscle was observed between diet 4 and the other diets mostly due to dissimilarities in free amino acids (FAAs). Higher levels of FAAs including tyrosine, cysteine, phenylalanine, histidine, tryptophan, threonine, isoleucine, valine, methionine, glutamine, creatinine and threonine may be attributed to enhanced food delivery, digestive proteolysis and amino acid uptake in abalone fed with diet 4. This view is supported by previous studies in which the amino acid profile in abalone tissue is reported to be directly affected by the animal's diet (Mai, 2002; Tung, 2010). Amino acids play important roles in metabolic pathways involved in growth, defence mechanisms, stress responses, health, food intake and nutrient utilisation (Santiago, 1988; Li , Mai & Trushenski, 2009; Guoyao, 2010; Effendy, Hutabarat, Ambariyanto, & Basuki, 2018). A higher level of free amino acids was reported in probiotic-fed abalone as a results of better feed digestion due to the proteolytic activity of probiotics (Grandiosa et al., 2018). Other studies reported the importance of glutamine in growth and disproportional relation of free glutamine with

stressors (Walsh, Blannin & Robson, 1998; Aledo, 2004; Travers et al., 2010; Cardinaud et al., 2014). Therefore, it can be suggested that the lower levels of haemocyte ROS and a higher amount of FAAs in abalone tissues may indicate a better health of animals fed with diet 4 (Viant, 2003; Rosenblum et al., 2005).

6.5 Conclusion

The feeding trial conducted in this chapter proved that probiotics and nutrients can be delivered successfully in chitosan-coated alginate (CCALG) beads to black-footed abalone (*Haliotis iris*). A significantly high growth performance of juvenile abalone clearly demonstrated that the CCALG can be an efficient delivery vehicle for nutrients to abalone. The beads encapsulating both probiotics and nutrients may be an ideal option for future feeding trials in larger scale. This diet resulted in a high growth rate of abalone, low feed wastage and desirable FCR that can potentially improve abalone production while minimising feed costs and improve sustainability of this important aquaculture species.

Chapter 7

Overall discussion, recommendations and conclusions

“The most sustainable way is to not make things. The second most sustainable way is to make something very useful, to solve a problem that hasn’t been solved”.

Thomas Sigsgaard

7.1 Thesis overview

Aquaculture as an important source of food for the world's growing population, has been a great asset to compensate for environmental impacts of fishing industries such as pollution, habitat destruction and depletion of fish stocks. Additionally, it can address the significant increase in protein demands from seafood resources (FAO, 2018). This has led aquaculture industries to adopt high-density culturing practices which can maximise the production in a shorter time frame and per unit of culture area. The intensive-oriented farming pattern would be entangled with disease outbreaks and inconsistent production if it is not accompanied with technological advances.

Scientists have tried to enhance productivity and environmental sustainability of aquaculture in different ways. For example, use of probiotics for marine animals as a green strategy has been tried to replace antibiotics and chemotherapeutics. Having been researched as a new potential approach to promote aquaculture sustainability, probiotics have yet to be efficiently and practically used in farms. The cost of continuous production of probiotics and lack of an efficient delivery method are limiting factors that still need to be improved. Administration of probiotics in form of feed additives or through culture water will generate undesirable impacts on the quality of culture water. Therefore, delivery method of probiotics to farmed aquatic animals must be deliberately considered to avoid destructive effects on environment and marine ecosystems.

In the experimental chapters of this thesis, New Zealand abalone industry was particularly targeted due to the rapid growth of abalone market and the need for innovative technologies. The highly competitive international market of abalone, the commitment of abalone industry to adopt environmentally sustainable methods, the slow growth rate of abalone and occasional mortality due to disease and high temperature are among the reasons that suggest the need for new advances in abalone farming. Additionally, having a high amount of feed wastage due to physical disintegration of conventional feed, nutrients leach and low feed consumption rates can cause further deterioration of water quality, increase the feed cost and hence reduce the total production of the farm. The main aim of this thesis was to develop an

encapsulated probiotic feed to improve the growth rate of New Zealand black-footed abalone (*Haliotis iris*). Development of an encapsulated feed that can help the industry to obtain a better growth of abalone was performed stepwise through Chapters 3, 4, 5 and 6.

7.2 General discussion

Chapter 2-Literature review

A critical step toward development of aquaculture is a mutual collaboration between farmers and scientists. This leads to current aquaculture issues to be conveyed to scientists and farm operators to be educated on advanced technologies. The review, presented in Chapter 2 of this thesis, was carried out to introduce the potential of encapsulation techniques to design targeted delivery methods of bioactives for aquaculture applications. There have been considerable advances in designing formulations capable of providing controlled and targeted delivery of bioactives in pharmaceutical industries while, not many seem to be aware of the great potential of these concepts in aquaculture. This literature review was conducted to give an overview of potential applications of encapsulation technology in aquaculture. Suggestions and recommendations on selecting suitable materials and methods of encapsulation, a comprehensive summary of previous research and practical considerations were provided. It is hoped that this review will draw attentions toward more efficient delivery methods of bioactives to aquaculture processes and lead to future incorporation of innovative techniques for more advanced and sustainable practices in aquaculture.

Chapter 3: Characterisation of probiotics isolated from *Haliotis iris*

Chapter 3 aimed to characterise previously isolated probiotic bacteria from healthy adult abalone. The three bacterial species were previously isolated and characterised by Hadi (2012), however, the failure to obtain expected growth rates using these probiotics during subsequent studies (Tuterangiwhiu, 2015; Grandiosa et al., 2018), questioned the ability of those probiotics to retain their characteristics after a long chilled-storage (-80˚C). Increase in feed digestibility is one of the important modes of actions of probiotics. Therefore, the objective of this chapter was to ensure the presence of

bacterial features, mainly alginate, protein and starch hydrolysis, which should help with feed digestion.

The results obtained confirmed that the three species of probiotics have characteristics that may help abalone in feed digestion such as alginolytic activity, proteolytic activity and acid production associated with *Vibrio* sp., *Exiguobacterium* sp. and *Enterococcus* sp. respectively.

Chapter 4: Development of a microencapsulated probiotic delivery system for *Haliotis iris* using an emulsion technique

In this chapter, chitosan-coated alginate microparticles produced using an emulsion technique were proposed as probiotics delivery vehicles to abalone. High encapsulation efficiencies for all the three probiotics indicated the efficiency of the developed microparticles to maintain the viability of encapsulated bacteria. Microparticles were further imbedded in alginate beads to facilitate delivery to bottom-feeding abalone. High consumption rates were achieved due to alginate being a feed attractant. Furthermore, lack of bacterial discharge in the first 6 hrs of incubating microparticles in seawater showed the potential of this delivery system in minimising environmental contamination and retaining the quality of culture water compared to conventional methods of probiotic delivery. The developed system was also capable of providing a target release mechanism where low numbers of bacteria were initially released in simulated gastric juice followed by high amounts released in simulated intestinal juice. Tracking the fluorescent labelled probiotics through the GIT of probiotic-fed abalone indicated successful delivery of probiotics into the GIT followed by digestion of microparticles.

Chapter 5: Probiotic encapsulation and characterisation of chitosan-coated alginate beads for oral administration to abalone

Although the results obtained in the previous chapter were satisfactory, Chapter 5 aimed to develop fast-sinking beads that can be easily scaled-up for manufacturing in-farm as an alternative approach to using embedded microparticles.

An extrusion technique was used to produce chitosan-coated alginate beads. This technique is inexpensive and easily applicable for industrial purposes. A step-by-step optimisation was carried out to produce beads with desired features such as lower bacterial release and higher physical stability in sea water. The developed beads showed a fast sinking rate making them easily accessible to bottom-feeding abalone. Monitoring abalone fed with alginate beads revealed a high palatability and consumption rate. A significantly higher bacterial load observed in probiotic-fed animals showed the efficiency of chitosan-coated alginate beads in delivering probiotics to abalone.

Chapter 6: Effect of encapsulated feed and probiotics on the growth and immunity of *Haliotis iris*

The aim of this chapter was to assess the growth performance of abalone in response to encapsulated feed and probiotics compared to conventional feed and probiotic delivery methods. Therefore, four different diets were tested with an ultimate aim to select one encapsulated diet that confers a better growth performance to abalone. Chitosan coated alginate (CCALG) beads containing both nutrients and probiotics demonstrated significantly better growth performance, lower FCR and feed wastage which potentially can increase abalone production and reduce the feed cost in abalone farming. Considering the increase in shell length and weight gain obtained for abalone fed with encapsulated feed and probiotics, market sized abalone (80 mm) can be obtained over 30.66 months as opposed to 52.87 months for abalone fed with conventional feed. Furthermore, the weight of abalone fed with encapsulated diet can reach to 40.88 g within 30.66 months while this figure may reach to only 15.86 g for abalone fed with conventional diet over the same period. This would make a significant improvement in the productivity of abalone farming. However, it should be considered that these figures were calculated based on the results of laboratory-based feeding trial and is reported here only to show the potential of encapsulated feed and probiotics diet compared to conventional feed. More accurate estimations can be achieved after an on-farm trial.

7.3 Study limitations and future recommendations

Chapter 3:

An attempt was made to determine the probiotic bacteria to species level; however, more than one species was identified for the probiotic bacteria due to the limitations of 16s rRNA gene sequencing method. It would be beneficial to identify the strains of the probiotic bacteria before using them in a larger scale. The capability of probiotics to help in feed digestion was evaluated using qualitative and quantitative screening microbiological methods. It would be interesting to study the microbial digestive enzymes and their activity in simulated conditions of the abalone digestive tract.

Chapter 4 and 5:

The aim of these two chapters was to develop a carrier to deliver the three probiotic bacteria to black-footed abalone. However, some of the experiments, including bacterial release evaluation, determination of probiotic load in the GIT of abalone, and *in-vivo* tracking of encapsulated bacteria were carried out using only one probiotic bacterium. *Exiguobacterium* sp. was used in the bacterial release study and tracking experiments. The reason was the limitation in detecting *Enterococcus* sp. under fluorescent microscopy due to the very small size of the bacterial cells. *Vibrio* sp. also showed a very low viability after staining with a fluorescent dye. Therefore, to increase the replicability of the results, *Exiguobacterium* sp. was used as a model bacterium. Furthermore, only *Enterococcus* sp. was utilised to assess the bacterial load content in probiotic-fed animals, as a selective medium to isolate *Enterococcus* sp. was accessible to this study.

Chapter 6:

The aim of this chapter was to examine the effect of encapsulated probiotics and nutrients on health and growth of juvenile abalone. Although it was clearly demonstrated that the developed alginate-based carrier can be an efficient delivery system of bioactives to abalone, the wider application of these results is subjected to some limitations. One of the limitations was confinement to laboratory settings that did

not allow to use the same density of animals as in abalone farm. Also, animals were kept in a static condition during the feeding trial as opposed to a recirculating system. An additional limiting factor was the unavailability of seawater for a daily replacement in the experimental tanks; therefore, the cleaning and changing of water were performed every second day.

On-farm trials are to be considered in future studies in order to assess the efficiency of CCLAG beads to deliver feed and probiotics to abalone in flow-through systems as they usually perform better in farm compared to static conditions. It is recommended to optimise the amount of feed ratio which is delivered using CCALG beads in future studies to determine the minimum amount of feed that can result in an optimum growth rate. This may reduce the feed wastage to even less than the 18% that was obtained using diet 4 while maintaining a desirable growth performance. It would be of value to perform a sensory evaluation to understand any possible changes in abalone meat quality parameters especially taste and texture before moving toward commercialisation. The long-term storage stability of beads is also an important factor which should be further investigated as a critical step toward commercialisation.

Another area for further research could be investigating the effect of dietary-encapsulated probiotic feed over a longer period as abalone may have different feeding behaviour and growth rate at different times of the year.

Preliminary bead consumption observations demonstrated that abalone are highly attracted to alginate beads much more than the conventional feed. It would be interesting to perform in-depth behavioural studies on the eating habits of abalone and how they may be changed using spherical beads.

Using CCALG beads for feed delivery would be helpful to compensate the environmental impacts of current conventional feed. Therefore, valuable information may be obtained by conducting an elaborate study to investigate the environmental impact of CCALG beads compared to current conventional feed.

A preliminary metabolomics study was performed for the animals fed with different diets which showed a clear distinction between the animals fed with diet 4 and the other

dietary treatments. More in-depth analysis of metabolomics profiles and involved metabolomics pathways would reveal a better picture of the effect of different diets on growth performance and health of abalone.

7.4 Thesis impact and conclusion

Abalone is considered as one of the most expensive seafood worldwide and is mainly produced from aquaculture with a production value of more than 150,000 tonnes per year. A high market demand for abalone has pushed abalone growers for high intensity production. However, limiting factors such as the slow growth rate of animals has restrained the total production. Similarly, the abalone industry is an important economic sector in New Zealand with almost 85% of the total production exported. The high quality of New Zealand abalone (pāua) has promoted this species in many Asian countries including Hong Kong and China. However, the international abalone market is extremely competitive. Therefore, it is essential to improve pāua farming using advanced and affordable technologies. In this study, the developed CCALG beads that were produced using a simple extrusion technique using natural polymers, showed a great potential in delivering probiotics and nutrients to pāua. Using encapsulated feed instead of using conventional feed pellets can help the industry to enhance their production in a sustainable manner while reducing costs and feed wastage. This thesis was conducted considering various pillars of sustainability and is in line with the Government of New Zealand's new aquaculture strategy specially taking the following objectives into account: maximising the value of existing farms through innovation and extending to high value land-based aquaculture (New Zealand Government, 2019).

To conclude, this thesis provides the first coherent scientific report on developing a chitosan-coated alginate-based encapsulated nutrition/probiotic delivery system with high palatability and stability in seawater. An encapsulation technique was developed to encapsulate feed and probiotics to improve the delivery of bioactives to black-footed abalone. This is the first report of using CCALG encapsulated probiotic-feed for abalone which resulted in a substantial growth rate, improvement in FCR and reduction in feed wastage. The developed delivery system can be potentially scaled up for industrial

applications as it is biocompatible, easy to produce and efficient in delivery of feed and probiotics to juvenile abalone. This research provides the baseline for adopting encapsulation approach to improve delivery methods of not only feed and probiotics but also other bioactives such as immunostimulants to farmed abalone.

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Appendix

Sequencing results of the 16s rRNA for the probiotic bacteria

Enterococcus sp.:

CACCGGAAAAAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAG
GGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTT
GAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACT
GAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAG
TCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGA
GAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCT
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GTCGCAAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGC
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Exiguobacterium sp.:

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Vibrio sp.:
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