Use of Probiotic Bacteria to Improve the Growth of Farmed New Zealand Abalone (*Haliotis iris*)

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Dedicated to my wonderful late parents

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LIST OF ABBREVIATIONS

TACC	Total Allowable Commercial Catch
CFU	Colony forming unit
GIT	Gastrointestinal tract
LAB	Lactic acid bacteria
MRS	De Man–Rogosa–Sharpe Medium
TCBS	Thiosulphate citrate bile sucrose medium
UV	Ultraviolet
P.a.	Per annum
FAO	United Nations Food and Agriculture Organization
WHO	World Health Organization

ATTESTATION OF AUTHORSHIP

“I hereby declare that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirement for a degree.
I also certify that the thesis has been written by me. Any help that I have received in my research has been acknowledged.

Signed:

Date:

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CONFIDENTIAL MATERIAL

This thesis contains confidential information which if publicly available may jeopardise the future intellectual property rights of the author.

Abstract

Abalone are known to have a very slow growth rate that results in significant financial constraints for its cultivation. Commercially farmed abalone which are given formulated feed consisting of soy flour and seaweed still require 4 to 5 years to attain a market size of (80-100 mm) for shell length.

To improve growth in farmed New Zealand abalone (paua), *Haliotis iris*, potential probiotic isolates were isolated from healthy adult abalone obtained from OceaNZ Blue (Bream Bay, New Zealand) and from farm tanks. The isolates were screened qualitatively according to their ability to hydrolyze feed nutrients (such as proteins, starch, and alginate), produce lactic acid, and resist bile salts. Phenotypic and 16s rRNA techniques were used to identify the potential probiotic isolates. Biochemical analyses to determine which isolate exerted the strongest proteolytic, amylolytic, and alginolytic activities were carried out.

This study has developed a multi-strain conglomerate of 2- and 3- probiotic bacterial strains that have been supplemented into a commercial abalone feed to determine if probiotic microorganisms can increase the growth rate of farmed *H. iris.* The 2-probiotic conglomerate consisted of *Exiguobacterium* JHEb1 and *Vibrio* JH1, the 3-probiotic conglomerate consisted of *Enterococcus* JHLDc in addition to *Exiguobacterium* JHEb1 and *Vibrio* JH1.

The probiotic feeds were used in a laboratory feeding trial involving abalone juveniles (sized 20-30 mm) to determine if probiotic microorganisms can increase the growth rate of farmed *H. iris*. Two groups of abalone (in 3 replicates) were fed 2- probiotic supplemented, and 3- probiotic supplemented feed were compared with the control group (3 replicates) administered with un-supplemented feed. Proximate analysis of abalone faeces were performed to determine differences in proteins, carbohydrate, and lipid in all abalone groups and determine if these nutrients were more efficiently metabolized in the presence of probiotic bacteria.

A significant growth improvement was obtained with the 3-probiotic supplemented feed that produced a significant shell length increase of 20.9%, wet weight gain of 19.8% and reduced mortality (3.33%) ($p<0.05$). The 2-probiotic supplemented feed also resulted in significant increase in shell length and survival ($p<0.05$) but not in weight gain.

This study is the first to report of the application of *Exiguobacterium* JHEb1in abalone. This species was incorporated in both the 2-probiotic and the 3-probiotic feeds. This study is also the first to report that a combination of three probiotic species supplemented into the commercial feed of farmed *H. iris* increased growth measured by shell length and wet weight.

Chapter 1

Introduction

1.1 Introduction

The black-footed abalone *Haliotis iris* is the main commercial species of abalone in New Zealand; it is called “pāua” in New Zealand Maori language (Dutton, 1986). At present, there are about 20 species of abalone harvested commercially worldwide inclusive of the New Zealand species (Hahn, 1989 a). The main abalone fishing countries are Japan, Australia, New Zealand, South Africa, Mexico and the United States (Tung, 2010).

This kind of abalone is infrequently distributed all along the coasts of both main islands of New Zealand, Chatham Islands, Stewart Island, and the Snares Islands. Maori people have harvested *H. iris* for hundreds of years as far back as 1150 AD. Traditionally they harvested and traded abalone for their flesh and their decorative shells. The abalone flesh (the muscular foot) is eaten as a delicacy, while the polished shells are used for decorative purposes and to make jewelry. Abalone meat is exported and sold locally as both fresh and canned products (Sainsbury, 1982).

H. iris can live in both sheltered and exposed shores, and is considered as a shallow-water species which is habitually most abundant at <5 m depth. However its distribution may extend to 20 m in depth (Sainsbury, 1982; Schiel, 1991). It inhabits rocky substrates by clinging firmly to the rocks, particularly flat boulder bottoms, and like other abalone species prefers well oxygenated sites where there is sea water with stable salinity (Hahn, 1989 b). They also grow much larger in colder waters around the coast of Southland of New Zealand and Stewart Island where can be found in much more abundant (Sainsbury, 1982).

Research in aquaculture has recently focused on abalone since the supply of wild abalone seems to be dwindling due to overfishing, their slow growth rate and their tendency to aggregate in one region (Heath & Moss, 2009). To increase the supply of abalone, land-based aquaculture of abalone was started in New Zealand in the 1980’s using developed techniques to gain sustainable yield. There are now about 40 farms around the country cultivating paua for meat, shell, and pearl production (Heath & Moss, 2009).

Abalone aquaculture is an economically important industry in New Zealand where aquaculture has been identified by the government as the country's fastest growing primary industry. Aquaculture is believed to bring New Zealand significant economic growth in an environmentally sustainable approach.

Abalone is considered to be a highly prized Mollusk delicacy and fetches a high price at the market. The New Zealand export of abalone reached a peak of NZ$80 million in 2001, **Figure 1-1**. Although contributing to the top ten seafood exports, valued in excess of NZ$62 million in 2010, **Table 1-1**, this value has obviously declined probably due to reasons stated previously. The slow growth rate of abalone could be a significant problem in the abalone industry. Commercially farmed abalone which are given formulated feed grow faster than wild abalone but they still require 4 to 5 years to attain a market size of 80-100 mm in shell length. Reducing this time period would result in a profitable operation.

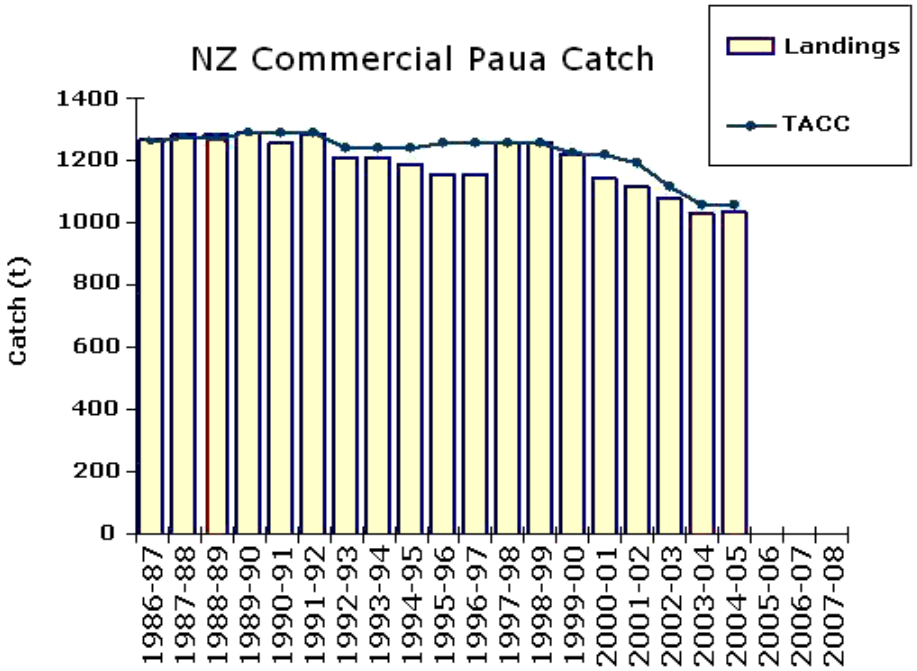

Figure1-1 Statistics of commercial catch of abalone in New Zealand (PICL, 2005)

Species	Export Value (FoB)
Rock Lobster	$229,221,123
Hoki	$172,747,270
Mussels	$171,467,731
Squid	$87,440,753
Salmon (All)	$85,676,382
Paua	$62,683,097
Fish prod unfit for human	$49,297,624
Orange Roughy	$48,709,284
Jack Mackerel	$42,702,397
Ling	$42,105,679

Table 1-1 Top ten New Zealand seafood exports in the year 2010 (SeaFIC, 2010)

To address the issue of the slow growth rate of abalone, studies have focused on the development of nutrient-rich artificial diets. Further, the concept of probiotic bacteria which aid digestion in humans and livestock has been adopted in aquaculture and its application has been rapidly increasing. The use of probiotics to improve growth in abalone presents an enormous potential.

1.2 Probiotics Concept

The use of probiotics in aquaculture, in general, has become more popular due to increasing demand to use safe, environment-friendly additives to the feed to improve nutrition and growth of aquaculture animals (Irianto & Austin, 2002; Macey & Coyne, 2005). Gatesoupe (1999) defined 'probiotics' as "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive with the aim of improving health". Studies have shown evidence of remarkable improvement in health and survival of aquaculture animals in intensive rearing system, which have been administered diet containing

probiotics. Manipulating the gut microflora with probiotics could lead to great benefit to host health and improving their growth rate (Olafsen, 2001a).

The use of probiotics is now commonplace in 'functional foods' not only for humans but also for animals as therapeutic and prophylactic feed supplements (Kailasapathy & Chin, 2000; Stanton et al., 2005). The lactic acid bacteria (LAB) are commonly used in probiotic dairy products and terrestrial animal feeds (Ringø & Gatesoupe, 1998). LAB are characterized by their resistance to acidic and bile environment in the intestinal tract. Sugars are fermented by LAB to lactic acid and other organic acids thus lowering the pH in the intestinal tract which could inhibit many bacteria including intestinal pathogens. LAB have been shown to produce antimicrobials which again could inhibit pathogens (Ringø et al., 2005).

Yeasts and the spore-forming *Bacillus* have also been used as probiotics, (Hong et al., 2005; Kesarcodi-Watson et al., 2008). Several studies indicated in their study on shrimp *(Litopenaeus vannamei* and *Penaeus monodon)* that the use of *Bacillus* sp. in the diet, have generally increased the shrimp survivals and immune parameter when challenged with pathogenic *Vibrio harveyi* and white spot syndrome virus (Li et al., 2009; Rengpipat et al., 2000). Yeasts, such as *Saccharomyces*, and *Debaryomyces* have been used widely in aquaculture, they showed great immunostimulatory activity and high production of inhibitory substances (Irianto & Austin, 2002; Van der Aa Kühle et al., 2005). Growth rate improvement noticed in abalone *Haliotis midae*, when they fed supplemented feed with two yeast and one bacterial strain (Macey & Coyne, 2005).

Several modes of action have been suggested to explain how probiotics could be beneficial to the host's health:

1. Enzymatic assistance to digestion and absorption of nutrients.
   Many studies suggested that probiotic microorganisms can improve the digestion of nutrients in GIT of the host by production of protein and carbohydrates hydrolysis enzymes (Erasmus et al., 1997; Krishnaprakash et al., 2009; Sahu et al., 2008).

2. Antagonism towards pathogens

   Several studies have reported the effect of probiotics to be strongly inhibitory against pathogens in aquaculture e.g. *Vibrio anguillarum*. Several strains of the bacterium *Roseobacter* are inhibitory toward other pathogenic bacteria namely *Vibrio splendidus* and *Vibrio anguillarum, Roseobacter* strain T5 can produce inhibitory compound such as the sulfur containing tropodithietic acid (Balcázar et al., 2007 b; Bruhn et al., 2006).

3. Competitive exclusion

   Microbial interactions play an effective role between competing beneficial and potentially pathogenic microorganisms. Probiotics can prevent bacterial diseases in aquaculture through specific competition for pathogen receptor sites on the mucus of the gut of the host (Balcázar et al., 2006; Verschuere et al., 2000).

4. Improvement of water quality

   It has been reported that improved water quality in aquaculture associated with the use of probiotics, such as *Bacillus* species. Probiotics can reduce the concentration of nitrogen and phosphorus in the pond, consequently increased the survival rate and yield of aquaculture animals, e.g. shrimp *Penaeus monodon* (Balcázar et al., 2006; Dalmin et al., 2001).

5. Immunostimulatory function.

   Probiotic microorganisms may benefit the host by stimulating the immune system in both cellular and humoral immune defense. Stimulating the host immune system could occur through the probionts production of specific substances called immunostimulants (Balcázar et al., 2007 a; Rengpipat et al., 2000; Sahu et al., 2008).

   More details for each mode of action of probiotic microorganisms are in the next Chapter (Literature Review).

   A single probiotic species could exert either one or a combination of these modes of action. The use of multi-strain or multi-species probiotics would serve as an approach by which all the potential benefits from probiotic microorganism

could be obtained. Few studies used multi-strain probiotic in supplementation of feed for aquaculture animals (Macey & Coyne, 2005)

Bairagi et al. (2004) reported the advantage of the addition of two *Bacillus* spp. (*B. subtilis* and *B. circulans*) to the diet of rohu *Labeo rohita*. Their study showed that application of the two fish intestinal *Bacillus* to the leaf meal (instead of fish meal), improved the growth, conversion ratio, and protein uptake ratio. These effects attributed to amylolytic and cellulolytic enzymes of the additive bacteria. A study carried out by Balcázar et al. (2007a), showed the use of three LAB (*Lactococcus lactis ssp., Leuconostoc mesenteroides and Lactobacillus sakei*) enhanced the cellular and humoral immune functions in the host rainbow trout.

Macy and Coyne (2005) reported that using multi-strains feed supplement for abalone (*H. midae*), improved the growth rate. The combination of three probionts enhanced the immunity of abalone by increasing the survival rate after challenging with the pathogen bacteria *Vibrio anguillarum*.

1.3 Aims and objectives of the study

New Zealand aquaculture operations such as OceaNZ Blue (Bream Bay, Bay of Islands) are committed to naturally processed paua of the highest quality (PAUA, 2011).They prefer not to use any chemicals or unnatural substances in the feeding, breeding and protection against diseases. Therefore, supplementation of probiotic microorganism into abalone feed is an attractive strategy to enhance abalone growth and improve profitability in the industry.

The aim of this study is to develop a probiotic supplement for *Haliotis iris* to improve the growth rate.

The study is designed to:

1. Isolate potential probiotic strains from the digestive tract of healthy adult abalone, and from abalone farm tanks.

2. Select potential probiotics according to their ability to hydrolyze feed nutrients such as proteins, starch, and alginate.
3. Apply multi-strain probiotics into formulated feed to assess their effects on growth of aquacultured abalone.
4. Analyze the fecal matter of 2 and 3-probiotic supplemented abalone and compare the composition of protein, carbohydrate and lipid in feces with those of unsupplemented feed abalone.

1.4 Research Questions and Hypotheses

The general purpose of this study is mainly to examine the probiotic-supplemented feed and observe improvement in the growth of abalone (*H. iris*) by testing the following hypotheses:

- The selected probiotic bacteria can enhance the digestion of nutrients components of the commercial feed such as protein, carbohydrate, and alginate.

- The use of probiotic-supplemented feed can increase the growth rate (body weight, shell length) of *Haliotis iris* over the period of the feeding trial.

Chapter 2

Literature Review

2.1 New Zealand Abalone *Haliotis iris*

Paua are highly valuable resource in New Zealand, both as food and raw material for jewelry and other handcraft products. Paua meat is considered a healthy food with its high protein (71.99%) and low fat content (Imai, 1997).

Abalone are univalve mollusks. The New Zealand abalone are classified as (Tung, 2010):

Phylum Mollusca
Class *Gastropoda*
Family *Haliotidae*
Haliotis
Haliotis iris (Paua)

"Abalone" is a name derived from Spanish name "Abulon", which means "sea ear" (Tung, 2010), used for a variety of species of single-shelled mollusks from the *Haliotidae* family genera *Haliotis.*

In New Zealand, there are three documented species of abalone. The first species the black-footed abalone *H. iris (*Martyn 1784), known as 'paua' are the main commercial species (Dutton & Tong, 1981). The yellow-foot abalone, *H. australis*Gmelin,1790 commonly named the queen, are another species smaller than paua but also of good commercial value (O'Halloran, 1986). The white-footed abalone, *H. virginea (*Gmelin, 1790) commonly named the virgin, are the smallest species with the least commercial values, thus not harvested commercially (Dutton & Tong, 1986). The lifespan of all three species can reach more than ten years (Dutton & Tong, 1981; Tung, 2010).

2.2 Abalone Nutrition

Abalone are herbivorous mollusks. Wild abalone naturally feed on macroalgae (Tanaka & Sugimura, 2003). *H. iris*, similar to *H. australis*, are known to prefer and grow faster on red algae (*Hymenocladia*) (Poore, 1972). The smaller abalone juveniles (<5 mm) graze microscopic diatoms and ingest bacterial cells The sedentary, larger juveniles feed mainly on drifting macroalgae (Dutton &Tong, 1981; Garland et al., 1985).

Most farmed abalone are fed also with algae and depend on the availability of these algae. Marine algae consist mainly of high carbohydrate: protein ratio, with carbohydrate making up to 70% of the dry weight, in some cases (Pakulski & Benner, 1992). The wild algae *Ulva lactuca* contains lower protein concentration (3.7-19.9%) (Tung, 2010).

Abalone are known to grow slowly *H. iris* reaches its commercial size of 100 mm in 5 years (Sales & Britz, 2001) The slow growth rate of abalone in the wild and those of farmed abalone fed with only algae has been attributed to the low protein: carbohydrate ratio in marine algae. A diet consisting mainly of algae can hardly meet the 30-40% protein requirement of the New Zealand abalone (Tung, 2010). Schiel (1993) and Erasmus et al. (1997) similarly realized that farmed abalone fed on the kelp *Ecklonia radiata* did not exhibit high growth rates. Kelp and other macroalgae contain low protein at approximately 15% and an unbalanced amino acid profile. These low nutrient levels could not supply the proteins required for growth in some abalone species (Tung, 2010: Erasmus et al, 1997).

2.3 Probiotic: Definition and Principle

The word' probiotic' was derived from Greek words meaning 'for life' (Gismondo et al., 1999). This is in contrast to 'antibiotic', which literally means 'against life'. Probiotic as a term has evolved throughout the years.

The first definition for probiotics was by (Lilly, 1965), who described probiotic as "Substances produced by microorganisms which promote the growth of other

microorganisms". Later, a new definition included both substance and microorganism as probiotic where (Parker, 1974) defined probiotics as "Organisms and substances which contribute to intestinal microbial balance". Fuller (1989) reported that probiotic should be live microorganisms that can improve microbial balance in the host gut, hence, defining probiotic as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". After Fuller's definition, additional terms were commonly used to describe probiotics such as "friendly", "healthy", and "beneficial" (Wang & Li, 2008).

In aquaculture, the term "probiont" is used synonymously with the term "probiotic". This is done likewise in this study.

Havenaar and Huis In't Veld (1992) have indicated that one or more live microorganisms can benefit the host through improving gastrointestinal microflora, thus "A viable mono- or mixed-culture of microorganisms which when applied to animals or humans, beneficially affects the host by improving the properties of the indigenous microflora". Schaafsma (1996) pointed for the first time the importance of the amount and concentration of microorganisms, thus probiotics are: "Living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition". Naidu and Bidlack (1999) added more effects of probiotics to the host such as; improving physiology and immunity which extended the definition to "A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract". Scherzenmeir and de Vrese (2001) have strongly recommended that probiotic should be well-known microorganisms which when applied in adequate amount can colonize the host microflora, thus: "A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host"

Lately, the most acceptable definition approved by the United Nations Food and Agriculture Organization (FAO), and the World Health Organization (WHO) has

been “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (WHO/FAO, 2001).

The concept of probiotic was first applied to human foods but is now widely accepted and utilized in animal feed in aquaculture where the immediate ambient environment has even larger influence on the health status than in terrestrial animals or humans (Verschuere et al., 2000), Within the context of aquaculture, the term probiotic not only refers to live microorganisms in feed but also to those which are added to the water (i.e. water additives) used to farm fish and shellfish (Gatesoupe, 1999), and as a biocontrol agent. Thus the 2001 WHO definition of WHO may be modified to include organisms and supplement to the host, environment or feed (Verschuere et al., 2000).

2.4 The Rationale for the Use of Probiotics in Aquaculture

The application of probiotics in aquaculture, particularly in mollusk aquaculture, has mostly been towards disease management with special reference to molluscan culture (Kesarcode-Watson et al., 2008). Few studies demonstrated the rationale of using probiotics from the perspective of the digestion process in the host and the evaluation of safety of use (Wang & Li, 2008).

Krishnaprakash et al., (2009) recognized that in addition to reducing the effect of pathogenic organisms in the gut of shrimp, the incorporation of probiotics in shrimp diet could improve intestinal microbial balance, resulting in enhancement of food absorption and increased activity of digestive enzymes.

Irianto & Austin (2002) have suggested that the beneficial effects attributed to the use of probiotics in aquaculture could be due to competitive exclusion and inhibition of pathogenic microorganisms, assistance in the metabolism of the host and stimulation of the host immunity. Studies have shown that probiotic strains could inhibit pathogenic bacteria both *in vitro* and *in vivo* through several different mechanisms. These mechanisms include production of inhibitory compounds, such as bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide,

formation of ammonia, diacetyl, and alteration of pH values by organic acids (Verschuere et al., 2000).

The use of probiotics in aquaculture represents a potentially ideal alternative to the use of antibiotics in the industry, thus preventing the development of antibiotic resistance in both humans and microorganisms. Without the use of antibiotics, improved disease resistance against a pathogenic strain of *Vibrio anguillarum* was obtainedwhen atlantic cod fry were fed on dry feed containing lactic acid bacteria (*Carnobacterium divergens*) (Gómez, 2007).

Bruhn (2006) tried to define the beneficial effect of the probiotics in aquaculture, and how to determinate if the potential microorganism is a successful probiotic to use. He suggested that live microorganisms may be administrated for different purposes; disease prevention, water quality improvement, or as a feed, and all of these can endorse directly or indirectly health and /or survival of the farmed animals (Bruhn, 2006). Other suggestions of how to measure the effect of probiotic could be via its ability to decrease frequency of disease and/or increase survival from lethal diseases (Gram & Ringø, 2005).

2.5 Kinds of Probiotics

Most probiotics tested for use in aquaculture:

1) Gram-positive bacteria; such as *Bacillus* and lactic acid bacteria LAB ; *Carnobacterium, Lactobacillus, Lactococcus* (Gatesoupe, 1999).
2) Gram-negative bacteria such as *Aeromonas, Pseudoalteromonas, Pseudomonas, Roseobacter* and *Vibrio* (Gatesoupe, 1999; Verschuere et al., 2000)
3) Yeasts; such as Saccharomyces, and Debaryomyces (Irianto & Austin, 2002).

LAB are the most common probiotics used in the food industry, they are nonmotile, nonsporulating, generally catalase-negative, Gram-positive bacteria that produce lactic acid as a major or the only product of fermentation. LAB are commonly found in the gastrointestinal tract of various endothermic animals, in

milk, dairy products, seafood products, and on some plant surfaces. Probiotic LAB can colonize fish and crustacean intestinal tracts and improve the survival and growth of host fish species (Balcázar et al., 2008; Iehata, 2009). *Enterococcus faecium* as LAB also shown significant improve of the survival rates of European eels (*Anguilla Anguilla L.*) when added to the feed compared with control groups after challenged with pathogenic *Edwardsiella tarda* (Chang & Liu, 2002).

Lactic acid bacteria (LAB) are used regularly as probiotics in aquaculture, due to their production of benefical substances other than lactic acid, e.g. bacteriocins and other chemicals that have inhibitory activity against pathogenic microorganisms. The production of organic acids such as lactic acid primarily benefits the host by lowering the pH of the GIT that naturally prevent pathogenic bacteria from colonization (Kesarcodi-Watson et al., 2008). Recently, Sarkono et al. (2010) has pointed that two of ten LAB strains isolated from the gut of abalone *Haliotis asinine*; *Lactobacillus* OPA4 and AL1, showed the ability to suppress the growth of pathogenic bacteria namely *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*. The two strains showed high tolerance to bile salts and acidity (Sarkono et al., 2010).

In the past, LAB have not been considered as part of the indigenous microflora of some aquatic animals like fish, therefore, it was thought that including these bacteria to the fish feed could be useless. However, LAB have been observed to colonize the GIT of several wild species of marine fish as well as farmed Atlantic salmon (Gildberg,1997). LAB were observed to benefit the host through colonization in the gut and improving digestion, immunity, growth rate, and survival rate of a wide range of host species (Iehata et al., 2009).

Other frequently probiotics to use in aquaculture are the benefical *Bacillus* species. They are spore-forming bacteria that produce the antimicrobial peptide bacteriocin. *Bacillus* spp. showed the ability for adhesion and provide immunostimulation of the host, they have been used in aquaculture to improve growth rate, survival, and water quality (Cherif et al., 2001; Cladera-Olivera et al., 2004). The spores of Bacillus have good storage property (Hong et al, 2005).

These bacteria improved the health status of juvenile shrimp *Penaeus monodon* either by reducing or preventing the effect of the pathogenic *Vibrio* spp. (Dalmin et al., 2001).

Decamp and Moriarty (2006) showed that *Bacillus* species and yeasts have the same effect as antimicrobial agents when applied during shrimp cultivation. Bairagi et al.(2004) found that adding two fish intestinal *B. subtilis* and *B. circulans* to the diet of Indian Carp fish rohu *Labeo rohita* improved feed conversion ratio and protein efficiency ratio resulting in improved growth. The two species produce extracellular cellulolytic and amylolytic enzymes(Bairagi et al., 2004; Van der Aa Kühle et al., 2005)**.**

Vibrio species have shown significant benefits to aquaculture animals. For example *Vibrio halioticoli* increased the activity of the digestive enzymes in abalone (Sawabe et al., 2003).*Vibrio* and *Pseudomonas* are common used genera associated with aquatic environments. Both genera have been recommended as potential probiotic bacteria for cultivating fish, mollusks, and crustaceans (Sawabe & Sugimura, 1998). Balcázar et al. (2007 b) reported that *Vibrio alginolyticus* UTM 102, *Pseudomonas aestumarina* SLV22 with combination of *Bacillus subtilis* UTM 126, *Roseobacter gallaeciensis* SLV03 were antagonistic against the shrimp-pathogenic bacterium, *Vibrio parahaemolyticus* (Balcázar et al., 2007 b).

2.6 Probiotics Mechanisms and Mode of Actions

2.6.1 Competition for Adhesion Sites

Competing for adhesion sites on the mucus of GIT and other tissue surfaces is a possible mechanism to prevent pathogen colonization. Bacterial adhesion to tissue surface is a major stage for pathogenic infection, therefore, the first course of action for probiotics would be competing with the pathogens for adhesion sites (Verschuere et al., 2000).

Chabrillón et al. (2006) observed that the selected potential probiotic *Vibrio* strain Pdp11 adhered in high numbers to the intestinal mucus of the fish Gilthead sea bream. Although *Vibrio* Pdp11 showed no antagonistic activity against *Vibrio anguillarum in vitro*, the mortality rate was significantly low for the fish when challenging with *V. anguillarum*, These result suggested that the mode of the potential probiotic in the study was the colonization of GIT mucus and preventing the pathogenic *Vibrio anguillarum* from invasion (Bruhn, 2005; Chabrillón et al., 2006). However, colonization of potential probionts in the GIT of the animal host has always been questionable. Several studies could not detect the probiotics adhering to the mucus *in vitro* and *in vivo*. For example, some studies have been reporting the success of certain bacteria to adhere to intestinal mucus *in vitro,* however, the same bacteria failed to adhere to the animal intestinal mucus *in vivo* (Hansen & Olafsen,1999). Fuller (1992) suggested that colonization of the GIT of animals by probiotics is probable only after birth, and only high doses of addition of probiotics can cause its temporary domination. Mature animals that have been introduced to probiotics, showed quick decrease of probiotics populations in GIT within days after treatment has stopped (Fuller, 1992).

2.6.2 Competitive exclusion

Antagonism between bacteria commonly occurs in nature such as in the GIT of animals. This interaction keeps the ecological balance between beneficial and harmful microorganisms. Manipulation of the microflora in the GIT can be achieved by introduction of probiotics resulting in the reduction or elimination of pathogenic bacteria (Balcázar et al., 2006). Rosenfeld & Zobell (1947) reported the first study on marine microorganisms that can either compete with opportunistic pathogens or produce antibiotic compounds. Recent studies have again demonstrated the inhibitory role of beneficial microorganisms against pathogens in aquaculture (Verschuere et al., 2000; Vine et al., 2006).

2.6.3 Competition for Iron

Most animal pathogens require iron for growth. However, iron is not only limited in the tissues and body fluids of animals but also is present as insoluble Ferric iron, Fe3+ (Verschure et al 2000). Some pathogens produce siderophores to acquire their essential iron. Siderophores are low molecular- weight substances that act as iron chelators. There are probiotic bacteria that can similarly produce siderophores. In highly iron-stressed tissues and body fluids of aquatic animals, the siderophore-producing probiotics could deprive pathogens of iron (Kesarcodi-Watson et al, 2008; Verschure et al 2000) The first evidence of this mechanism was presented by Gram et al. (1999). *V*. *anguillarum* was observed to be inhibited by a culture supernatant of *Pseudomonas fluorescens* grown under iron-limitation, while no inhibition occurred with *P. fluorescens* grown in high iron concentration (Gram et al., 1999).

2.6.4 Supply of Nutrients and Digestive enzymes

Benefical microorganisms could aid the digestive processes in aquatic animals. It has been reported that *Bacteroides* species. and *Clostridium* Species contributed to fish nutrition, through supplying fatty acids and vitamins (Sakata, 1990). Krishnaprakash (2009) suggested that using probiotics in the diet of shrimp can improve intestinal microbial balance, causing increased activity of digestive enzymes and enhance food absorption in the gut.

Bacteria isolated from the gut of the sea hare, sea urchins, the minke whale, and abalone, have been observed to produce enzymes capable of hydrolyzing complex polysaccharides present in the host's feed (Erasmus et al., 1997). In agreement, Sahu et al. (2008)added that the use of beneficial microorganisms may speed up the rate of breaking down food to free amino acids and glucose. Mutual benefits between bacteria and hosts such as shrimp would occur in the presence of simple products resulting in improved health of shrimp.

Altering enzyme production in the host gut via probiotic application can increase the yield of animals. A study in China showed that the application of effective microorganisms such as probiotics to commercial freshwater prawn (*Penaeus orientalis*) cultures achieved an increased harvest at 103% (Qi et al., 2009).

2.6.5 Disease Resistance

An outbreak of cholera in 1991-1994 occurred in Ecuador involving multi-drug resistance in *Vibrio cholera*e. The outbreak started with people working in shrimp farms. The strain *V. cholera* strain 01 was sensitive to 12 antimicrobial agents. In Ecuador, the same strain developed multiple resistances through gene transfer from non-cholera *Vibrio* pathogens of shrimp (Weber et al., 1994). With the reluctance in using antibiotics in aquaculture, there has been a heightened need for alternative to antibiotics (Verschure et al, 2000; Vine et al 2006). Several studies have reviewed the use of probiotics in aquaculture as a potential alternative to antibiotics in controlling pathogens (Fuller, 1992; Rinkinen et al., 2003).

The mechanism by which probiotic microorganism improve disease resistance in aquaculture animals is a subject of several studies. Disease resistance may be attributed to improved health, growth performance, feed utilization, and stress response when the host's microflora is modulated by probiotics (Merrifield et al., 2010 b).

The abundance of lactic acid bacteria (LAB) in rainbow trout during a furunculosis outbreak provided a clear evidence of the contribution of LAB to the elimination of the causal organism of furunculus, *Vibrios* that caused furunculosis. Three LAB species, *Lactococcus lactis, Lactobacillus plantarum*, and *Lactobacillus fermentum,* were observed in healthy fish during the outbreak. These bacteria are non-pathogenic and have not been reported to cause any infectious diseases in fish. *Carnobacterium*, *Vibrio alginolyticus*, the genera *Pseudomonas*, *Aeromonas* and *Flavobacterium* have been used as biological control agents (Balcázar et al., 2006; Balcázar et al., 2007 a).

That could indicate that these bacteria can increase resistance against pathogens. LAB are known to produce bacteriocins and other antimicrobial that are inhibitory to other bacteria (Kesarcodi-Watson et al., 2008). Probiotic bacteria have been evaluated on their activity against fish aquaculture diseases, however, commercial vaccines are available for the majority of pathogens in adult fish. Hence, the use of probiotic bacteria is promising to prevent infectious diseases in fish larvae, mollusks, and crustaceans. In which there are no available vaccines. In general, probiotic bacteria may not have wide ranging application in aquaculture but may be effective in a specific production of animals (Bruhn, 2006).

2.6.6 Enhancement of Immune response

Probiotic microorganisms can stimulate non- specific immune system of the host. Administration of probiotic bacteria *Clostridium butyricum* to rainbow trout increased the phagocytic activity of leucocytes in the blood, resulting in enhanced resistance of the fish against vibriosis (Sakai et al., 1995).

The mechanism of probiotics to stimulate the host immune response is usually through producing immunostimulants. These products enhance the defense system against pathogens by induce the host to increase phagocytosis, antibodies, chemiluminescent response and by producing superoxide anion (Sakai,1998). Immunostimulants are varied and depend on the type of probiotics; e.g. lipopolysaccharides (cell wall component) of Gram-negative bacteria have been demonstrated to increase macrophage phagocytic activity in red sea bream *Pagrus major*. Bacterin, from *Vibrio anguillarum* is the most successful vaccine, spores from *Clostridium butyricum*, and glucan from yeast cell walls. All these Immunostimulants have been used in aquaculture (Sakai, 1998).

Balcázar (2003) explained that application of mixed culture of *Vibrio* and *Bacillus* sp. enhanced the resistance of juvenile white shrimp against pathogenic *Vibrio harveyi* and the virus that causes white spot syndrome by stimulating the immune system and increasing phagocytosis (Balca´zar, 2003). This increased the survival and growth rate of white shrimp. Further the use of *Bacillus.* sp.

strain S11 activated both cellular (phagocytes) and humoral (antibodies) immune defenses in tiger shrimp, *P. mondon* (Rengpipat et al., 2000; Sahu et al., 2008).

2.6.7 Improving Water Quality

Probiotics in aquaculture can be applied as live, dead or a component of microbial cell via the feed or into the rearing water from which both the host and the ambient environment benefit (Balcázar et al., 2006; Merrifield et al., 2010 a; Olmos et al., 2011).

Commercial probiotics used in shrimp *Penaeus vannamei* rearing ponds were found to reduce concentrations of dissolved nitrogen and phosphorus leading to an increase in white shrimp yields (Tegner, 1985; Wang & Li, 2008; Wang et al., 2005). Further, Sahu et al.(2008) stated that this role of the benefical bacteria is very important in reducing slime and sludge formation, foul odours are eliminated by degradation of organic materials. Beneficial bacteria also consume the inorganic forms of nitrogen; such as ammonia, nitrate, and nitrite. These activities improve water quality and reduce diseases caused by *Vibrio* sp., *Aeromonas* sp., and viruses as well as increasing zooplankton numbers which benefits the animals feeding on live feed (Krishnaprakash et al., 2009; Sahu et al., 2008).

Application of high density of *Bacillus* spp. during cultivation of juvenile shrimp *Penaeus monodon* improved water quality in the farm by degradation of organic matter to CO_2 (Balcázar et al., 2006). Recent study showed that the newly known *Exiguobacterium* spp. has been used as probiotic in shrimp culture and contributed to improving the water quality of the shrimp earthen pond (Sombatjinda et al., 2011). Most benefical bacteria, such as *NItrosomonas* can convert ammonia to nitrite while others, such as. *Nitrobacter*, can further mineralizing nitrite into nitrate (Krishnaprakash et al., 2009).

2.7 Probiotics Criteria and Selection of Candidate Probionts

It is fundamental to understand the mechanism of action of selected probionts. The criteria of selection is based mainly on biosafety, method of administration and the target part of the host body the probiont is expected to affect (Huis in't Veld et al., 1994).

Candidate probiotic species should have one or more of the properties below that benefit the host. There is no single probiont that has all appropriate properties but the more present would make the probiont a good candidate for selection (Jöborn et al., 1997; Merrifield et al., 2010 a; Nikoskelainen et al., 2001; Qi, 2009):

- Must not be pathogenic, not only to the host species, but also to other aquatic animals and humans
- Must not possess plasmid-encoded antibiotic resistance genes.
- Must be able to adhere and grow well on intestinal mucus, and be able to colonize in large numbers
- Should have tolerance against high acidity inside the host stomach, and against bile salt in other parts of the gut like hepatopancreas.
- Should be able to produce extracellular digestive enzymes and/or vitamins.
- Should be native to the host, and host environment.
- Should be registered for use as safe feed additive (**Table 2-1**).
- Should remain viable during processing and normal storage.
- Should be able to multiply well with short doubling time in the host rearing temperature.
- Should possess antagonistic properties against one or more key pathogens.

Probiotics
Bacillus cereus var. toyoi
Bacillus licheniformis
Bacillus subtilis
Enterococcus faecium
Lactobacillus casei
Lactobacillus farciminis
Lactobacillus plantarum
Lactobacillus rhamnosus
Pediococcus acidilactici
Saccharomyces cerevisiae
Streptococcus infantarius

Table 2-1 Authorized microorganism as probiotics in feeding stuffs under Council Directive 70/524/EEC (Balcázar et al., 2006)

Selecting the appropriate probiont has not been easy for most studies due to factors such as uncertainty of how the probiont would react under a stressful condition. Selection of *Vibrio* species as probiont can be controversial since the genus has been associated with pathogenicity. *V. alginolyticus* has been suggested as probiont in shrimp *Litopenaeus vannamei* larvae. but some strains have been the causal organism of vibriosis in shrimps (Gomez-Gil & Roque, 2000).

2.8 Probiotics in Abalone

2.8.1 Effect of Microflora on Abalone Digestibility and Growth

The endogenous microflora found in the GIT of the South African abalone *Haliotis midae* is diverse consisting of Gram- positive bacteria, Gram- negative bacteria, and yeasts (Erasmus, 1996; Goosen, 2007; Macey & Coyne, 2005).

Mollusks are considered exclusive accumulators of specific microorganisms that lead to unique interaction between the animals and microorganisms (Romanenkoa et al., 2008). It is important to study the microbial diversity of microflora of marine animals to understand the role they play in their host, and to determine the effects of modulating the microflora the host animal (Olafsen, 2001b).

Bacteria isolated from the gut of the sea hare *Aplysia juliana*, sea urchins, minke whale, and abalone, have been shown to produce enzymes capable of hydrolysing complex polysaccharides present in the host's feed. Seaweed contains agar, carrageenan, laminarin, and alginate. Erasmus et al (1997) suggested that bacteria in the digestive tract improved the digestive efficiency of a host by supplying polysaccharolytic enzymes. Bacteria contribute to degradation of seaweed that consists of agar, carrageenan, laminarin, and alginate to simpler sugars easily assimilated by the host (Erasmus et al., 1997).

Coyne and Doeschate (2008) reported that 70–90% of enzyme activity in *H. midae* was extracellular enzymes secreted by bacteria into the lumen of the gut. It appears that enzyme production is an important role of microorganisms present in abalone gut (ten Doeschate & Coyne, 2008).

Benefical bacteria comprise 40–65% of the microflora detected in three species of the Japanese abalone, *Haliotis discus, Haliotis diversicolor aquatilis*, *Haliotis diversicolor diversicolor*, and a South African abalone, *Haliotis midae.* Most of these bacteria were *Vibrio halioticoli.* The abundance of *Vibrio halioticoli* in the gut of Japanese and South African abalone presents a strong evidence that *Vibrio halioticoli* provides vital contributions to abalone digestive process (Sawabe et al., 2003).

Vibrio bacteria are commonly associated with abalone species; several studies showed that they can have a helpful influence on the health of host abalone. *Vibrio midae* was confirmed as a probiotic organism in abalone *Haliotis midae* (Macey &Coyne, 2005; Macey & Coyne, 2006b).

2.8.2 Probiotics Use in Abalone Aquaculture

In contrast to other aquaculture animals, studies on application of probiotics in abalone have been directed towards growth enhancement rather than disease control. ten Doeschate & Coyne (2008) suggested three mechanisms by which improved growth in abalone can be achieved with probiotic supplement. Firstly, by increasing the concentration of simple nutrients which can easily be absorbed in the gut. Secondly, increasing the pool of important enzymes like amylase and protease in the abalone digestive gut. And thirdly, the host can use the bacterial supplement as an additional source of nutrients (ten Doeschate & Coyne, 2008). Probiotic bacterial to abalone artificial feed containing extracts of two algae (*E. maxima* and *G. gracilis*) have shown to increase the feed nutritional value and consequently improve the growth of abalone (Troell et al., 2006). Such supplementation can work well with (LAB) which can increase digestive enzyme activities and increase production of volatile short-chain fatty acids (VSCFA) that may potentially enhance the abalone growth (Iehata et al., 2009).

To increase the growth rate of the South African abalone, *Haliotis midae*, a probiotic-supplemented diet was administered (Macey & Coyne, 2005). The probiotic microorganisms included a Gram-negative species, *Vibrio midae* (SY9) and two yeast species namely *Cryptococcus* sp. (SS1) and *Debaryomyces hansenii* (AY1). SY 9 and SS1 were described as proteolytic and amylolytic while no specific characteristics were ascribed with AY1.

Increased levels of protease and amylase activity were observed in *H. midae* fed with a diet supplemented with the three microorganisms. These enzyme activities resulted in increased growth rate of small and large abalone by 8% and 34%,

respectively, after 8 months of feeding with a probiotic feed (Macey & Coyne, 2005).

H. midae showed significant improvement in growth rate when fed a kelp diet supplemented with *Pseudoalteromonas* C4 compared with unsupplemented group in both laboratory and farm feeding trials. In the study antibiotics were used to clear the abalone GIT from its microflora before conducting feeding trial, thus any changes of shell length or weight gain can be related to the treatment with P Pseudoalteromonas C4. Result later were shown that animals fed C4-supplemented diet after pre antibiotics treatment a significant improvement in shell length and weight gain by 38% and 39% respectively over 163 days of feedings trial, compared with the animals neither treated with C4 strain nor with antibiotics. These results can be correlated with role of C4 strain in increasing the alginolytic activity in the gut (ten Doeschate & Coyne, 2008).

The dominant species observed in the gut of *Haliotis discus hannai* were non-motile fermenter like *Vibrio halioticoli* clade and *Vibrio* spp. These bacteria may enhance the digestive efficiency, and increase , accordingly, improve the growth rate of a host by supplying polysaccharolytic enzymes (El-Shanshoury et al., 1994). In a study, the administration of *Pediococcus* sp. Ab1, a potential probiotic, increased the production of volatile short chain fatty acids (VSCFAs). The bacteria colonized the gut of *H. gigantea* for 12 days after termination of feeding trial. In addition, abalone fed with commercial feed supplemented with *P. sp*. Ab1 exhibited dominance of other probiotic bacteria namely *Vibrio halioticoli* clade in the gut. Other *Vibrio* species were dominant in the gut of abalone that received unsupplemented feed with *P.* sp. AB1. These results can be explained by a suggestion of Merrifield et al. (2010 b) that the “presence of a probiotic strain within the digestive tract may modulate the complex microbial communities and promote a more functional population”.

Endogenous polysaccharases enzymes in abalone *H. midae* fed either kelp (*Ecklonia maxima)* or red algae (*Gracilaria verrucosa*), were found to vary in response to diet, which may be related to the bacterial that are capable of hydrolysing different complex of polysaccharides in algae (Erasmus et al., 1997).

Probiotic bacterial isolates included in diets of *H. midae* containing *E. maxima* and *G. gracilis* extracts have shown to improve nutritional value of artificial feed, then improve growth of abalone (Macey & Coyne, 2005; Troell, 2006).

Goosen (2007) and Tuomola et al. (2001) reported that probiotics can produce organic acid as growth-inhibiting metabolites, these acids and their salts can promote growth of abalone in South Africa *H. midae*. They can play as manipulators of microflora in the GIT of abalone (Goosen, 2007)

Multispecies probiotics have demonstrated that it is possible to provide synergistic bacteria to abalone with complementary modes of action to develop protection against pathogens or improve growth rate (Timmermana et al., 2004)

Chapter 3

Isolation, Screening and Identification of Potential Probiotic Bacteria for Abalone (Paua)

3.1 Introduction

Increased growth rate in cultured abalone is typically achieved by using specially formulated diets (Britz, 1994). However, probiotic bacteria were observed to increase abalone growth rate by assisting the digestive system in *H. midae* via increasing the pool of hydrolysis enzymes (Erasmus et al., 1997). Olafsen (2001) pointed to the importance of the initial study of the microbial diversity in the gut of marine animals to determine the effects of an alteration of the microflora on the host growth and health.

The aim of this experiment is to isolate and screen naturally-occurring bacteria from healthy cultured paua *(H. iris*), that could assist in the digestion of nutrients present in AbMax 16 ,the commercial feed used in paua farming at OceaNZ Blue (Bream bay, Bay of Islands). The isolates could be considered as potential probiotic which could be administered with the feed in land-based paua aquaculture.
The potential probiotic bacteria were initially screened based on the following selection criteria:

1. Ability to hydrolyze protein (proteolytic)
2. Ability to break down starch (amylolytic)
3. Ability to hydrolyze alginate (alginolytic)
4. Ability to tolerate bile salts
5. Ability to tolerate high acidity
6. Ability to produce acid

Isolates were subjected to initial basic screening of potential probiotic bacteria. Qualitative screening was performed using plate or tube assay to determine hydrolysis of protein, carbohydrate, and alginate. Tolerance experiments for all isolates against bile salts and acidity were conducted in different concentration for further qualitative screening.

Samples were treated according to specific methods for environmental bacteria although some methods were adopted from diagnostic laboratories due to lack of methods suitable for marine bacteria.

3.2 Materials and Methods

3.2.1 Isolation of potential probiotic bacteria

3. 2.1.1 Dissection of Abalone

Isolation of potential probiotic bacteria from healthy adult abalone was initiated by dissecting abalone samples. Twenty, adult abalone sized 75-85mm, were dissected aseptically on two occasions using dissection board and flame-sterilized scalpel. Initially the adductor muscle (foot) was removed to expose the parts of the whole gut using **Fig. 3-1** and **3-2** as guide.

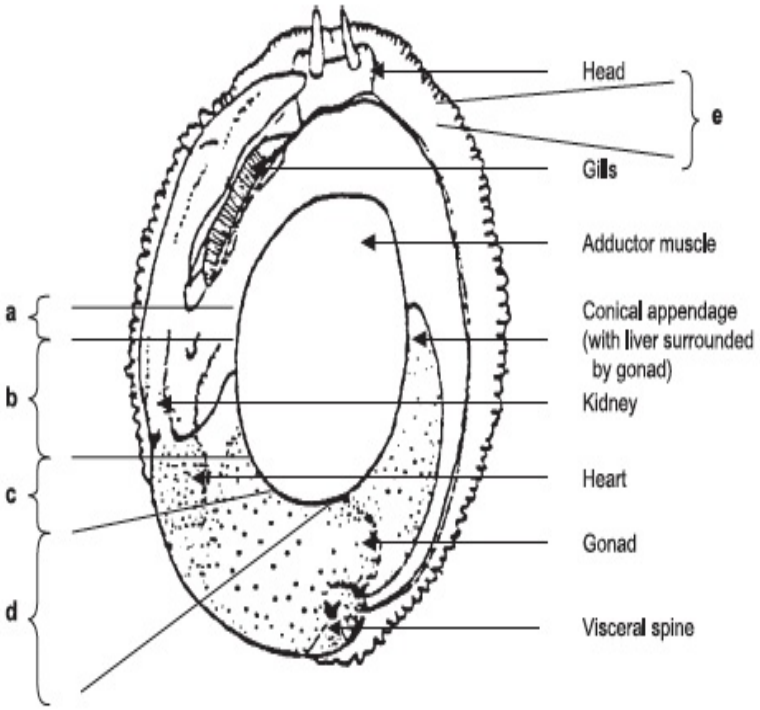

Figure 3-1 Anterior view of the abalone *Haliotis* showing the main organs; (a) gills and intestine, (b) kidney and heart, (c) heart and stomach, (d) stomach and crop, (e) foot (Macey & Coyne, 2005)

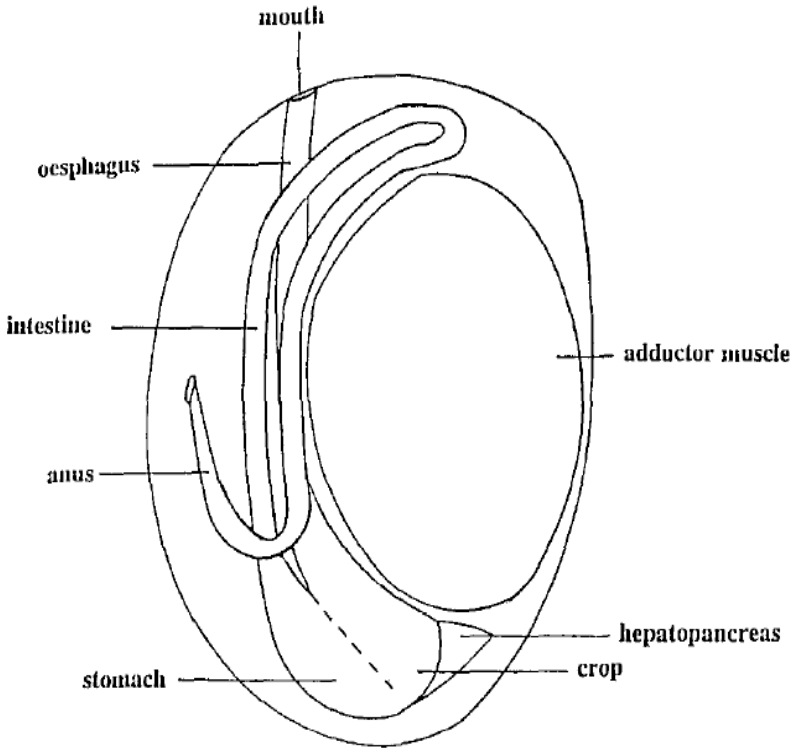

Figure 3-2 Abalone digestive tract (Erasmus et al., 1997)

Isolation of bacterial strains was following the method of Vijayabaskar & Somasundaram (2008). Samples were taken each from the stomach, intestine, digestive gland, hepatopancreas, rectum, and anus by swabbing using sterile cotton swabs. Samples from juice in the stomach and hepatopancreas were collected aseptically. The samples were then suspended in sterile Marine Broth. Each sample suspension was serially diluted and spread plated in Marine Agar and Plate Count Agar (DIFCO) with supplementation of 2% NaCl, to obtain the viable count and to allow growth of most bacterial isolates present in each abalone part. Aerobic and anaerobic using (GasPak^{TM} EZ) plates were prepared for incubation at two temperatures, 16^{o}C and 22^{o}C until colonies were obtained.

To obtain potential probiotics including lactic acid producing bacteria, samples from Marine Broth suspension above were spread plated in MacConkey (DIFCO), TCBS (DIFCO), and MRS (DIFCO) Agar with adjusting salinity to 2%NaCl. Two sets of plates were prepared for incubation at 16^{o}C and 22^{o}C. Pure cultures of isolates were obtained by a series of five-phase streak plating on appropriate medium.

3.2.1.2 Collection of Environmental Isolates

Potential probiotic bacteria were isolated from faecal samples and water from OceaNZ abalone tanks. Isolates were also collected from the bottom of the rearing tanks of adult and juvenile abalone by swabbing the surface with sterile cotton swab which was then suspended in sterile Marine broth (DIFCO). The samples were diluted and plated for isolation and viable count as described in Section 3.2.1.3. Isolation using MacConkey, TCBS, and MRS Agar was done as described in the previous section. A series of five-phase streak plating was used to obtain pure cultures of desired isolates.

3.2.1.3 Viable Count of Bacteria in the Abalone Gut and Rearing Water

The purpose of conducting viable count experiment is to determine abundance and species composition of culturable microorganisms in the host gut and rearing water (Iehata et al., 2010). Viable cell count, usually reported as the number of colony-forming units (CFU) of the organism of interest per unit area, volume, or weight of sample (Macey & Coyne, 2006a).

Viable cell count was obtained by spread plating aliquots from a series of dilutions into Marine agar (DIFCO), MacConkey agar (DIFCO), TCBS agar (DIFCO), and De Man–Rogosa–Sharpe agar MRS (DIFCO).

Bacterial isolates were preserved for long term storage at -80^{o}C in Marine broth (Difco) supplemented with 15% Glycerol (Vishnivetskaya & Kathariou, 2009).

3.2.2 Screening of Potential Probiotic Isolates

3.2.2.1Screening for Protein Hydrolysis

Isolates were cultured on 0.2% casein agar plates, described by Gutierrez-Maddox (2002) with modification, in order to determine protein hydrolysis. Casein agar was prepared by mixing (1.0g) sodium caseinate (ACROS organics), (0.1g) magnesium sulphate, (0.5g) glucose, (0.005g) ferrous sulphate, 0.10g di-potassium phosphate ($K_2 HPo_4$), (7.5g) agar, and (500ml) deionized water. The medium was autoclaved at 121^{o}C for 15 min. After inoculation the cultures were incubated at (22^{o}C) until visible colonies were present. Hydrolysis of protein (casein) is indicated by the presence of clear halo around the colonies. Isolates were compared based on the diameter of clear halo surrounding a single colony. In case of hydrolysis not easily observed against natural light, the plates were flooded with 1% HCl acid, then pouring off the excess. The acid should precipitate any unhydrolyzed casein.

3.2.2.2 Screening for Starch Hydrolysis

Isolates were screened for their ability to hydrolyze starch by spread-plating on 1% starch Marine Agar plates (Gutierrez-Maddox, 2002). Starch agar was prepared by mixing starch dextrin 10g, nutrient agar, and NaCl 19.4% dissolved in 1L of deionized water, then autoclaved.

Cultures were incubated at 22^{o}C until colonies were visible. Starch-hydrolyzing (amylolytic) colonies were distinguished from non-hydrolyzers with the application of Gram iodine solution into the cultures. Amylolytic colonies produced clear zones while non-amylolytic colonies did not remove the blue colour of the starch-iodo complex.

3.2.2.3 Screening for Alginate Hydrolysis

3.2.2.3.1. Alginate Agar Plate Method

Screening for alginate hydrolysis in solid medium was carried out using a modified method of (Nakamura, 1987). Isolates were spread plated on 2.0% alginate agar medium. The medium was prepared by adding (20g) sodium alginate (SIGMA), (5g) yeast extract (DIFCO), (10g) agar (DIFCO), (0.2g) MgSO4, (1g) NH4H2PO4, (1g) K2HPO4, (19.4g) NaCl, to 1L of deionized water, in addition of and 0.003%bromothymolblue as pH indicator. All ingredients were dissolved in 1L of deionized water, then autoclaved.

The plates were incubated at 22^{o} C until colonies were visible (2-5 days). Colonies producing acids from alginate hydrolysis were surrounded by a yellow zone. Confirmation of results was carried out by the addition of 70% ethanol for 1 hour and observation of precipitation of alginate (Hu et al., 2006).

3.2.2.3.2. Alginate Broth Method

Potential probiotic isolates were screened for alginate hydrolysis by cultivation in (5 ml) of 0.1% sodium alginate broth consisting of 0.5% peptone, 0.1% yeast extract, 3.0% NaCl and 0.1% sodium alginate (Kitamikado, 1990). The pH of the medium was adjusted to 7.6 for cultivation of marine bacteria. The cultures were incubated at 22^{o}C for 1-5 days.

Alginate hydrolysis was determined every 24 hours using precipitation test with acidic albumin (Kitamikado, 1990). The principle of the test is the development of turbidity (precipitation) when alginic acid and bovine albumin are mixed in an acidic condition. Acidic albumin was prepared by dissolving 1g of bovine albumin fraction V (Thermo Scientific, USA) in 1L of deionized water before adding 3.26g of sodium acetate and 4.56ml of glacial acetic acid. The pH of the solution was adjusted to pH 3.72-3.78 using 2M HCl. After every 24 hours of incubation, 0.5 ml of each bacterial culture was withdrawn and centrifuged at 3,000-4,000 rpm for 10 min. A 0.2 ml fraction of the supernatant was collected and transferred into a small cuvette. Acidic albumin (0.2ml) was added just

before reading the transmission at 600nm in a spectrophotometer (Ultrospec™2100 pro UV/Visible). The control for the experiment was prepared by using sterile 0.1% sodium alginate broth (without bacteria). This was centrifuged and tested with acidic albumin in the same way as the test solutions.

3.2.2.4 Screening for Bile Salts Tolerance

Evaluation of bile salts tolerance for the bacterial isolates was performed using a modified method of (Hyronimus et al., 2000). The isolates were grown on Marine Agar containing different concentrations of Oxgall bile salt (BDH). The Gram-positive bacteria were evaluated in Marine Agar containing bile salts at 0.1%, 0.2%, 0.3%, 0.5%, 1%, and 1.5%. The same concentrations of Oxgall were added to MRS agar medium for screening lactic acid bacteria. For Gram-negative bacteria obtained from TCBS agar, higher concentrations of bile salt were used; 1%, 1.2%, 1.4%, 1.6%, and 1.8%. The rationale behind this was that TCBS agar medium already contained 0.8% Oxgall and the isolates were able to grow on it, thus the screening for Oxgall (bile salt) tolerance used a range with the lowest at 1%. The plates were streak plated with each isolate and incubated at 22^{o}C until growth was present.

3.2.2.5 Screening for Acid Tolerance

Bacterial isolates were assessed for their ability to tolerate high acidity following the method of Iehata et al.(2009), and Lin et al.(2006) with modification to fit the range of acidity inside the gut of paua which is pH 1.5 - 5.5. Tubes of Marine broth (9 ml) at pH 1.5, pH 2.0, and pH 5.0 were prepared using 5M HCl. The broths were inoculated with a colony and incubated for 3 hours at 22^{o}C. Viability at each pH value was determined by comparing the plate count obtained after 0 hour with that after 3 hour incubation.

3.2.2.6 Screening for Acid Production

For detection of acid production, the acid-base titration method was utilized (ADMI, 1971). Marine broth with 5% glucose was prepared in 100 ml of capped bottles and steriled by autoclaving for 15 min in 121^{o}C. A colony of bacterial isolate was inoculated into modified Marine broth in two replicates.

The cultures were centrifuged at room temperature for 15 min at 4,000 x g. The supernatant (50 ml) was collected in a 250 ml flask and titrated with 0.1M NaOH using phenolphthalein indicator until a light pink colour appeared. Lactic acid concentration was calculated using the equation (ADMI, 1971):

$$\text{Lactic acid \%} = \frac{(\text{ml NaOH}) \times (\text{M NaOH}) \times (\text{mol. weight lactic acid}) \times 100}{\text{volume of sample} \times 100}$$

3.2.3 Phenotypic Characterization

Initial phenotypic analysis was performed by assigning the isolates to major phenotypic groups using standard Gram staining (Bergey et al., 1994). Cultivation in selective media, such as MacConkey Agar ,TCBS Agar and *Pseudomonas* Agar was done as complementary tests for Gram-negative isolates (Hansen & Sorheim, 1991). The isolates were then subjected for further morphological, cultural, physiological and biochemical analyses described later in this Chapter.

3.2.3.1 Cell and Colony Morphology

The morphological cell characteristics of the isolates were determined by Gram-staining using ethyl alcohol as decolourizing agent (Bergey et al., 1994). Spore formation was determined by (5%) malachite green (BDH) staining (Bergey et al., 1994). Motility was studied using (0.3% agar) motility medium was included; 10g beef heart infusion, 10g tryptose, 19.4g sodium chloride, 53.4g gelatin, 3.0g agar, and 1Lof deionized water. The medium was prepared by boiling for 1 min to dissolve the ingredients then autoclaved (Gutierrez-Maddox, 2002; Kaiser,

2011). The characterisation of colonies of the isolates was carried out by culturing on Marine Agar, TCBS agar, and MRS agar (refer to Section 3. 2.1.1).

3.2.3.2 Cultural Characteristics

The temperature requirement of the isolates was determined by incubation of isolates grown in Marine Agar at 16^{o}C, and 22^{o}C. Incubation time was 48-72 hours, except for alginate agar culture which required longer period (3-5 days). Cultures were incubated in both aerobic and anaerobic conditions.

3.2.3.3 Biochemical Characteristics

The following tests were used for initial biochemical phenotyping of the isolates, all the methods were conducted according to and Bergey's Manual (Bergey& Holt, 1994) and Systemic Microbiology Manual (Gutierrez-Maddox, 2002) :

- Catalase test using 3% H_2O_2 (BDH) was performed on colonies in Marine Agar to detect production of catalase which breaks down H_2O_2 into H_2O and O_2.Catalase producers would produce gas bubbles upon reaction with H_2O_2.
- Oxidase test for the presence of cytochrome oxidase was performed on 24 hour old colonies grown in Marine Agar. Tetra-methyl-p-phenylenedi-amine di-hydrochloride (ACROS Organics) was used as oxidase reagent. Oxidase positive isolates would produce a blue-purple color change within 30 sec.
- Blood haemolysis was tested on isolates streak plated on Colombia agar supplemented with 5% Sheep Blood agar (Fort Richard Laboratories). Cultures which are ß-haemolytic would remove the red colour of the medium completely, while α-haemolytic isolates have incomplete heamolysis. Ɣ-haemolytic isolates have no haemolysis.
- Gelatine hydrolysis test was performed by inoculating gelatine stabs (DIFCO) Isolates capable of gelatine hydrolysis would liquefy and not allow gelatine agar to set at refrigeration temperature.

- Oxidation/Fermentation (O/F) test was used to determine whether the isolates oxidize or ferment glucose. (Hugh & Leifson O/F agar (DIFCO) was used to determine glucose metabolism.
- Triple Sugar Iron test (TSI) from (DIFCO) was used to determine sugar fermentation (glucose, sucrose and fructose) and hydrogen sulphide production in the Gram-negative isolates. Sugar fermenter would produce yellow colour while hydrogen sulphide producers would produce black colonies.
- DNase agar (Fort Richard Laboratories) was used for the detection of deoxyribonuclease activity. DNA is a polynucleotide which binds to methyl green and gives stable colour in alkaline pH. In the presence of DNase enzyme, DNA will be degraded and methyl green releases and loses its colour in lower pH. Thus DNase-positive bacteria show clear zones around colonies. .
- Baird-Parker agar containing egg yolk emulsion (Fort Richard Laboratories) was used for detection of lipase production in Gram-positive isolates.
- XLD agar (Fort Richard Laboratories) was streak plated for degradation of xylose, lactose, and sucrose to acid. Acid from fermentation lowers the pH and changes phenol red color to yellow.
- Bile Eesculine Agar, BEA (Fort Richard Laboratories), a selective, differential medium, was used to isolate and identify members of the genus *Enterococcus.* All members of the *Enterobacteriaceae* family grow on this medium, yet certain bacteria can hydrolyze esculine to 6, 7-dihydroxycoumarin, which reacts with iron to produce a characteristic blackening of the medium.
- Tryptic Nitrate broth (Fort Richard Laboratories): Was used for detection of nitrate reduction to nitrite. Nitrate-reducing isolates which produce nitrite produce a red colour with sulphanilic acid and α- naphthylamine.

3.2.4. Identification of the Isolates

All bacterial isolates were identified according to Gram-positive and Gram-negative bacteria flow charts that mentioned in Bergey's Manual of Determinative Bacteriology (9th Edition)(Bergey et al., 1994). In addition API20 E® strips and API10 S® strips (from BioMerieux) were used for further identification of Gram-negative isolates. The suspension of one colony was inoculated into mini cuvette of the strip, and incubated for 24-48 hour. Reading andinterpreting the results was through Bio Mérieux website [www.apiweb.biomerienx.com].

3.2.5 Phylogenetic Analysis for the Isolates

Bacterial isolates were sent to Landcare Research (Auckland 1072) for 16S rRNA classification and to confirm the phenotypic identification carried out earlier. The primers used to amplify the 16S rRNA gene were 27F (forward) and 1525R (reverse) primers for 16S rRNA (Lane, 1991).

3.3 Results

3.3.1 Isolation and Enumeration of Potential Probiotic Bacteria

The purpose of conducting viable count experiment is to determine abundance and species composition of culturable microorganisms (Section 3.2.1.3) found in the paua digestive tract and rearing tank (**Table 3-2**).Viable counts obtained after incubation for 48 hr in different media are shown in **Table 3-1**; viable count from Marine agar reflects the total number of culturable bacteria in the sample. The intestine region with pH 7.0 showed the greatest abundance of microorganisms, while the anus with pH 8.2 had the least number (1.1×10^8 and 3.0×10^3, respectively). The highest number of presumptive lactic acid bacteria LAB at 3.8×10^5, were found in the stomach. There was a low number of presumptive LAB in the intestine. Bile salt resistant bacteria that were presumptive Gram-negative and presumptive *Vibrio* bacteria were obtained in MacConkey Agar and TCBS Agar, respectively.

A lower number of microorganisms were sampled from the rearing tank and water compared to those from the animals.

Sample	pH value of the gut region	Viable count CFU/ml (average)			
		Marine agar	MacConkey agar	MRS agar	TCBS agar
Stomach, digestive gland	5.5	2.5×10^6	3.0×10^3	3.8×10^5	3.0×10^5
Intestine	7.0	1.1×10^8	3.0×10^3	1.2×10^3	3.0×10^5
Hepatopancreas	8.0	5.0×10^4	2.2×10^4	< 1	2.5×10^4
Anus	8.2	3.0×10^3	1.2×10^4	6.7×10^3	
Tank swab	-	1.15×10^6	2.0×10^3	<1	1.8×10^3
Adult tank water	8.3	8.8×10^3	$<2.0 \times 10^3$	<1	3.0×10^3
Juvenile tank water	8.2	3.0×10^2	$< 2.0 \times 10^3$	<1	1.4×10^3

Table 3-1 Viable count (CFU/ml) of bacterial population in abalone (GIT), and rearing water

Isolates	Culture Medium	Source of isolates
JHDC (LAB)	MRS	Stomach, digestive gland
JHEb1	Marine	Intestine, V tank
JHBa2	Marine	Intestine, Rectum
JH1	TCBS	Stomach, Water sample
JH2	TCBS	Intestine, Tub water
JH3	TCBS	Intestine
JH4	TCBS	Juveniles abalone Tank
JH5	TCBS	Intestine
JHC	Marine	Stomach, Hepatopancreas, Intestine, Adult abalone tank

Table 3-2 Bacterial strains isolated from abalone (GIT), and rearing water

3.3.2 Screening of Potential Probiotic Isolates

3.3.2.1 Protein Hydrolysis

Results of screening for protein hydrolysis showed that JHEb1had the highest ability to degrade casein present in the medium. JHBac2 and JH1 showed moderate proteolytic ability whereas, JHLDc and JHC, showed no protein hydrolysis activity (**Table 3-3**).

Bacterial Isolates	Casein hydrolysis zone (mm)
JHEb1	5
JHBac2	3-4
JHLDc	0
JH1	4
JH2	1-1.5
JH3	2-3
JH4	3
JH5	2
JHC	0

Table 3-3 Casein hydrolysis of bacterial isolates

3.3.2.2 Starch Hydrolysis

Five out of nine isolates were capable of hydrolyzing starch. JHBac2 was observed to have the greatest amylolytic ability shown as the largest clear zone in starch agar. JHEb1, JH1, and JH3 produced smaller clear zones than JHBac2 and demonstrated comparable starch-hydrolysing property to each other (**Table 3-4**).

Bacterial Isolates	Starch hydrolysis zone (mm)
JHEb1	3
JHBac2	5
JHLDc	0
JH1	3
JH2	0
JH3	3
JH4	2-3
JH5	0
JHC	0

Table 3-4 Starch- hydrolysing property of the isolates

3.3.2.3 Alginate Hydrolysis

3.3.2.3.1 Alginate Agar plate Method

Out of the nine isolates, three isolate grew in alginate agar plates were able to grow in alginate-containing plate after 5 days at 22^0C. Three isolates, JH1, JH3, and JH5 were observed to hydrolyze the alginate in the medium as indicated by yellow colonies surrounded by clear zones upon addition of ethanol (**Fig. 3-3**). Isolates, such as JHBa1, that neither changed the colour of the agar nor produced a clear zone around colonies were not able to break down alginate but grew on the nutrients present in medium such as yeast exact (**Fig. 3-4**).

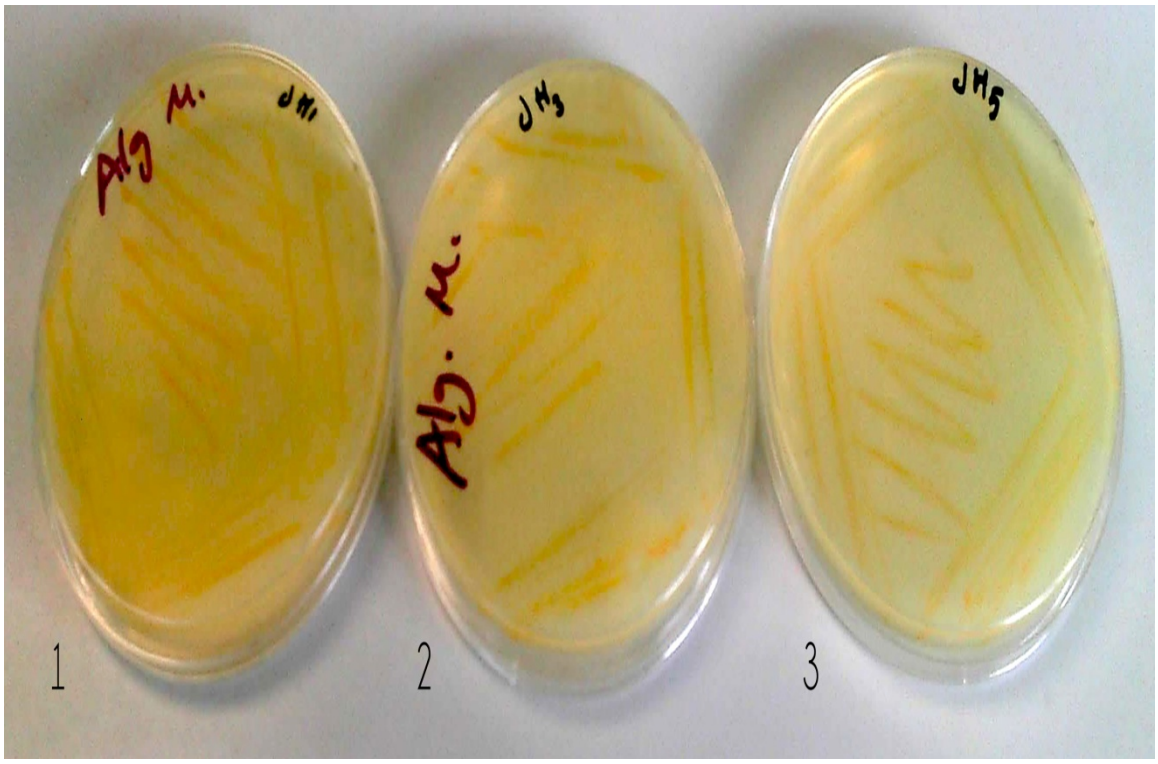

Figure 3-3 Alginate agar plates with change in colour (positive) and clear zone of hydrolysis (1- JHI, 2- JH3, and 3- JH5)

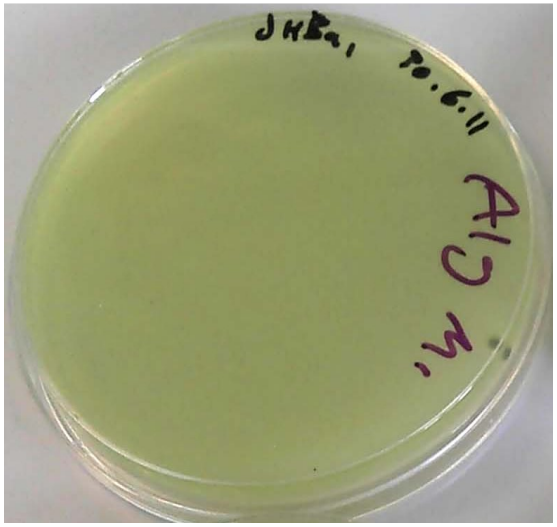

Figure 3-4 Alginate agar plate with no change in colour and no clear zone around the colonies indicative of absence of alginate hydrolysis

3.3.2.3.2 Alginate broth tube method

Results obtained at day 2, and day 5 of a five day- incubation period are shown in Table 3.5. The supernatant of the isolates were each added to acidic albumin-alginate solution and then compared with the control. Isolates were evaluated on their ability to clear the albumin-alginate precipitate (**Fig. 3-5**). Transmission was measured by spectrophotometer at 650 nm. The control solution produced a transmission value at 10.2%. JH1 was the only isolate which completely cleared the precipitate and produced a transmission value of 100% after 2 days of incubation (**Table 3-5**) . On this basis, JH1 exhibited the fastest ability to break down alginate. Lower transmission values were obtained with JH3 and JH5 at day 2, but these isolates later produced 100% transmission, hence, complete hydrolysis after 5days. JH3 and JH5 were slower alginate degraders. The results obtained with this method confirmed those with the alginate agar plate method.

Bacterial code	Transmission (%) at 650 nm control reading = 10.2%		
	2 d. incub.	**3 d. incub.**	**5 d. incub.**
JHEb1	9.0	14.2	_
JHBa2	9.2	10.1	_
JH1	100.0	_	_
JH2	10.7	20.7	_
JH3	12.8	18.4	100.0
JH4	12.0	_	_
JH5	6.3	27.9	100.0
JHDC	_	_	_
JHLC	_	_	_

Table 3-5 Alginate hydrolysis of the bacterial isolates, measured by transmission (%)

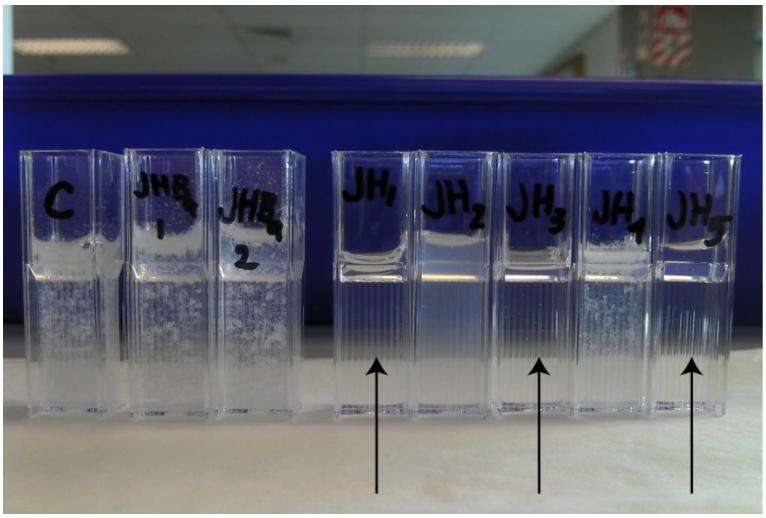

Figure 3-5 Experiment showing clear solution for alginate hydrolysis in JH1, JH3, and JH5, (left to right with arrow). Turbid solutions indicate absence of the alginate hydrolysis as in control (first from left)

3.3.2.4 Bile Salts Tolerance

Isolate	Agar Medium	0.1%	0.2%	0.3%	0.5%	1%	1.5%
JHC	Marine	+	+	+	+	+	+
JHDC	MRS	+	+	+	+	+	+
JHEb1	Marine	+	+	+	+	+	+
JHBa2	Marine	_	_	_	_	_	_

Table 3-6 Bile salt tolerance of isolates that grew on Marine and MRS agar.
+ = growth; — = no growth

Sample	0.8%	1%	1.2%	1.4%	1.6%	1.8%
JH1	+	+	+	+	+	+
JH2	+	_	_	_	_	_
JH3	+	_	_	_	_	_
JH4	+	+	+	+	+	+
JH5	+	+	+	+	+	+

Table 3-7 Bile salt tolerance for isolates that grew on TCBS agar.
+ = growth; — = no growth

Table 3-6 shows that all Gram-positive and the Gram-negative JHC isolates could tolerate bile salts up to 1.5%. JHBa2 failed to grow in all concentrations of bile salts used in the study. Out of the Gram-negative isolates, JH1, JH4, and JH5could grow in bile salts as high as 1.8%**.** JH2 and JH3 were not able to resist concentration higher than 0.8% (**Table 3-7**).

3.3.2.5 Acidity Tolerance

Viable counts expressed as CFU/ml of culture at 0 hour and after 3 hour of incubation at pH 5, pH2, and pH1.5 are shown in **Table 3-8**. Viable counts of all isolates were stable at pH 5.0 and pH 2.0 for 3 hours. JHEb1, JH1, JH2, and JH 3 showed a reduction in number in pH 2.0 by 1 log unit after incubation. JH 5 and JHC were the least tolerant in pH 2.0. JH2 and JH 4 were completely inhibited at pH 1.5 with the acid challenge while all the other isolates remained viable for 3 hours although with a slight reduction in number.

Isolate	Viable count in pH 5, (CFU/ml)		Viable count in pH 2, (CFU/ml)		Viable count in pH1.5 (CFU/ml)	
	0h.	3h.	0h.	3h.	0h.	3h.
JHEb1	5.0×10^8	5.5×10^8	2.3×10^5	2.8×10^4	1.4×10^5	2.0×10^3
JHBac2	2.7×10^4	2.0×10^4	6.8×10^3	1.1×10^3	8×10^3	18×10^3
JHLDc	2.2×10^7	2.0×10^7	8.2×10^5	1.4×10^5	6.0×10^4	1.6×10^3
JH1	2.0×10^5	2.9×10^4	3.0×10^6	2.0×10^5	6.5×10^4	4.0×10^3
JH2	4.8×10^3	1.4×10^3	4.0×10^4	2.0×10^3	3.0×10^3	0
JH3	5.0×10^4	5.0×10^4	7.8×10^6	5.4×10^5	3.0×10^4	2.4×10^3
JH4	1.2×10^4	10×10^4	5.0×10^6	3.3×10^5	2.5×10^6	3.0×10^3
JH5	3.0×10^4	2.8×10^4	4.0×10^5	2×10^3	1.0×10^5	0
JHC	5.0×10^6	4.7×10^6	5.2×10^5	4.0×10^3	6.0×10^6	3.2×10^5

Table 3-8 Acid tolerance of isolates measured as viable count (average) at 0 hr and 3 hr

3.3.2.6 Acid production

Acid production through sugar fermentation, amongst the nine isolates was varied. JHLDc produced the highest concentration of lactic acid (0.67%). JHC and JH4 were the second highest producers with 0.46% and 0.42%, respectively. JHEb1, JH1, and JH3 produced comparable acid concentrations while JH2 and JH5 were the lowest producing isolates (**Table 3-9**).

Isolates	**Lactic Acid %** (average)
JHEb1	0.18
JHBa2	0.36
JHLDc	0.67
JHC	0.46
JH1	0.18
JH2	0.09
JH3	0.22
JH4	0.42
JH5	0.11

Table 3-9 Acid production in potential probiotic isolates

3.3.2 Phenotypic characterization

3.3.2.1 Cell and Colony Morphology

Tests	JHEba1	JHBa2	JHLDc
Colony morphology	Circular	Irregular	Circular
Margin	Entire	Undulate	Entire
Elevation	Raised	Flat	Raised
Surface	Smooth	Rough	Smooth
Density	Opaque	Opaque	Translucent
Pigments	Orange	White	-
Gram reaction	+	+	+
Cell shape	Rods	Rods	Cocci
Size	Moderate size	Big boxy shape	big
Motility	+	+	-
Catalase	+	+	-
Oxidase	-	-	-
Spore formation	-	+	-

Table 3-10 Cell and colony morphology of the Gram-positive isolates

Tests	JHC	JH1	JH2	JH3	JH4	JH5
Colony morphology	Circular	Circular	Circular	Circular	Irregular	Circular
Margin	Entire	Entire	Entire	Entire	Undulate	Entire
Elevation	Raised	Raised	Raised	Raised	Flat	Flat
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Density	Opaque	Opaque	Opaque	Translucent	Opaque	Translucent
Pigments on marine agar	Beige	Beige	Beige	Orange	Beige	Beige
Pigments on TCBS agar	-	Yellow	Yellow	Dark green	Yellow	Light green
Gram reaction	-	-	-	-	-	-
Shape	Rods	Rods, curved & straight	Rods, curved,& straight	Long thin rods, curved & straight	Rods, small, pointed ends	Rods small, thick, curved
Motility	+	-	+	+	+	+
Catalase	-	+	+	+	+	+
Oxidase	-	+	+	+	+	+

Table 3-11 Cell and colony morphology of the Gram-negative isolates

3.3.2.2 Biochemical Characteristics

The biochemical characteristics of bacterial isolates are shown in the **Table 3-12.**

Isolates / Test	**JHEb1**	**JHBa2**	**JHLDc**	**JHC**	**JH1**	**JH2**	**JH3**	**JH4**	**JH5**
Glucose	+ (no gas)	+ (no gas)	+ (no gas)	+ (no gas)	+ (no gas)	+ (no gas)	-	+(no gas)	+ (no gas)
Sucrose	+	-	+	+	+	+	-	+	-
Lactose	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	-	+	+
Arabinose	-	-	-	-	-	-	-	+	-
TSI(Triple sugar iron)	A / K	A / K	+	+	+	+	- (+H_2S)	+	+
CTA Xylose	+	+/-	-	-	+	+	-	-	-
CTA Lactose	-	-	-	-	-	-	-	-	-
Tryptic Nitrate	+	+	+	+	+	+	+	+	+
Bile esculine agar	-	-	+	+	-	-	-	-	-
DNase agar	+	+	-	-	+	+	+	+	-
Lipid hydrolysis	+	-	-	-	-	-	-	-	-
Blood hydrolysis	ɣ	**ß**	ɣ	ɣ	ɣ	**α**	ɣ	**ß**	ɣ
Starch hydrolysis	+	+	-	-	+	-	+	+	-
Casein hydrolysis	+	+	-	-	+	+	+	+	+
Gelatin hydrolysis	+	+	-	+	-	-	-	-	-
XLD agar	**-**	**-**	**-**	**-**	**+**	**-**	**-**	**-**	**-**
Pseudomonas agar	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**

Table 3-12 Biochemical characteristics of bacterial isolates on different media

Keys**: A=** acid, **K=** alkaline, **α=** incomplete haemolysis, **ß=** complete haemolysis, **ɣ=** no haemolysis

3.3.3 Identification

3.3.3.1 Identification Using Dichotomous key

The flow charts of dichotomous key adopted from Bergey's Manual of Systematic Bacteriology (Brenner et al., 2005; DeVos et al., 2009) were used in identifying the isolates.

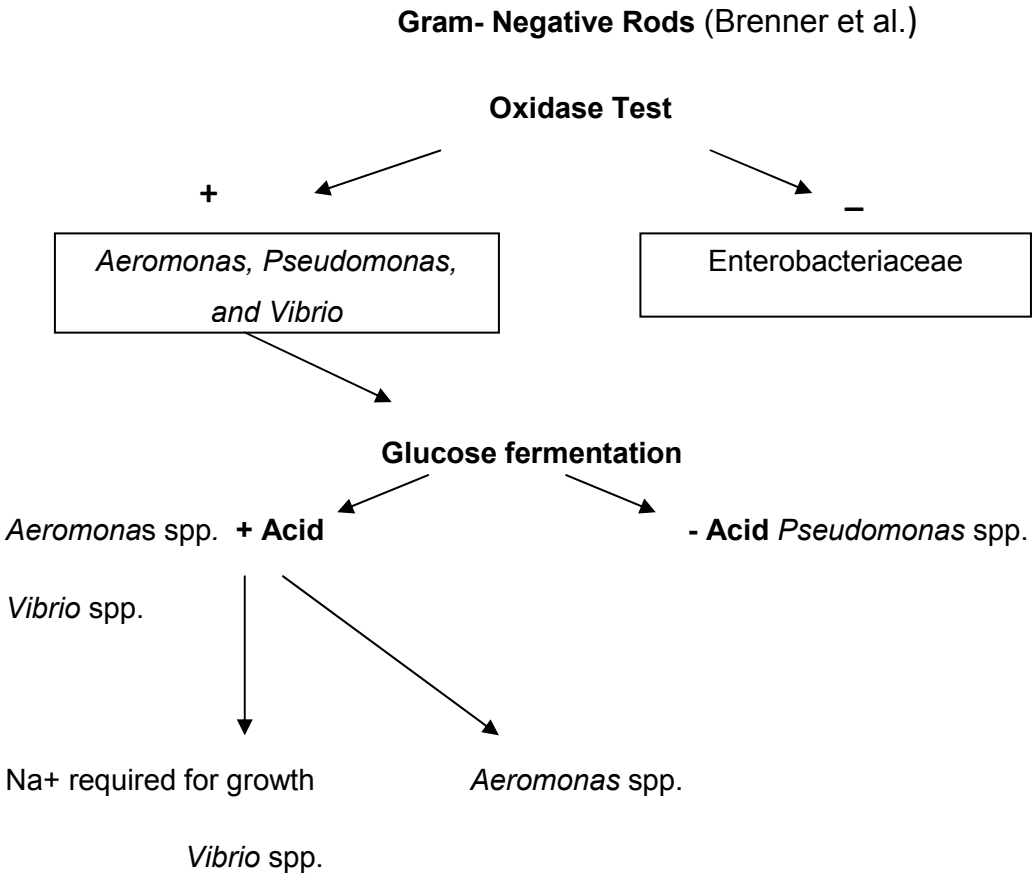

Figure 3-6 Flow chart for Gram-negative bacteria isolates

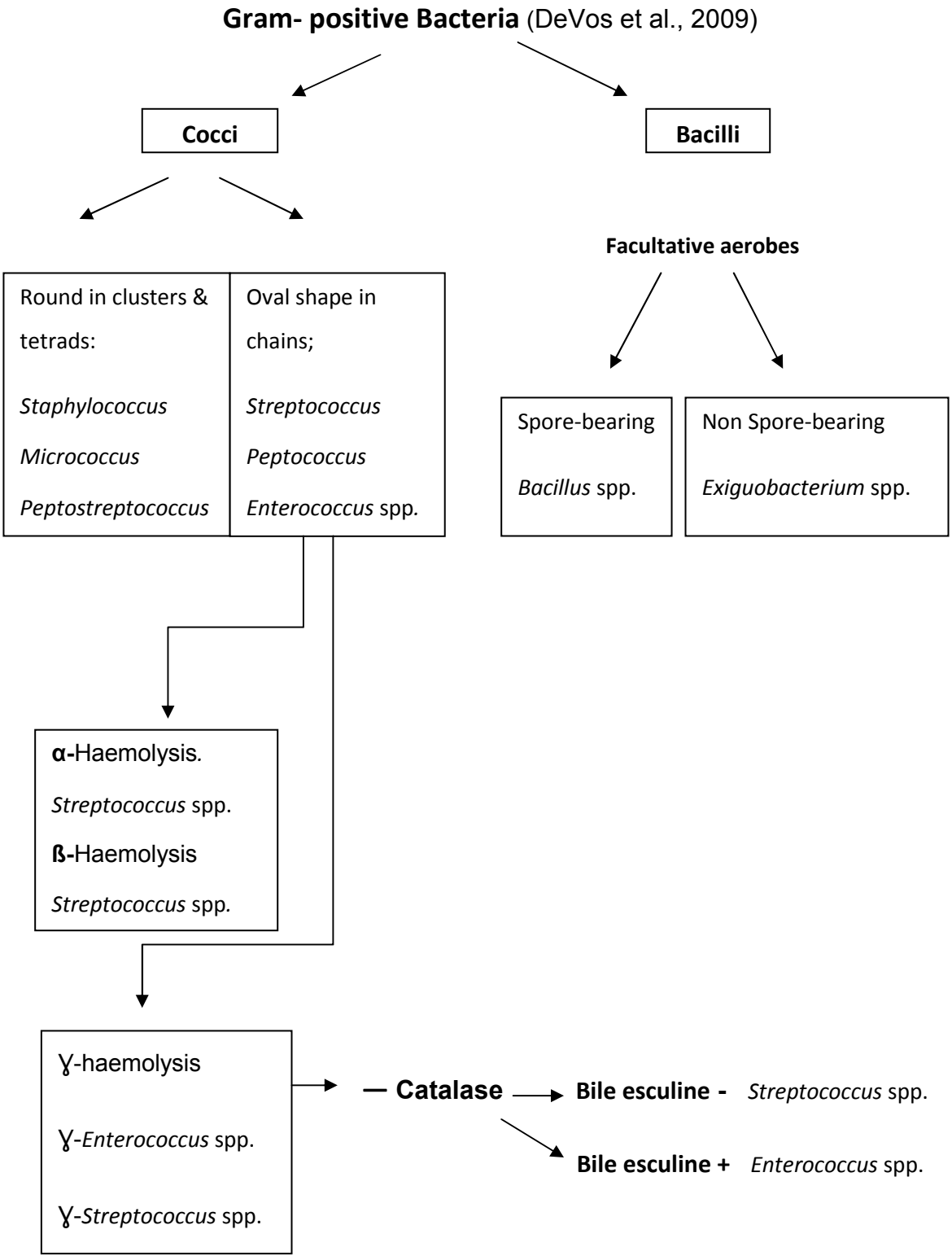

Figure 3-7 Flow chart for Gram- positive bacterial isolates

3.3.3.2 API 20E Strip Results for Gram-negative Isolates

Isolate code	Isolation medium	Colony	API code	Bacteria ID	Id. Assessment
JH1	TCBS	Big yellow	1027735	*Aeromonas hydrophila*	possibility; *Vibrio fluvialis*
JH2	TCBS	Small yellow	0004014	*Aeromonas spp.*	possibility; *Vibrio fluvialis*
JH3	TCBS	Dark green	0004772	*Shewanella putrefaciens or Citrobacter freudii*	Good identification
JH4	TCBS	Big yellow	2206066	*Pseudomonas*	99.9% with supplementary tests (2206066 73)
JH5	TCBS	Light green	00047734	*Pasteurella multocida*	Unacceptable profile
JHC	Marine	Beige	7306521	*Serratia marcescens*	Unacceptable profile

Table 3-13 API 20E results for Gram-negative isolates

The Gram-negative isolates JH1, JH2, JH4, and JH5 were easily identified as *Vibrio* species using the dichotomous key and were distinguished from *Enterobacteriaceae* due to the presence of oxidase. API 20E system failed to confirm the identification of 4 out of 5 Gram-negative isolates in this study. These results were similar to what was reported by Santos et al. (1993) when the API system could not confirm the identification of bacterial isolates from fish and shellfish. JH3 was identified as *Shewanella* with a positive confirmation using API 20E kit.

The Gram-positive isolates were identified using the dichotomous key with JHLDc belonging to genus *Enterococcus*. JHBa2 was *Bacillus* and JHEb1 was *Exiguobacterium.* JHEb1 could be distinguished from lactic acid bacteria by its positive catalase production and orange big colonies and from *Bacillus* by the absence of spore formation.

3.3.3.3 Identification Based on 16S rRNA Gene Sequence

Results of the 16S rRNA sequencing revealed matching identification from classical phenotypic characterization except for *Exiguobacterium* and *Cobetia* (**Table 3-14)**; Cobetia is not included in the API system.

Bacterial Isolates code	Bacterium scientific Name
1. JHEb1	*Exiguobacterium* sp.
2. JHBa2	*Bacillus* sp.
3. JHLDc	*Enterococcus* sp.
4. JHC	*Cobetia* sp.
5. JH1	*Vibrio* sp.
6. JH2	*Vibrio* sp.
7. JH3	*Shewanella* sp.
8. JH4	*Vibrio* sp.
9. JH5	*Vibrio* sp.

Table 3-14 Identification of the isolates by 16S rRNA sequencing

3.4 Discussion

Viable counts obtained from MRS and TCBS media suggest that putative *Vibrio* species and putative *Enterococcus* were dominant and probably colonizing in the stomach, intestine and hepatopancreas of paua. The concentrations of isolates in the water of rearing tanks and in faecal matter suggest that the potential probiotic microorganisms could interact with the animals externally. These two factors, namely, colonizing property and being local to the rearing environment are one of the important considerations in isolating potential probionts (Merrifield et al., 2010 a). Since the objective of this study is to increase the growth rate of paua by introducing beneficial probiotic microorganisms, it was important to screen the isolates for properties which could enhance digestion of nutrients present in the artificial feed used in the farm. Hence, the isolates were screened qualitatively in this experiment for protein hydrolysis, starch hydrolysis and alginate hydrolysis. In addition, acid production, acid tolerance and bile tolerance of the isolates were evaluated.

3.5 Conclusion

Nine isolates demonstrated predominance in the gastrointestinal tract of paua. These isolates displayed the characteristics which were required for probiotics in paua. The isolates exhibited the ability to hydrolyze protein (proteolytic) hydrolyze alginate (alginolytic), tolerate bile salts and high acidity, and produce lactic acid. Using phenotypic characterization and 16S rRNA classification, the isolates were identified as *Exiguobacterium* JHEb1, B*acillus* JHBa2, *Enterococcus* JHLDc, Cobetia JHC, *Vibrio* JH1, *Vibrio* JH2, *Vibrio* JH4, *Vibrio* JH5, and Shewanella JH3.

Using qualitative screening, the isolates that answered the criteria with the highest value were *Exiguobacterium* JHEb1, *Vibrio* JH1, and *Enterococcus* JHLDc. The properties which make these isolates potential probiotic are as follow:

- *Exiguobacterium* JHEb1: starch hydrolysis, protein hydrolysis, resistance to high bile salts and acid concentrations and production of high lactic acid concentration.
- *Vibrio* JH1: starch hydrolysis, protein hydrolysis, fast alginate hydrolysis, resistance to high bile salts and acid concentrations
- *Enterococcus* JHLDc: high lactic acid production, resistance to high bile salts and acid concentrations.

All isolates that exhibited proteolytic protein, starch, and alginate hydrolysis activities, including the three isolates above, were subjected to further quantitative biochemical studies in the next chapter to evaluate enzyme activities involved in digestion of nutrients in paua feed.

Profiles of Candidate Probionts for Feeding Trial

***Exiguobacterium* spp.**

The genus first described by Collins in 1983 is afacultative anaerobic, asporogenous (non-sporeforming), irregularly shaped cells which vary from rods in the exponential phase to short, almost coccoid forms in the stationary phase (Collins et al., 1983). *Exiguobacterium* colonies grown on nutrient agar are circular, entire, raised, smooth, and orange in color. It has the ability to grow in a wide range of pH (5.5-10.5), temperature (5-40^{o}C) aerobically and anaerobically. Moreover, this genus can grow in medium containing salt (NaCl) within the range of 0-5% (Kasana & Yadav, 2007).

Cells are Gram-positive, non-acid fast, motile with peritrichous flagella (GEE et al., 1980) catalase positive and oxidase negative. Acid is formed from glucose, sucrose, galactose, and some other sugars. In anaerobic conditions with glucose as substrate, lactate, acetate, and formate are main end products (Collins et al., 1983). Classification of the genus is as follows:

Phylum Firmicutes

Class Bacilli

Order Bacillales

Family Bacillaceae

Genus ***Exiguobacterium*** (Collins et al., 1983)

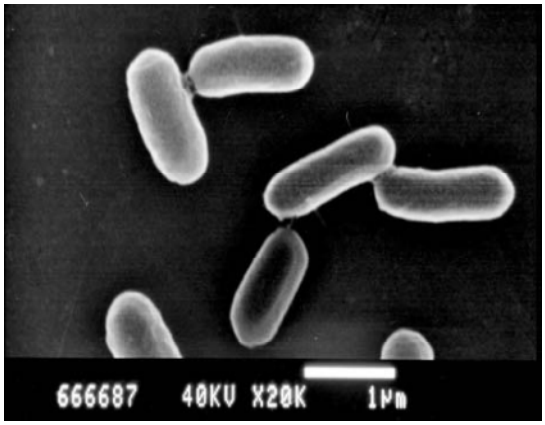

Figure 3-8 The scanning electron microscopy photograph of *Exiguobacterium* sp. (Tan et al., 2009)

Exiguobacterium has been found in a variety of environments; thermophilic, a slightly alkaline, and hot spring water with high carbonate concentrations, such as samples collected from Angel Terrace, which is known as part of Mammoth Terrace (Vishnivetskaya et al., 2011). *Exiguobacterium spp.* have been isolated from Greenland glacial ice, the hot springs at Yellowstone National Park, and the environment of food processing plants (Vishnivetskaya & Kathariou, 2009)

Some species of the genus *Exiguobacterium* have been found in aquaculture system, and they were considered to have the potential to improve the growth and survival rate of brine shrimps (*Artemia sp.).* Further, a production of beneficial polypeptide antibiotics e.g. bacitracin, gramicidin S, polymyxin, and tyrotricidin have been found in aquaculture water which included *Exiguobacterium* spp. Those antimicrobial substances were reported to be active against a wide range of bacteria (Sombatjinda et al., 2011).

In the present study *Exiguobacterium* JHEb1 (**Fig 3-9**) exhibited typical characteristics of the genus *Exiguobacterium.*

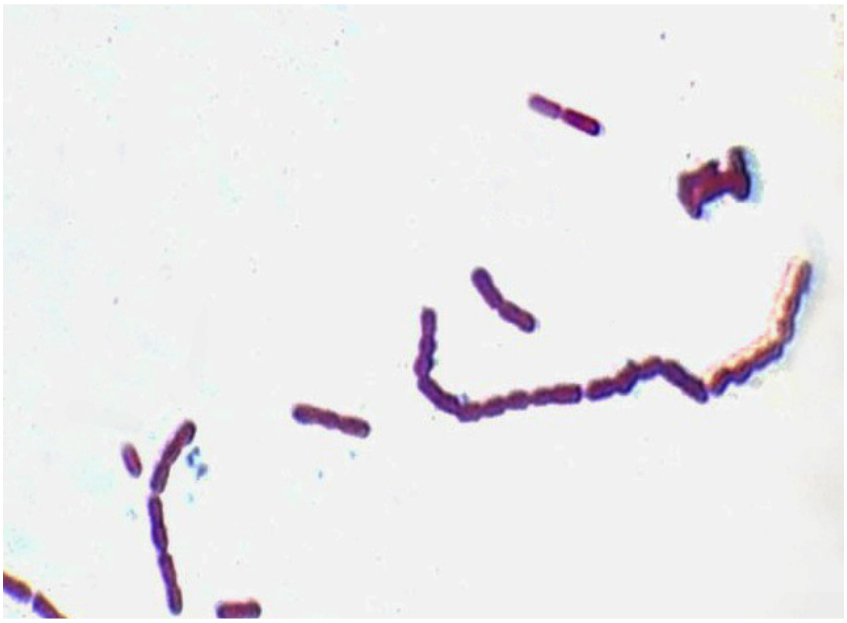

Figure 3-9 *Exiguobacterium* JHEb1 under 1000 X

***Vibrio* spp.**

Bacteria classified in the genus *Vibrio* are Gram-negative straight or curved rods which are motile with a polar flagellum *Vibrio* spp. however some species are non-motile. They grow on agar media supplemented with NaCl, hence halophilic are facultative anaerobes. *Vibrio* spp. show good growth on the selective medium thiosulphate citrate bile sucrose (TCBS). They metabolize glucose by fermentation, oxidase-positive, catalase–positive. They produce un-pigmented colonies on marine agar (Maci et al., 2001). The Taxonomy of Vibrio genus is as follows:

Phylum Proteobacteria

Class Gamma Proteobacteria

Order Vibrionales

Family Vibrionaceae

Genus ***Vibrio*** *(Bergey et al., 1994)*

In present study *Vibrio* JH1 exhibited optimal growth at 16^{o}C, pH 8 and 2-3% NaCl. The species showed the ability to degrade agar, forming a depression around the colonies as described by Leon et al. (1992), (**Fig. 3-10**).

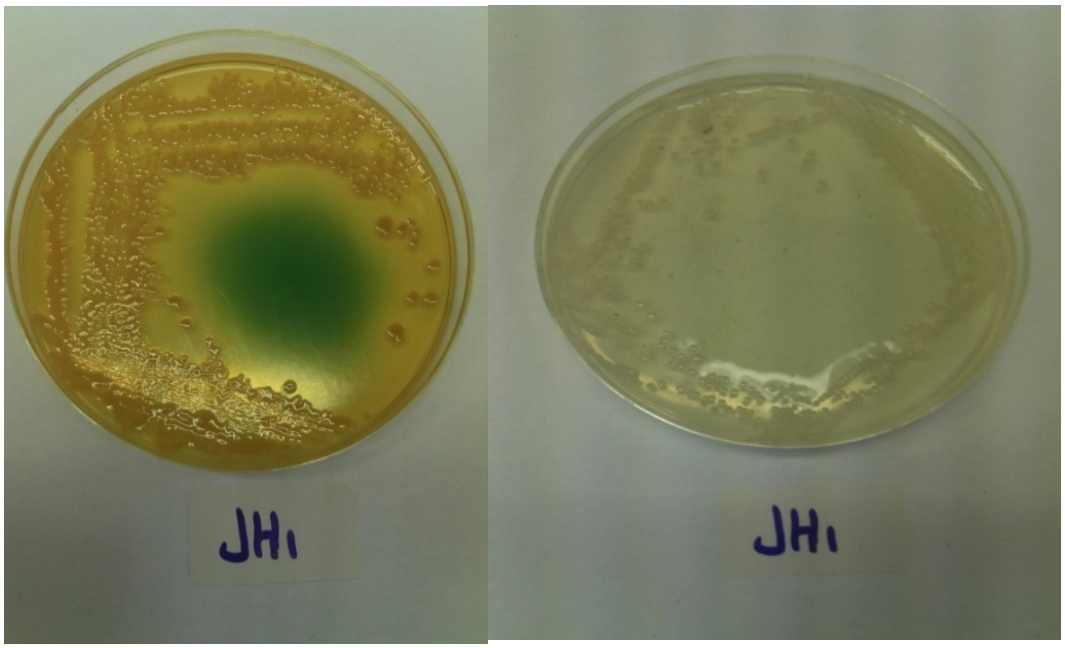

Figure 3-10 *Vibrio* JH1 growth on TCBS Agar (left), on Marine Agar as agarolytic bacterium (right)

***Enterococcus* spp.**

Enterococcus is a genus of lactic acid bacteria. Enterococci are Gram-positive cocci that usually occur in pairs (diplococci) or short chains, and are difficult to distinguish from streptococci by using just physical characteristics (Schleifer & Kilpper-Balz, 1984). The taxonomy of the genus is as below:

Phylum: Firmicutes

Class: Cocci

Order: Lactobacillales

Family: Enterococcaceae

Genus: ***Enterococcus*** (Thiercelin & Jouhaud, 1903)

(Schleifer & Kilpper-Balz, 1984)

LAB such as *Enterococcus* spp., are the most common probiotics used in the food industry. They are Gram-positive bacteria that are nonmotile, non-spore-forming, and commonly catalase-negative. They produce lactic acid as a sole product of fermentation (Ringø & Gatesoupe, 1998). *Enterococcus* JHLDc isolate in **Fig. 3-11** below.

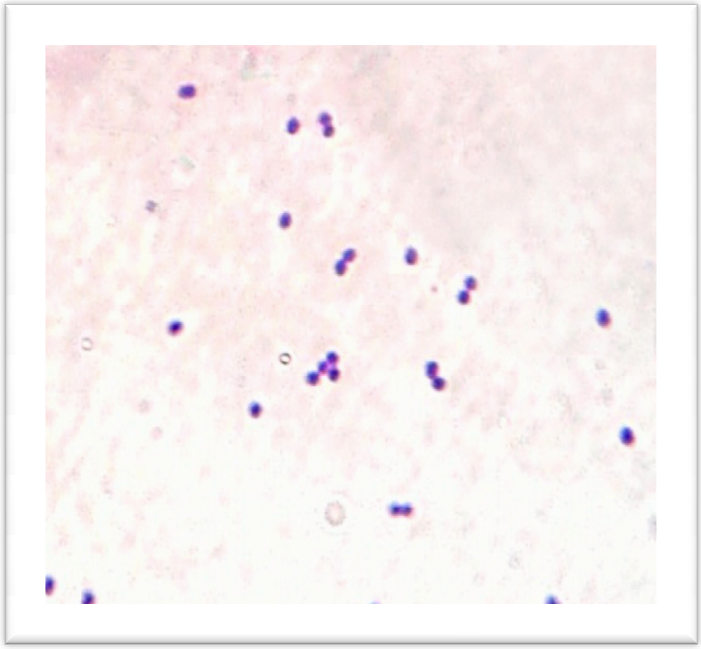

Figure 3-11 *Enterococcus* JHLDc under 100X

Chapter 4

Enzyme Activity Assays and Quantitative Screening for Potential Probiotics

4.1 Introduction

There has been an increasing number of evidence suggesting that the health and survival of organisms grown in intensive farming systems could be improved by modulating the intestinal microflora with probiotic microorganisms (Olafsen, 2001; Macey and Coyne, 2005). Enteric bacteria play an essential role in abalone nutrition, by hydrolysing complex polysaccharide components of macroalgae to simple polymers. The majority of enzyme activities in abalone gut were microbial extracellular enzymes (Erasmus et al., 1997; ten Doeschate & Coyne, 2008).

Tanaka et al. (2004) investigated the numbers of microorganisms in the gut of abalone as a first step to understand their abilities and role in digestion. The gut microflora of abalone *Haliotis discus hannai* varied with the growth stage, yet it seemed to be stable in abalone older than 1 year (Tanaka & Sugimura, 2003).

Controlling microbial balance in the GIT of abalone by using probiotic microorganisms can lead to high feeding efficiencies (Erasmus, 1996). Increased levels of protease and amylase activity were observed in *Haliotis midae* fed with a diet supplemented with the three probiotic microorganisms. These enzyme activities resulted a significant improving in abalone growth rate (Macey & Coyne, 2005).

A recent experiment using a single strain of the Gram-positive *Bacillus* sp. to supplement the formulated feed of abalone *Haliotis iris* (paua) in OceaNZ Blue (Bream Bay, Bay of Islands) did not show any significant increase in the growth rate of juvenile paua [Bruno Cedat, (2009), personal communications].

The purpose of the experiments in this Chapter is to screen the potential probiotic isolates obtained in Chapter 3 by determining their enzyme activities on protein, starch, and alginate. The enzyme activities could provide a quantitative basis for selection of the appropriate probiotic supplement to add to the commercial feed in the feeding trial.

4.2 Material and methods

4.2.1 Bacterial cultures

The nine isolates obtained in Chapter 3 were streak plated in Marine Agar. The cultures were incubated at 22^{o}C for 48 hour, except cultures for alginate hydrolysis which needed longer incubation.

4.2.2 Preparation of crude extracellular enzyme

One colony from the bacterial isolates was inoculated into 1% casein-marine broth, 1% starch-marine broth, and 1% alginate-marine broth for protease, amylase, and alginase assay, respectively. All cultures were incubated at 22^{o}C with shaking at 100rpm for 48 hour except for alginate cultures which required longer incubation time (48-120 hour).

After incubation, the liquid broth cultures were transferred into sterile plastic centrifuge tubes (40-50ml) with lids, and were centrifuged for 10 min at 10,000 x g in a Sorval centrifuge at 4^{0}C to avoid denaturation of the enzymes. The cell-free supernatants were collected with care using test tubes that have been put on ice earlier, and were handled with gloves to optimize the conditions of collecting the enzymes and avoid denaturation. All samples were filtered using a 0.22 µm millipore filter (Millex® syringe filter units, SIGMA-ALDRIC) to remove any residual bacterial cells.

4.2.3 Enzyme Assays

4.2.3.1 Proteolytic Enzyme Assay

Azocasein assay was adopted to assess protein hydrolysis for bacterial isolates from abalone gut. Azocasein assay was observed to be a highly sensitive assay for detection of amino acids resulting from the hydrolysis of protein substrates (Bisswanger, 2004) Azocasein assay was preferred over casein assay to minimize the interfering elements experienced with casein. Azocasein is an artificial orange-

red, flaky shaped substance. It is a chemically modified protein, prepared by adding sulphanamide groups to casein; hence, azocasein is a chromophore-labeled protein. Azocasein solubilized in water with gentle heating appears clear, dark orange in colour. The method is very resistant to interference and is consequently appropriate for the detection of proteolytic activity in crude samples(Rowan &Buttle, 1994).

The azo group covalently bound to casein, which is liberated by proteolytic cleavage, and their intense color is determined in the form of trichloroacetic acid-soluble peptides after adding trichloroacetic acid (TCA). When azocasein is subjected to proteolytic action, short peptides and amino acids are liberated from the chain and stay in solution resulting in orange colour of the solution. The greater the proteolytic activity the more intense the orange colour of the solution. Therefore it is easy to be quantified in a spectrophotometer (Ultrospec™ 2100 *pro* UV/Visible) at 340 nm.

In the present study, azocasein was purchased from (Sigma-Aldrich NZ Ltd / Auckland 1030). It was prepared as a solution at 0.2% in 0.1M potassium phosphate buffer (pH 8.0) with gentle warming to about 40-50^{o}C. Reactions were carried out in 1.9 ml micro-centrifuge tubes. A 0.5ml of azocasein was mixed with 0.2 ml of protease sample (from culture supernatant) and incubated at 25^{o}C for 30 minutes. The reaction was stopped by adding 0.2ml of 10% trichloroacetic acid. The mixture was centrifuged at room temperature for 5 minutes at 12,000 rpm. The supernatant was transferred to a quartz cuvette and the absorbance was measured in a spectrophotometer at 340 nm, with two replicates for each sample of cultural supernatants. A standard curve for Trypsin amino acid was used to calibrate the amount of amino acids generated from azocasein hydrolysis in µg/ml of product.

Proteolytic enzyme (protease) activity was determined as previously described (Bisswanger, 2004; Iehata et al., 2009; Macey &Coyne, 2005) :

One unit of protease activity is defined as the amount of enzyme that gives an increase in optical density at 340 nm of 0.1 in 30 min at 25^{o}C.

Therefore, the absorbance reading for the fastest rate of proteolysis in the first 10 minutes of reaction was divided by 0.1 and multiplied by 3 for 30 minutes to calculate numbers of units of protease enzyme for each isolate. To express the enzyme activity as standard International Units/ml, values were multiplied by 5 to account for the 0.2 ml volume of culture supernatant used in the assay.

4.2.1.2 Amylolytic Enzyme Assay

The amylolytic ability of the isolates was determined by assaying the reducing sugars, produced from starch hydrolysis, using the Dinitrosalicylic acid (DNS) colorimetric method (Wang, 1988). The DNS method detects the presence of an aldehyde group and results in the oxidation of the aldehyde functional group in the reducing sugars, such as glucose, to a carboxylic acid group.

Aldehyde group 

carboxyl group

DNS (3, 5-dinitrosalicylic acid) in the presence of Carboxyl group is reduced to 3-amino, 5-nitrosalicylic acid and can be detected by the intensity of the developed color.

3, 5-dinitrosalicylic acid 

3-amino, 5-nitrosalicylic acid

Different color intensities relate to different reducing sugars, therefore it is essential to calibrate for each sugar. In this study, reducing sugars were calibrated with anhydrous glucose as glucose is the product of starch hydrolysis.

DNS reagent was prepared by adding 10g of Dinitrosalicylic acid powder, 0.5g of sodium sulfite, and 10g of sodium hydroxide pellets to one liter of deionized water. Potassium sodium tartrate solution (Rochelle salt) was prepared at 40% for colour stabilization. The starch substrate was prepared at 2% w/v. Soluble starch powder (Acros Organic) was dissolved in a small amount of cold deionized water and then boiled, after which phosphate buffer (pH 7.0) was added to take the starch solution to the required final concentration of 1% w/v.

The reaction mixture for the amylase assay was prepared by mixing the crude enzyme with the starch solution at 1:1 ratio in screw-capped test tubes. The tubes were incubated at 37 ^{o}C for 30 min. Two replicates were set up for each isolate. A 3 ml sample was collected every 10 min to which 3 ml of DNS reagent were added. The DNS-sample mixture was heated in a 90-100^{o}C boiling water bath for 10-15 min until red-brown colour has developed. One ml of Rochelle salt solution was added, then the tubes were cooled using cold water. The absorbance of the solution was determined in a spectrophotometer at 575 nm.

One international unit (IU) of amylase activity is defined as the amount of enzyme that released 1 µmol product (of reducing group) per min, as described by Ghose (1987) and Bisswanger (2004):

1 IU = 1 µMole / min of librated hydrolysis product

4.2.1.3 Alginolytic Enzyme Assay

Determination of alginate hydrolysis in the bacterial isolates was conducted using the same DNS colorimetric method above (Hu et al., 2006). Alginate is a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, respectively, covalenty linked together in different sequences or blocks.

Alginate solutions contain a polysaccharide anion with a negative charge for carboxylic acid group (d'Ayala et al., 2008). DNS (3, 5-dinitrosalicylic acid) in the presence of carboxyl group can be reduced to 3-amino, 5-nitrosalicylic acid which can be detected through measuring the developed color. Hence, the DNS method for reducing sugar was employed to detect the alginate hydrolysis.

The three isolates observed to demonstrate alginate hydrolysis in Section 3.2.2.3.2 were subjected to alginate hydrolysis assay, namely, *Vibrio* JH1, the fastest alginolytic isolate (1-2 day), *Shewanella* JH3, and *Vibrio* JH5 as two slow alginolytic (3-5 day) isolates. Determination of the amount of reducing sugars in each sample and the enzyme activity were calculated as in Section 4.2.1.2.

4.4 Results and Discussion

To assess the isolates for their activities, potential probiotics should meet one or more of the criteria shown in **Fig 4-1** below:

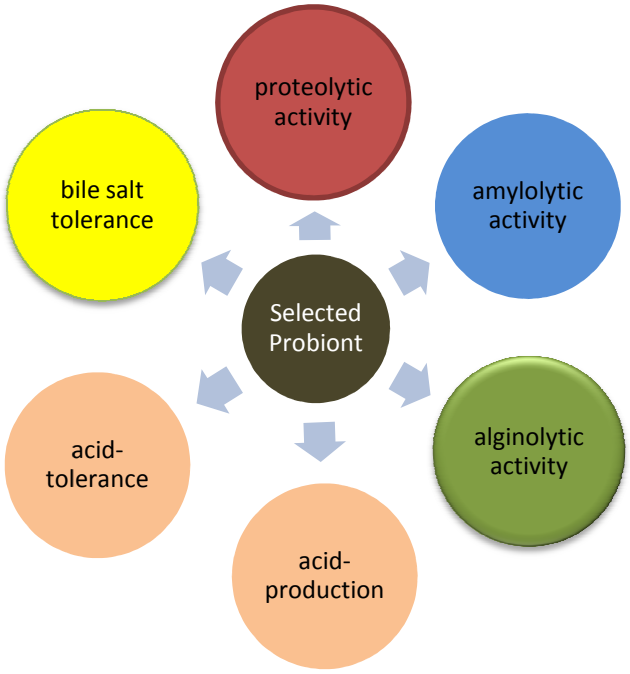

Figure 4-1 The main activities to assess the potential probiotic isolates

Screening the isolates for acid and bile salt tolerance, in addition to acid production was conducted in Chapter 3.

In the present Chapter the rest of screening was conducted through enzymatic assays for protein, starch, and alginate hydrolysis.

4.4.1 Proteolytic Enzyme Assays

Isolates	Amino acids concentration (30 min) µg/ml ± SE	Enzyme activity IU/ml ± SE
Exiguobacterium JHEb1	547 ± 10.8	128.3 ± 9
Bacillus JHBa2	182 ± 9	26.85 ± 1.6
Vibrio JH1	321.5 ± 6.5	62.4 ± 0.8
Vibrio JH2	34.7 ± 1.87	4.5 ± 0.4
Shewanella JH3	83 ± 14.6	3.75 ± 1.7
Vibrio JH4	630 ± 2.8	116 ± 8.5
Vibrio JH5	83.6 ± 0.37	10 ± 0.23

Table 4-1 Enzyme activity of proteolytic isolates

Results (**Table 4-1** and **Fig. 4-2**) showed that *Exiguobacterium* JHEb1, *Vibrio* JH4 and *Vibrio* JH1 had the highest proteolytic activity measured as IU/ml of cultural supernatant; (128.3 ± 9), (116 ± 8.5), and (62.4 ± 0.8), respectively. *Vibrio* JH5, *Shewanella* JH3 and *Vibrio* JH2 exhibited lower proteolytic enzyme activity (10 ± 0.23), (3.75 ± 1.7), and (4.5 ± 0.4), respectively. The proteolytic activity of *Bacillus* JHBa2 was mid-range (26.85 ± 1.6).

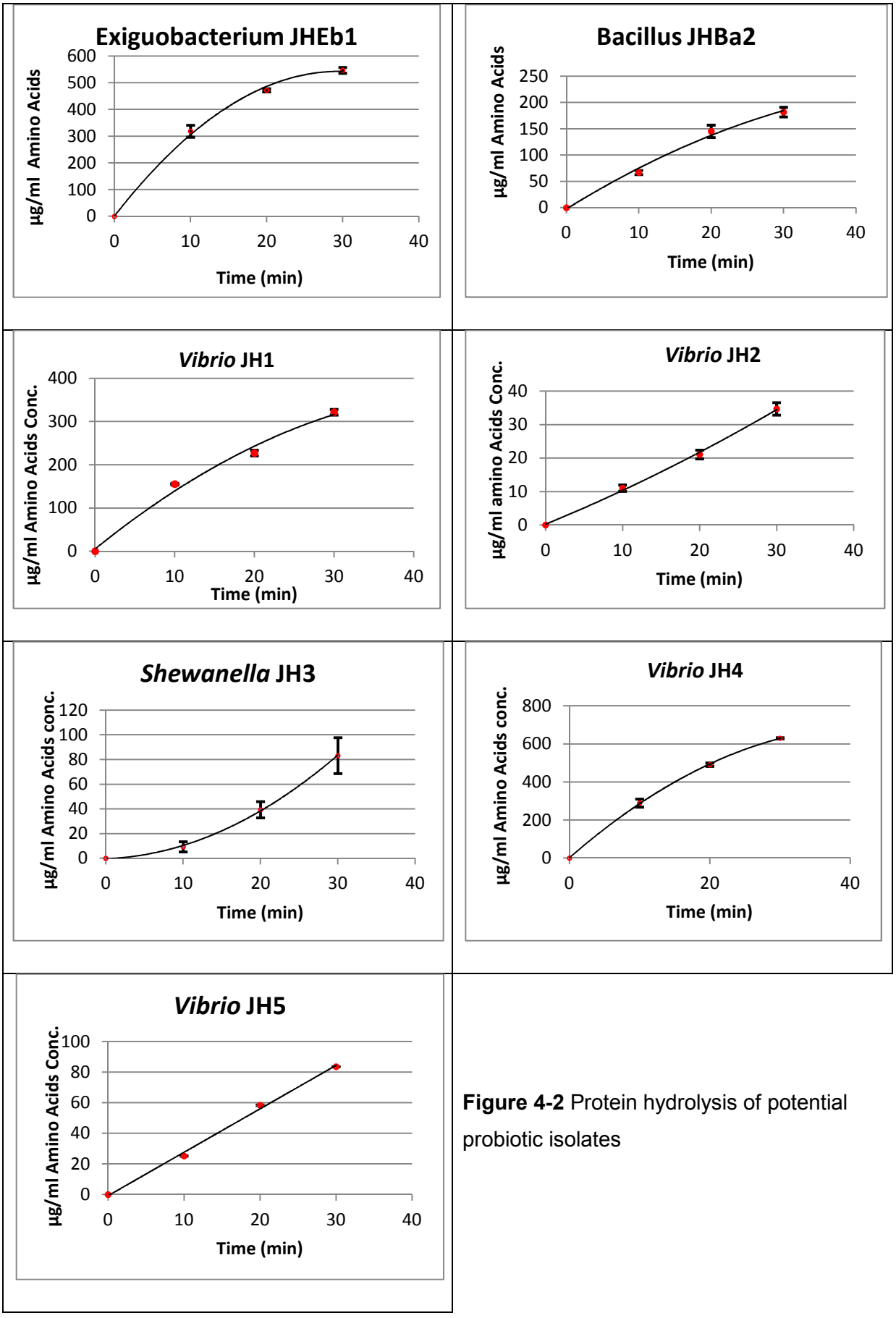

Figure 4-2 Protein hydrolysis of potential probiotic isolates

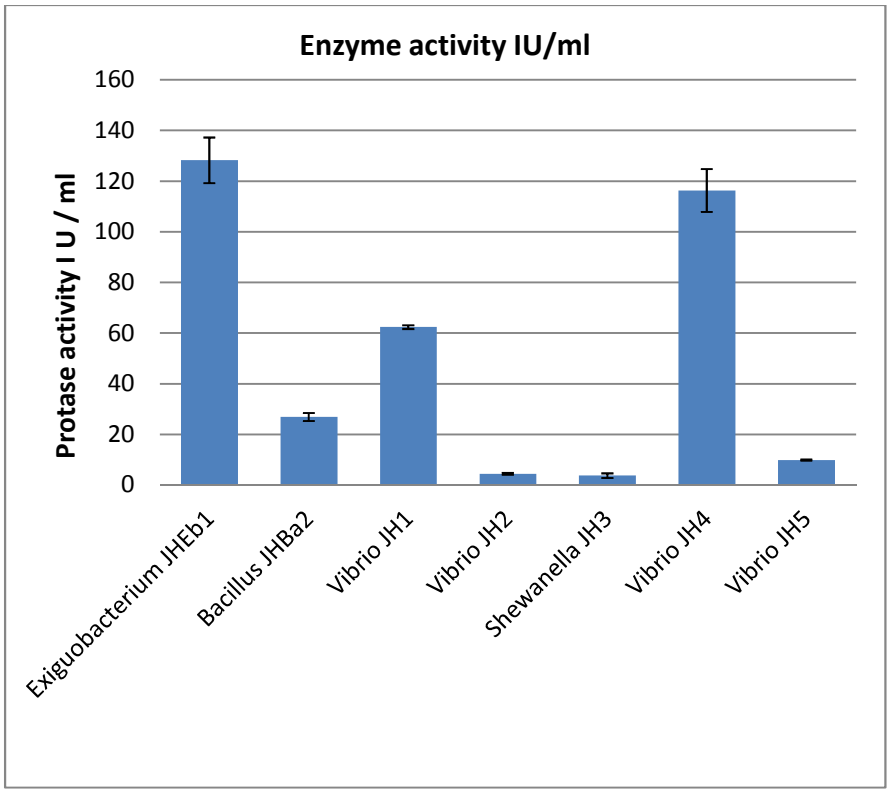

Figure 4-3 Enzyme activity of proteolytic isolates (IU /ml ± SE)

4.4.2 Amylolytic Enzyme Assays

Isolates	Glucose concentration (for first 15 min. of hydrolysis) µ Mol± SE	Enzyme activity IU ±SE	enzyme activity IU/ml ±SE
Exiguobacterium JHEb1	7827 ± 55.7	521.8 ± 3.8	347.8 ± 2.5
Bacillus JHBa2	5984 ± 518	398.6 ± 34.6	265.7 ± 23
Vibrio JH1	4214 ± 418	281 ± 27.9	186.6 ± 18.7
Shewanella JH3	2839 ± 345	189 ± 23	126.0 ± 15.4
Vibrio JH4	3824 ± 72	255 ± 5	169.7 ± 3.3

Table 4-2 Enzyme activity of amylolytic isolates

The rates of starch hydrolysis are shown in **Fig. 4-4**. All the amylolytic isolates exhibited high enzyme activity. *Exiguobacterium* JHEb1 was observed to have the highest amylolytic activity of 347.8 ± 2.5, followed in rank by *Bacillus* JHBa2 (265.7 ± 23) and Vibrio JH1 (186.6 ± 18.7), **Table 4-2**, *Shewanella* JH3 had the lowest activity of 126.0 ± 15.4

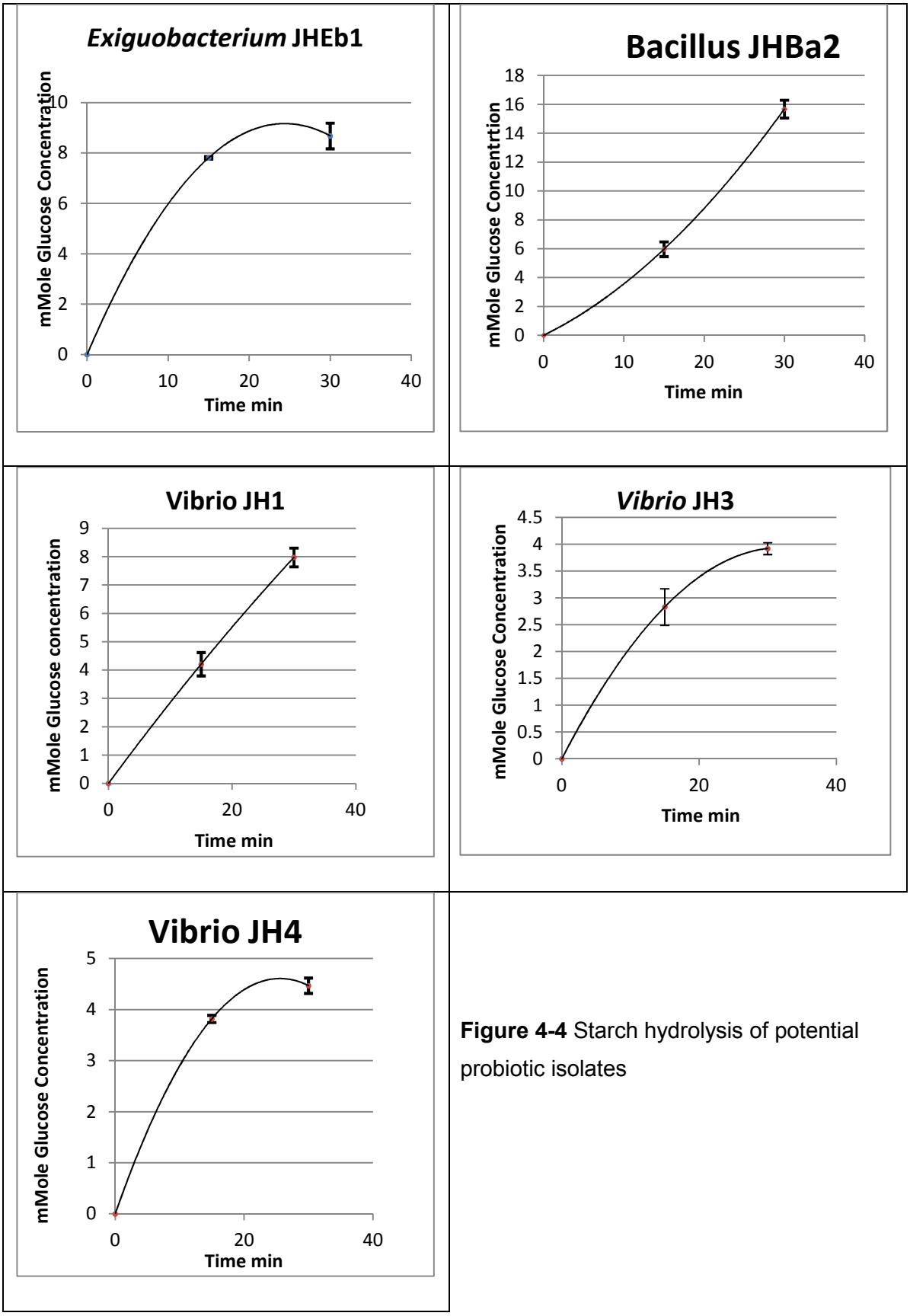

Figure 4-4 Starch hydrolysis of potential probiotic isolates

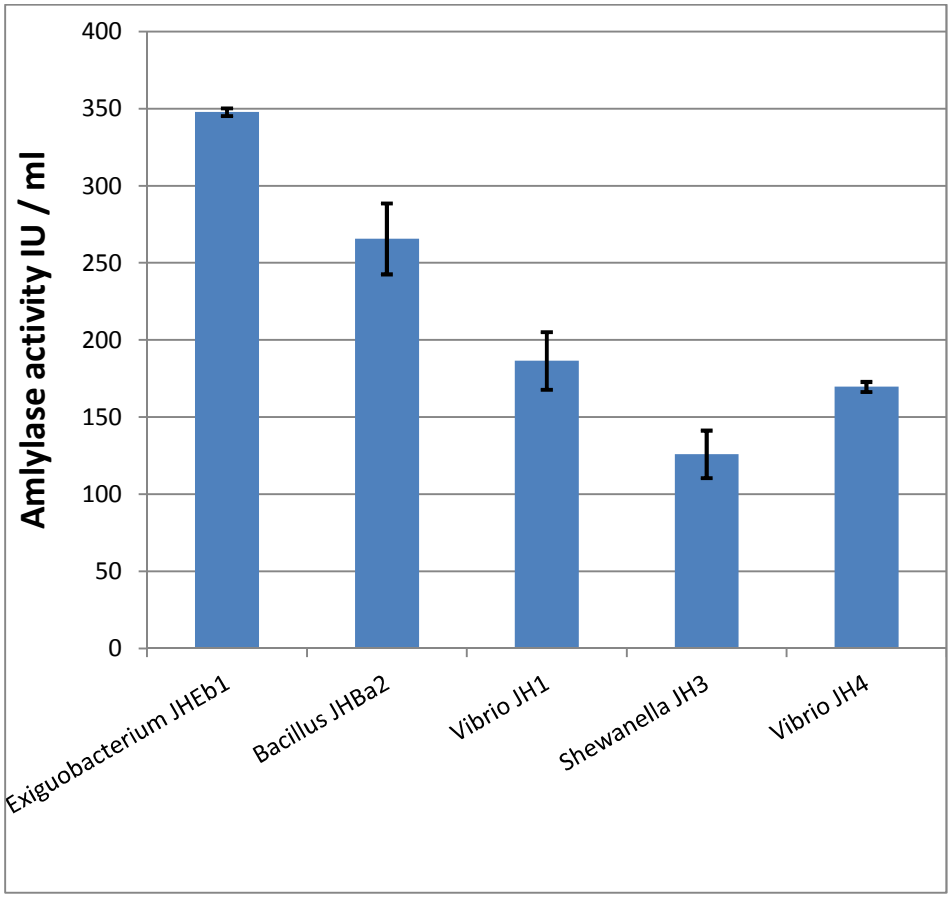

Figure 4-5 Enzyme activity (IU /ml ± SE) of amylolytic bacteria isolates

4.4.3 Alginolytic Enzyme Assays

Alginate hydrolysis detection for the three alginolytic isolates was shown that *Vibrio* JH1 is the highest in the rank, it was released (241±8.4IU/ml) of reduced sugar as product from alginate hydrolysis, comparing to *Shewanella* JH3, *Vibrio* JH5 which were released lower levels of product (86.7 ± 3.2 IU/ml) and (187.5 ±1.47 IU/ml) respectively, refer to **Fig. 4-6** & **4-7,** and **Table 4-3.**

The rates of alginate hydrolysis in the three alginolytic isolates are shown in **Fig. 4-6.** The highest alginolytic activity was observed in *Vibrio* JH1 (241±8.4IU/ml), **Table 4.3**, **Fig. 4-7**. *Vibrio* JH5 had only slightly lower activity than *Vibrio* JH 1 at 187 ± 1.47. *Shewanella* JH3 had the lowest alginolytic activity (86.7 ± 3.2).

Isolates	Reducing sugar concentration (for first 15 min. of hydrolysis) µ Mol± SE	Enzyme activity IU ±SE	Specific Enzyme activity IU/ml ±SE
Vibrio JH1	5422 ± 189	361.5 ± 12.6	241 ± 8.4
Shewanella JH3	1950 ± 72	130 ± 4.8	86.7 ± 3.2
Vibrio JH5	4211 ± 33	280.7 ±2.2	187 ± 1.47

Table 4-3 Enzyme activity of alginolytic isolates

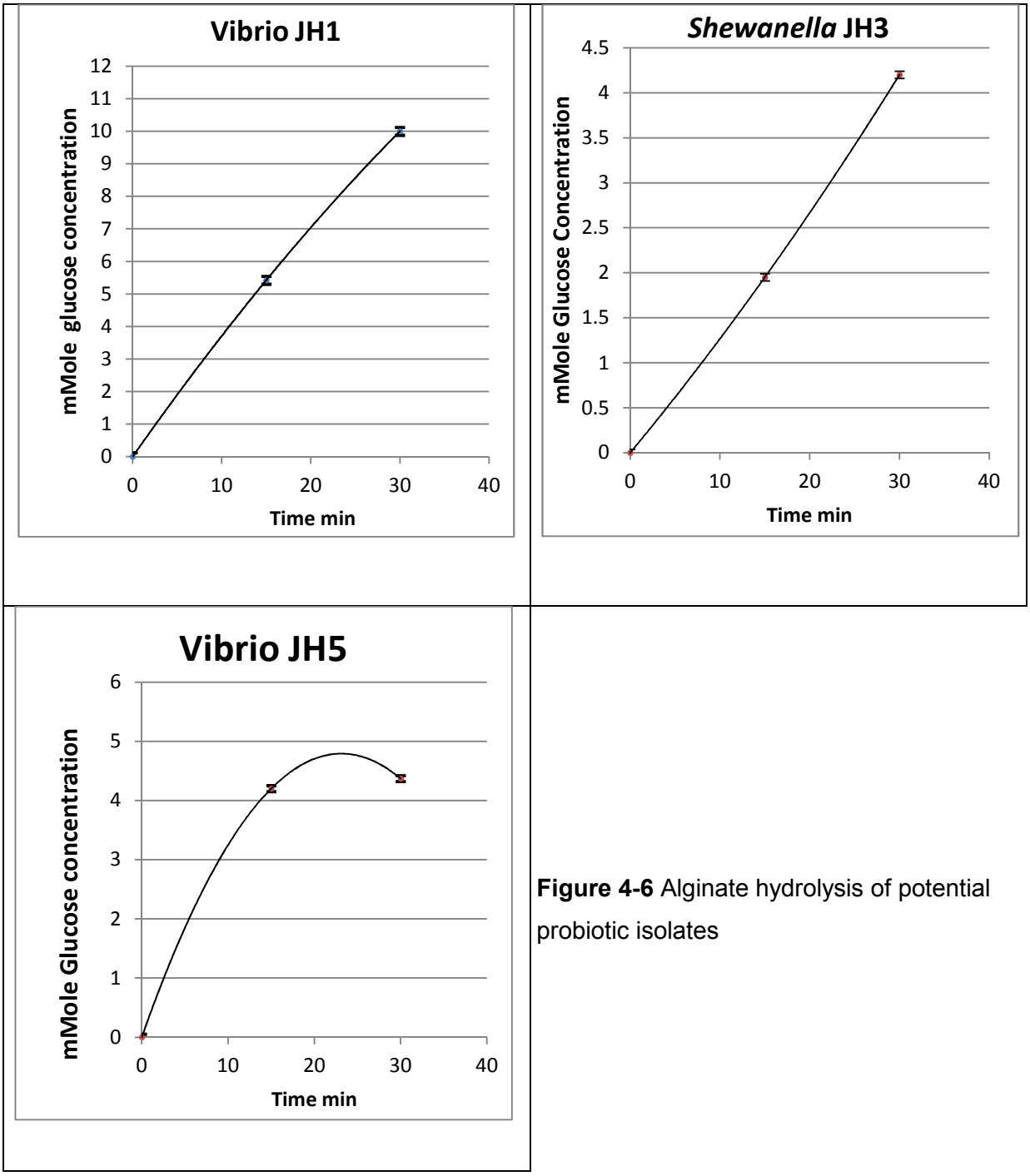

Figure 4-6 Alginate hydrolysis of potential probiotic isolates

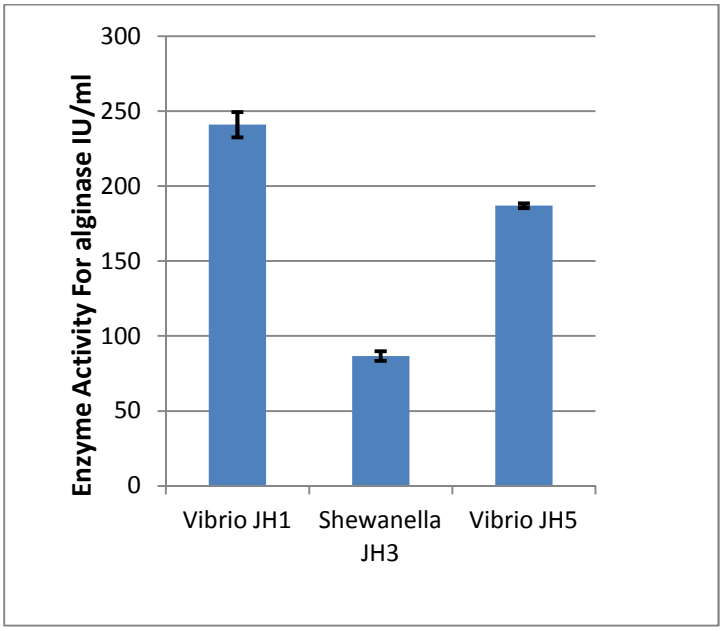

Figure 4-7 Enzyme activity (IU /ml± SE) of alginolytic isolates

The experiments in this Chapter were performed to provide a quantitative basis for the selection of the most suitable probionts to be used in the feeding trial. Enzyme activities from protein hydrolysis, starch hydrolysis and alginate hydrolysis were calculated. Similar assessment of digestive enzyme activities have been carried out in abalone and shrimps to evaluate the contribution of bacteria introduced to the animals (Iehata et al., 2010; Macey &Coyne, 2005; ten Doeschate & Coyne, 2008; Ziaei-Nejad et al., 2006)

The seven host-derived proteolytic isolates vary in their ability of protein hydrolysis, the higher proteolytic activity measured as specific protease units was observed by *Exiguobacterium* JHEb1 (128.3 ± 9 IU/ml), *Vibrio* JH4 (116 ± 8.5 IU/ml), and *Vibrio* JH1 (62.4 ± 0.8 IU/ml). They are more likely to be in the shortlist of the candidates of potential proteolytic probiotics for feeding trial. Unlike *Bacillus* JHBa2, *Vibrio* JH5, *Shewanella* JH3 and *Vibrio* JH2 that cannot be included in the probiotic treatment of feeding trial for their low production of proteolytic enzymes.

Out of the seven host-derived proteolytic isolates, *Exiguobacterium* JHEb1 *Vibrio* JH4 and *Vibrio* JH1 would be short-listed as probiotic candidates to be used in

the feeding trial. However, *Vibrio* JH4 produced ß- haemolysis on Colombia blood agar (Chapter 3, **Table 3-12**), a property which is associated with pathogenicity (Schulze et al., 2006). Choosing *Vibrio* JH4 could pose a potential hazard, hence, it was eliminated as a potential probiont.

To assess amylase activity for amylolytic isolates, *Exiguobacterium* JHEb, *Bacillus* JHBa2, and *Vibrio* JH1 were observed to have the strongest amylolytic ability. However *Bacillus* JHBa2 exhibited ß- haemolysis (**Fig 4-8**), thus it should not be included in the selection of potential probiotics.

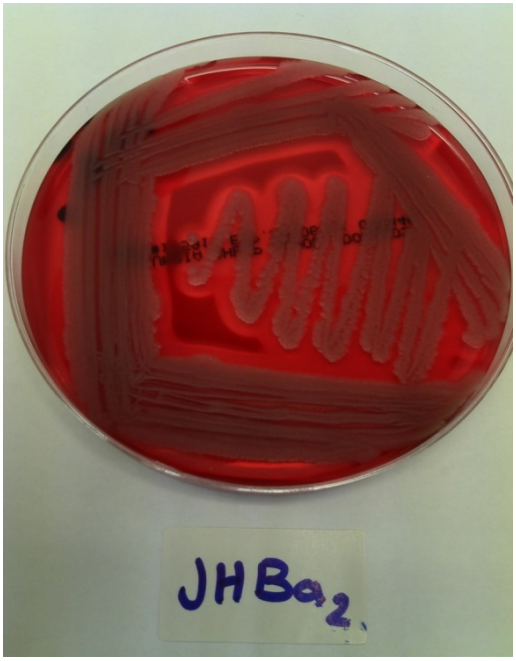

Figure 4-8 ß- haemolysis in *Bacillus* JHBa2

With regard to alginolytic enzymes, *Vibrio* JH1 showed the highest enzyme activity compared to *Shewanella* JH3 and *Vibrio* JH5. The enzyme activity obtained in this experiment confirmed the results of the qualitative alginate degradation in the previous Chapter where *Vibrio* JH1is was able to degrade alginate after 1-2 days compared to 4-5 days with *Shewanella* JH3 and *Vibrio* JH5.

The proteolytic activity of Vibrio JH1 is similar to those of the probiotic isolates used in *H. midae* (Macey & Coyne, 2005). Supplementation of the basal feed for *H. midae* with proteolytic isolates resulted in a significant increase in protein digestion and absorption within the intestine of abalone.

Vibrio JH1 also was the only isolate that exhibited fast rate of alginate hydrolysis, and agarolysis (pits around colonies on Marine agar plate, Chapter 3). The remaining two alginolytic isolates (*Shewanella* JH3, *Vibrio* JH5) could not be included in the feeding trial because of their poor, slow ability to degrade alginate. It is recommended to include isolate exhibiting fast rate of alginate degradation in the supplementation of the feed for feeding trial to aid the digestion of seaweed powder present in the commercial feed for abalone (Section 5.2.3).

The basis of including *Vibrio* JH1 in the feed supplement for *H. iris* is similar to that of Erasmus et al. (1997). It was suggested that the role of the enteric bacteria of abalone in pre-degradation of alginate that supplied a more easily digestible substrate for the host digestive enzymes. By decreasing the amount of energy required for digestion, there would be more available energy that can be utilized for growth. Similar results were obtained by ten Doeschate, V.E. Coyne (2008) who reported that *Pseudoalteromonas sp.* strain C4 increased the alginate lyase activity in the crop and stomach of *H. midae a*balone fed a kelp diet supplemented with strain C4. The alginate lyase activity was significantly greater compared to abalone fed a standard kelp diet. Erasmus et al. (1997) proposed that bacteria resident in the GIT of *H. midae* assist in the digestion of alginate, laminarin, agarose, carrageenan, and cellulose.

The screening experiment of bile salt tolerance; *Exiguobacterium* JHEb1, *Vibrio* JH1, and *Enterococcus* JHLDc were the most tolerant isolates with no potential pathogenicity (**Table 3-6**and **3-7,** Chapter 3). Screening for acidity tolerance showed that all isolates can tolerate acidity within the range 2-5 pH, and all isolates were able to tolerate to 1.5 pH except *Vibrio* JH2, and *Vibrio* JH5 (**Table 3-8**).

4.5 Conclusion

The goal of quantitative screening was to follow up on those isolates of interest to develop mixtures of probiotic bacteria for application to the feed of the abalone (paua) in the feeding trial. The most promising isolates according to quantitative enzyme screening are:

Exiguobacterium JHEb1; a proteolytic, amylolytic, lipolytic, bile salts tolerant, and high acidity tolerant isolate.

Vibrio JH1; a proteolytic, amylolytic, fast alginolytic, agarolytic, high bile salts tolerant, and high acidity tolerant isolate.

Enterococcus JHLDc; a high acid producing, high bile salts and acidity tolerant isolate.

This study proposes to a 2-probiotic-supplemented feed that include *Exiguobacterium* JHEb1 and *Vibrio* JH1.

A 3-probiotic-supplemented feed should include *Exiguobacterium* JHEb1, *Vibrio* JH1, and *Enterococcus* JHLDc.

Chapter 5

Feeding Trials Using Isolates of Probiotics

5.1 Introduction

Growth rate improvement in abalone may be achieved by the use of probiotic microorganisms. through these mechanisms: increasing the nutrients readily absorbed in the gastrointestinal tract; increasing the amount of digestive enzymes in the gut; or through use of the bacterial supplement as an additional nutrient source (ten Doeschate & Coyne, 2008).

The objective of this study was to determine if growth of abalone could be improved with application of the probiotics selected in the previous chapter.
The experiment was designed to incorporate probiotic bacteria with nutrient-degrading properties so that they could improve the digestion process inside the abalone gut through protein degradation and polysaccharide (starch and alginate) degradation that could result in increased shell length and weight of juvenile abalone.

The hypothesis of the study is the probiotic bacteria supplemented to the feed help the abalone benefit from the feed more than the unsupplemented feed resulting in lower concentration of protein, carbohydrate, and lipid in the faeces of the host.

Two multi-strain probiotic combinations were formulated as a conglomerate of 2 or 3 potential probiotic strains. This study also aimed to compare the effect of the two probiotic mixes on abalone growth.

The methods of administration of probiotic strains to aquaculture animals are several (Geovanny et al., 2007) in which the appropriate application with adequate concentration remains a critical issue. In this study, the application of the selected probionts was through supplementing them into the commercial feed. For effective and practical application of the probiotic mixes in the feeding trial, the following points were considered:

- Maintenance of the desired high concentration of viable bacteria.
- Retention of probiotic feed at the bottom of the tank during the feeding periods.

- Distinct difference between abalone faeces and probiotic feed for ease of collection of faecal sample for biochemical analyses.

5.2 Materials and Methods

5.2.1 Experimental Animals

Juvenile abalone, 20-30mm in shell length, were obtained from OceaNZ Blue Ltd (Paua farm), Ruakaka, northern New Zealand. The abalone were kept moist during transport from the paua farm to the aquaculture laboratory in AUT University by placing them in a cool box containing an ice bag. The ice bag was wrapped with thick pad of tissue to protect abalone from freezing and at the same time to maintain cold environment to allow survival during transport.

The abalone samples were tagged with water-proof tags with numbers to label each individual. The numbers of each individual and their shell length and weight measurement were record. The animals were placed into the tanks (20) abalone for each, and were acclimatized at aquaculture laboratory for one week prior to the experiment. During acclimatization time, they were fed commercial feed (AbMax 16).

5.2.2 Cultivation of Potential Probionts

The selected isolates from screening experiments were: *Exiguobacterium* JHEb1, *Vibrio* JH1, and *Enterococcus* JHLDc, (Section 4.5). The isolates were propagated in enriched media. *Exiguobacterium* JHEb1 was propagated in Marine broth supplemented with 1% yeast extract. The culture was incubated in a rotary shaker (100 rpm) at 22^{o}C for 48 hr. *Vibrio* JH1 was grown in Marine broth supplemented with 0.5% glucose in a rotary shaker (100 rpm) at 22^{o}C for 48 hr. *Enterococcus* JHLDc was propagated anaerobically in MRS broth containing 2% NaCl for 48 hr at 22^{o}C.

5.2.3 Abalone Commercial Feed

The commercial feed, AbMax 16, used in this study was manufactured by E. N. Hutchinson Ltd (Auckland, New Zealand). AbMax 16 was obtained from OceaNZ Blue Ltd and was maintained in AUT laboratory at 4^{o}C until required. The feed existed as dry, oval-shaped pellets (**Fig. 5-6**). The manufacturer ingredients and contents were listed as:

Wheat Flour 35%, Fish Meal (Milled NZ) 20%, Defatted Soy Flour & Isolate 13%, Seaweed Powder, Tapioca Starch, Propylene Glycol, Propylene Glycol, Sugar, Yeast – Saccharomyces, Wheat Gluten, Fish Oil (mixed), Phosphoric Acid (85%), Carrageenan, Lecithin Powder, Dicalcium Phosphate, Potassium Sorbate, Vitamin Mineral Mix (Abalone), Betaine, Stay-C (25%), and Vitamin E. Some components percentages are not given due to commercial sensitivity.

Proximate analyses (i.e., protein, carbohydrates, and lipid) were conducted on AbMax to determine its general composition.

5.2.4 Preparation of Probiotic- supplemented Feed

For effective delivery of the probionts and maintenance of adequate probiotic concentration three methods of application to the feed have been tested before the appropriate application was adopted. Firstly, preparation of feed cakes containing bacteria with agar, secondly, feed cakes containing immobilized bacteria with agar, and thirdly, application of bacterial broth added directly to the feed pellets.

In a pilot feeding trial, which conducted to check the appropriate application of feed and has not been mentioned in full details in this thesis, two options of feed cakes have been tested; feed cakes containing bacteria with agar at different concentrations (1.5 % w/v, 2% w/v, 2.5% w/v) and different amounts of feed pellets. The feed cakes were not stable and disintegrated into small pieces. The pieces of feed and broken agar were mixed with the slimy feces of abalone and made it difficult to separate the fecal matter for biochemical analysis. The same difficulty occurred with the second option of feed cakes which contained alginate beads of immobilized probiotic bacteria. However, the immobilized bacteria were beyond the reach of the abalone, for they had to graze onto the agar and the

feed pellets with immobilized beads this was not ideal for erratic feeders like abalone, where they were either fed on agar which was poor in nutrients or stopped eating. Application of broth cultures of the isolates added directly to the feed pellets was chosen for the feeding trial.

Three to five colonies of *Exiguobacterium* JHba1 grown in Marine Agar were inoculated into 50 ml of modified Marine Broth containing an additional 1% of yeast extract. *Vibrio* JH1 (3-5 colonies) from Marine Agar was transferred to 50 ml of modified Marine Broth containing 1% yeast extract and 0.5% glucose. Three to five colonies of *Enterococcus* JHLDc from MRS agar were transferred to MRS broth containing 2% NaCl. All cultures were incubated at 22^{o}C in a rotary shaker at 100 rpm.

Viable count was determined using spread plate count at day 0 and day 2. This enumeration was necessary to determine if a viable concentration between 10^6 CFU /g to 10^8 CFU/g is maintained in the feed (Gatesoupe, 1999; Macey & Coyne, 2006b; ten Doeschate & Coyne, 2008).

The cultures were transferred into sterile (50 ml) centrifuge tubes with caps. Centrifugation was conducted for 10 minutes and bacterial pellets (probiotic biomass) were collected and re-suspended in (25ml) sterile marine broth. For 2-probiotic supplemented feed, one bacterial pellet of Gram- positive isolate (*Exiguobacterium* JHBa1), two bacterial pellets of Gram- negative isolate (*Vibrio* JH1) were re-suspended in (25ml) of sterile marine broth, **Fig. 5-1**.

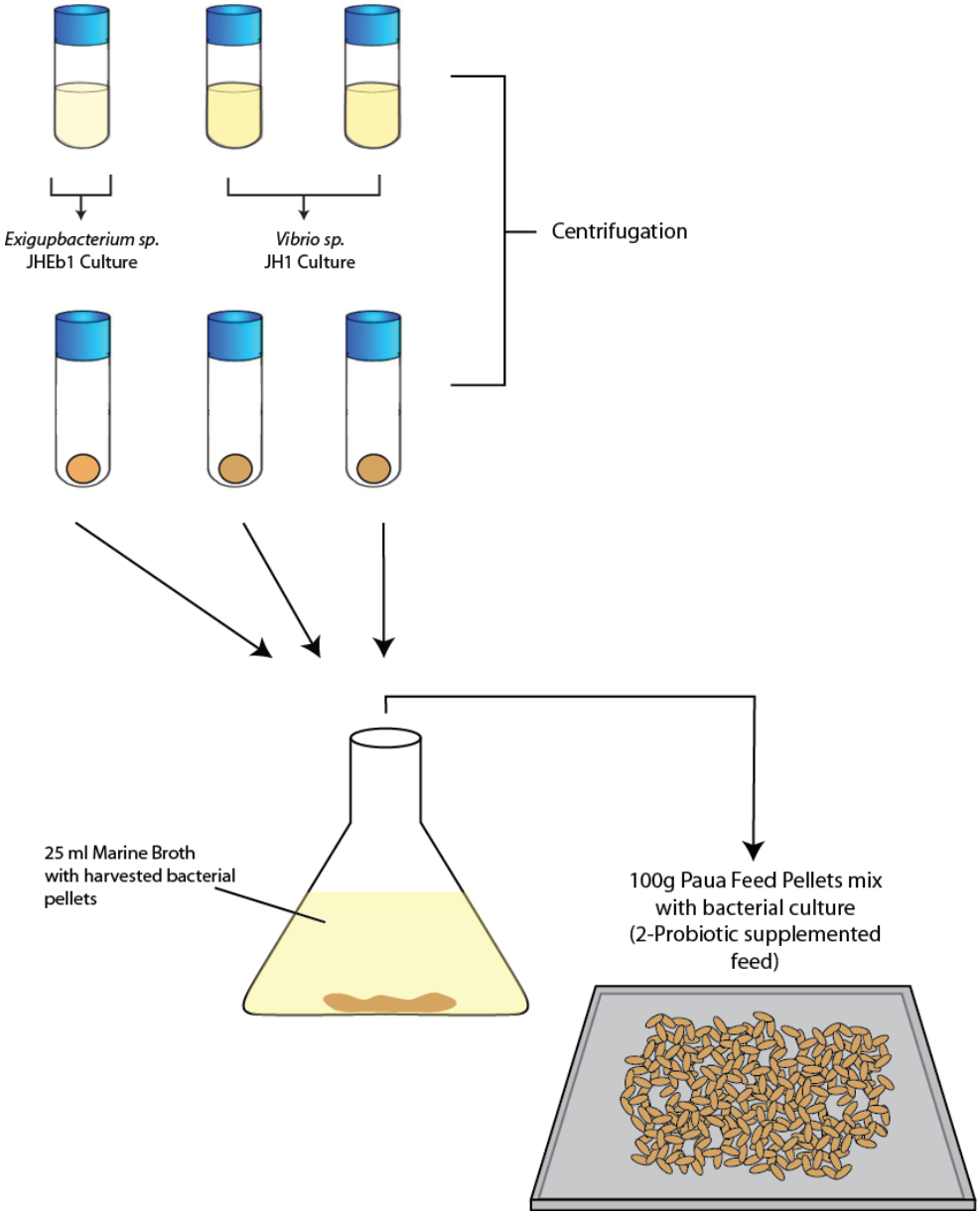

Figure 5-1 Preparation of 2-probiotic supplemented feed

For 3-probiotic supplemented feed, one bacterial pellet of *Exiguobacterium* JHBa1, two bacterial pellets of (*Vibrio* JH1, and two bacterial pellets of lactic acid bacteria (*Enterococcus* JHLDc) were included in 25ml of sterile marine broth. The rationale for this was *Exiguobacterium* JHBa1 is a very competitive bacteria and from previous experiments of viable counts of mixed culture of three isolates, it showed more growth than the two other isolates (*Vibrio* JH1 & *Enterococcus* JHLDc), **Fig. 5-2**.

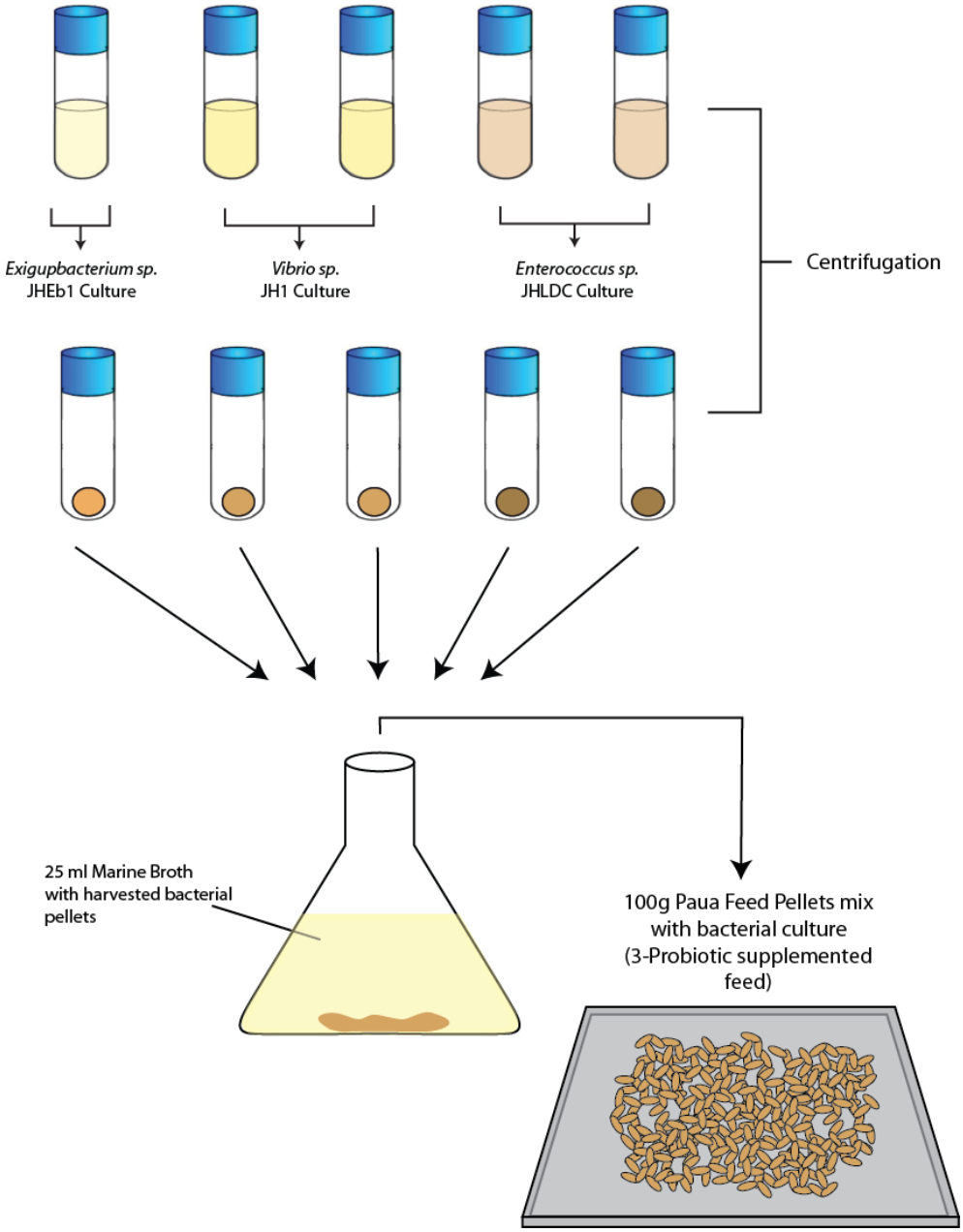

Figure 5-2 Preparation of 3-probiotic supplemented feed

About 100 gram of feed was placed on plastic tray sterilized previously by spraying with 70% ethanol and dried immediately. The well mixed bacterial suspension was spread all over the feed pellets using 20ml sterile syringe. The pellets were mixed manually with gloves sterilized with 70% ethanol. This procedure was to maintain even distribution and covering every single feed pellet with the bacterial suspension. The feed was air-dried in a laminar flow cabinet at room temperature for 2- 4 hr (Lauzon et al., 2010).

Storage for the prepared probiotics- supplemented feed was in 4^{o}C in covered glassware for no longer than one week to maintain probiotic viability (Gatesoupe, 1999; Wang & Li, 2008)

5.2.5 Measurements of Weight and Shell Length

Individual abalone weights were taken using a top pan balance (METTLER TOLEDO PB602-S, USA). First, individual abalone were blotted dry using tissue paper, and then they were placed on the balance pan. Measurements were taken to the nearest 0.01g at the start and end of the feeding trial to calculate weight gain and daily weight gain:

Weight gain/day = (final weight - initial weight) / 60 days

Shell length measurements were taken as maximum diameter (longest axis) of the shell, using digital vernier calipers (Mitutoyo, Japan made) to the nearest 0.01mm. Initial and final shell length measurements for each abalone, were recorded to use for the calculation of daily shell growth as mentioned by (Olmos et al., 2011):

Shell growth/day = (final shell length - initial shell length) / 60 days

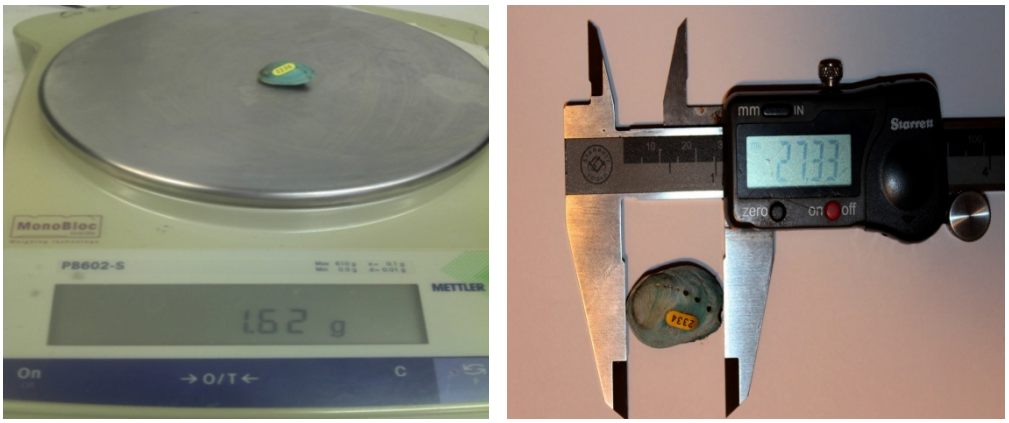

Figure 5-3 Abalone weighing and shell length measuring

5.2.6 Rearing Tanks

Three replicate of 42-L black plastic tanks (Bunnings Warehouse, Auckland) were used for each of three feeding treatments (diets) in abalone feeding trial. The treatments included a feed with 3- probiotic supplements, a feed with 2- probiotic supplements, and an un-supplemented feed (control). Twenty abalone were placed in three replicates for each probiotic diet, and for un-supplemented diet (control).

All nine tanks were covered with a weld mesh with openings of 12.5 mm^2 (Bunnings Warehouse, Auckland) to prevent the abalone from escaping. The tanks were covered with black plastic plates to provide darkness to promote abalone eating since they prefer to eat in the dark (Erasmus et al., 1997), **Fig. 5-4**.

Figure 5-4 Feeding trial tanks with mesh cover (left), and plastic covers (right)

5.2.7 Water System of Feeding Trial

The feeding trial was carried out at the AUT aquaculture laboratory. A static water system was employed to maintain the animals with seawater collected from Okahu Bay, Auckland. Immediately after collection, the seawater was filtered via (0.2 µm) filtration system and sterilized with a UV system. The sterile seawater was stored in a 2000 litre storage tank until needed within a week. Abalone were kept at a constant temperature of 16-16.9^{o}C in the aquaculture laboratory.

5.2.8 Feeding Trial

The trial period was for 2 months that started in October and ended December 2011. A total of 180 juvenile abalone were starved for 2-3 days in order to guarantee eating to full satisfaction in the start of the feeding trial. After starvation, the abalone were fed probiotic-supplemented feed (3 -combination, 2 -combination supplemented feed) and commercial feed for control group, all in three replications. The protocol of the feeding trail is outlined in **Fig. 5-5**.

Feeding abalone was conducted once a day, and according to the farm instructions with regard to feed rations, which depend on temperature. At 16-17°C, abalone of 25mm size usually feed at around 1.1% of body weight [Dr. Rodney Roberts (personal communication)].

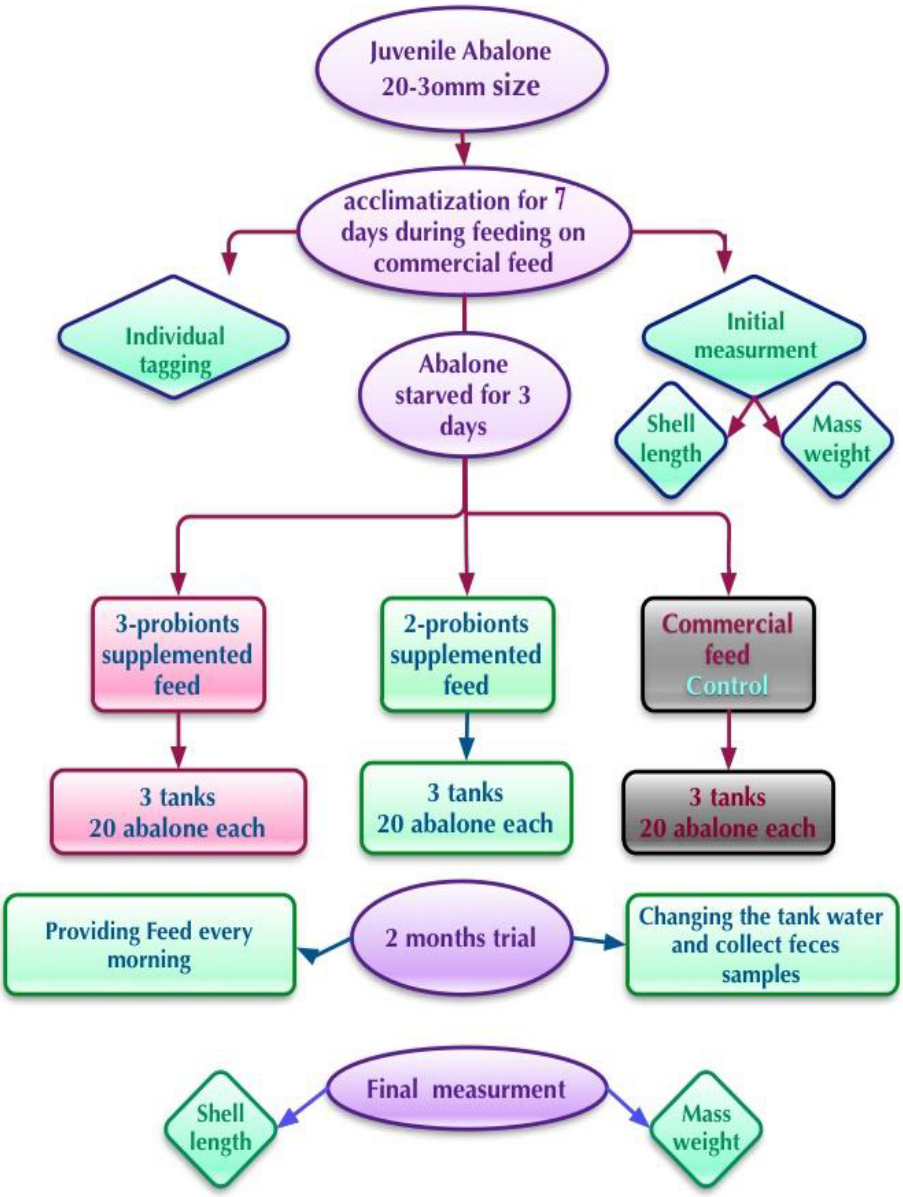

Figure 5-5 Protocol of feeding trial

Cleaning the tanks daily from uneaten feed pellet and faeces was done before applying fresh feed. Samples of feces had been collected for the last ten days of the trial. The relatively long period is referred to the nature of the feces which is slimy with thin consistency. Biochemical analysis of protein, lipid, carbohydrates, moisture, and ash required at least 2-3g of faeces to be collected, **Fig. 5-6**.

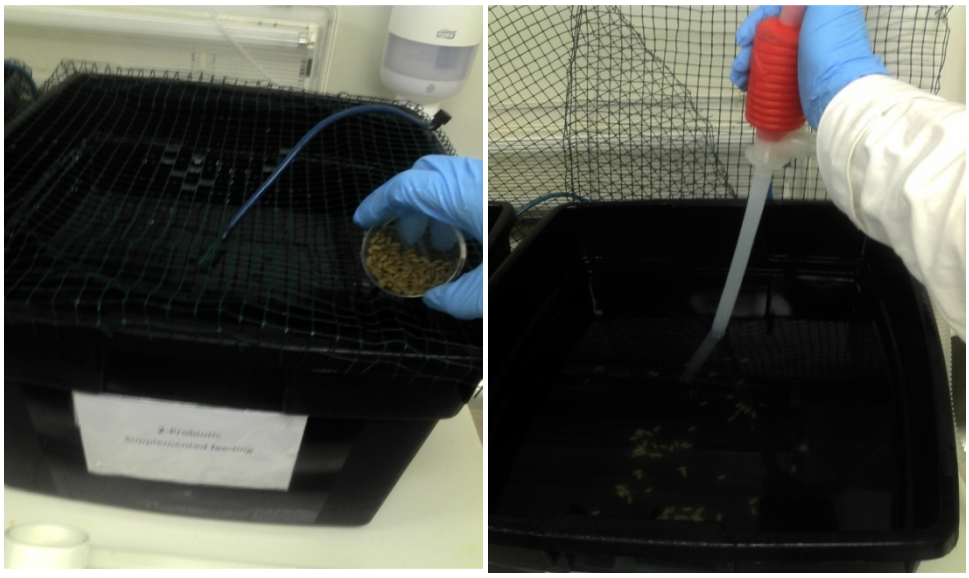

Figure 5-6 Application of feed to juvenile abalone (left), and collecting abalone faeces samples (right)

Tanks were vigorously aerated by using two air stones for each tank to optimize high level of dissolved oxygen close to 100%. Tank water was checked regularly for salinity, which should be (32-34 ppth), oxygen (90-100%), pH (7.9- 8.3), ammonia NH_3 (0.5mg/L), and Nitrite NO_2 around 5 mg /L [Dr. Rodney Roberts, (2011), (personal communications)].

In addition, bacterial viable counts were performed using Plate Count Agar (DIFCO) supplemented with (2% NaCl), for each of the water tanks every week to monitor probiotics concentrations.

A 50% seawater water exchange was performed daily (Irasema et al., 2011) for two days, and the whole amount of water was exchanged at the third day. While changing the whole amount of water, cleaning the tank was performed by wiping walls and tank bottom with tissue paper to remove the buildup of bacterial biofilm without using harmful chemicals that might affect the abalone health.

Dead individuals of abalone were removed immediately from the tank and replaced with other animals from the stock animals throughout the trial. The new

individuals were not included with the measurements of changes in growth. They were kept in the tanks to maintain the same number of abalone in all tanks.

At the end of the trial, mortality percentage was calculated and comparison between treatment groups was evaluated.

5.2.8.1 Biochemical Analysis

Samples of water from the bottom of each tank were placed in glass bakers, and then the faecal materials were separated from un-eaten feed pellets by collecting the upper layer of water. The collected samples were centrifuged and were dehydrated in an oven (80-100^{o}C) for 1 hr. The dried samples were grinded using a mortar and pestle to obtain fine powder. Analyses of total protein, lipid, and carbohydrate were conducted in AUT chemistry laboratory.

5.2.8.2 Protein analysis

Total protein for the abalone feed and faecal matter samples were estimated with the Kjeldahl method for total nitrogen (AOAC, 2005). The Kjeldahl method assumes that each gram of protein contains (0.16g) of nitrogen, which is equivalent to the factor 6.25. Consequently, total protein can be obtained by multiplying the nitrogen concentration by 6.25.

The method includes three main processes:

1) Digestion of the samples was conducted using sulphuric acid which converts protein to ammonia. Approximately 0.5g of each sample was placed in a 300ml Kjeldahl digestion tube. Then, 10ml of concentrated sulphuric acid (98%) was added. Tubes were then inserted into a digestion oven (420^{o}C). After about 45-60 minutes, hydrogen peroxide was added. Decomposition of samples was completed when the solutions became colorless compared to the initially dark color. Then, 50ml of deionized water were added.
2) Distillation with 35% sodium hydroxide to liberate ammonia from the mixture was performed, followed by distillation using a semi-automatic distillation unit, (UDK 139, VELP SCIENTIFICA). Digested materials were distilled with 25ml Boric acid

solution (4%). The final solutions were collected in 250ml Erlenmeyer flasks. Then, ten drops of green methyl indicator were added.

Titration with 0.1mol hydrochloric acid was used to quantify the amount of ammonia in the solution. Titration was stopped when the solution turned red. The results were recorded for calculations. Blank tubes were treated in the same manner as described above, but without the protein samples. The percent of total protein was determinate using the equation below:

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

5.2.8.3 Lipid analysis

A modified Bligh and Dyer (1959) extraction method was used to determine total lipid in feed and abalone faeces. Three replicates were prepared by blending the dried faeces samples and feed pellet using a blender. Approximately 0.5g of each powder sample was weighed using an analytical balance, capable of weighing to 0.0001g. Then, the samples were put into 15 ml centrifuge tubes and rehydrated with 2 ml deionized water. Three (3) ml of a mixture of two solvents (methanol and chloroform) at 2:1 ratio were added into the tubes with the samples and shaken to obtain a well homogenized mixture.

Additional chloroform (1 ml) was added to the tubes and mixed by shaking for about 10 minutes and then centrifuge for 10 minutes at 2000 rpm. Two phases of water and chloroform containing lipids were shown separated by the sample protein disc. Due to the high density of the chloroform, it was observed as yellow lower phase.

The lower chloroform layer was collected using a Pasteur pipette and transferred into clean glass tubes, which have been pre weighed. The tubes with the samples were reweighed after collecting chloroform mixture.

Tubes that contained the chloroform layer were exposed to the nitrogen gas flow for a few minutes to evaporate the chloroform to dryness and leaving the lipid

layer on the tubes wall. The tubes were reweighed to determine the weight of the lipid extract alone. The percent of total lipids was determinate using the equation below:

$$\text{Total lipid \%} = \frac{\text{Lipid extract (g)}}{\text{Lipid in chloroform solvent (g)}} \times 100$$

5.2.8.4 Carbohydrate analysis

According to (Porter &Earl, 1990), total contents of carbohydrates in both feed and faecal matter were estimated by difference. The sum of percent protein, lipid, ash, and moisture was subtracted from 100 to calculate the total percent of carbohydrates as in the equation:

Total estimated carbohydrates % = 100 - [%protein +% lipid +% moisture + %ash]

5.2.8.5 Moisture analysis

The moisture content of the samples was determined by taking the loss in weight (%) after oven drying at (100^{o}C) for 3 hours (De Knegt & Brink, 1998), according to the equation described by (McClements, 2003) as follows:

$$\text{Total moisture \%} = \frac{\text{M (initial)} - \text{M (dried)}}{\text{M (initial)}} \times 100$$

5.2.8.6 Ash analysis

A modified (AOAC, 1970) method was used to determine the ash content in the feed , and faecal matter samples. The samples were heated in a furnace at 520°C for approximately 2 hr. All samples with three replicates were weighed as: Weight of porcelain crucible and about 0.5g of dried sample and Weight of crucible with the sample after burning (ashing). Then, samples were cooled to room temperature in a dessicator. Percentage of ash was determined using the equation below:

$$\text{Total ash \%} = \frac{\text{M (ash)}}{\text{M (dried sample)}} \times 100$$

5.2.9 Statistical analyses

Collected data for biochemical analysis, growth changes (biomass and shell length), and mortality rate were analyzed using an SPSS statistics program (IBM SPSS version 20). One-way analyses of variances (ANOVA), and Tukey's *post hoc* tests at *p*-value = 0.05 were conducted to determine the differences between the means of the three groups of study (control, 2-probiotics, and 3-probiotics).

Percentage of growth improvement was calculated as follows:

Improvement in shell length % = (*P* - C) */* C X 100

Improvement in biomass % = (*P* - C) */* C X 100 (P; probiotic diet, C; control diet)

5.3 Results

5.3.1 Application of probiotic- supplemented feed

Preliminary trials, for testing three options of probiotic applications to the feed (Section 5.2.4), led to selecting the third one which was application of the bacterial cultures to the feed pellet directly. The feed pellets were easy to reach for the abalone and were of the same shape that they are used to in the farm. Further, fecal samples for biochemical analyses were easily collected and distinguished from the feed pellets. Checking the viable count in both feed (CFU/g) and rearing water of tanks (CFU/ml), **Table 5-1** below. The viable counts in the feeds and in the rearing water are shown in Table 5.7.

Results indicate that high probiont concentration was initially present in the feed before administration to the animals. The viable counts in the rearing water samples indicated that the probionts were present in viable condition. The microorganisms present in the control rearing water could be due to contamination from aerosols produced during aeration of the tanks positioned close to each other.

Diet	Viable count (average) in feed CFU/g	Viable count (average) in rearing water CFU/ml
Control	–	$1.5 – 2.0 \times 10^{2}$
2-Probiotics	$2.4– 2.5 \times 10^{8}$	$2.0– 2.8 \times 10^{4}$
3-Probiotics	$3.2– 3.5 \times 10^{9}$	$3. 0– 3.5 \times 10^{5}$

Table 5-1 Bacterial populations as viable count in feed and water samples

5.3.2 Growth trial

5.3.2.1 Changes in shell length

Increases in total shell length (mm) and daily shell length (µm) in response to supplementation of (AbMax 16) with probiotic bacteria are shown in **Fig. 5-7**. After 60 days of feeding, the greatest increase in shell length *H. Iris* was observed on juveniles fed with (AbMax) feed supplemented with a conglomerate of 3 probiotics, the 3-P treatment (4.03 ± 0.13).
The 2-probiotic-supplemented feed (2-P treatment) similarly produced an increase in the shell length of abalone to (3.84 ± 0.8). Hence, the 3-P treatment and 2-P treatment resulted in shell length gain of 20.9% and 15.4%, respectively. These results were found to be a significant improvement in shell length over those obtained from unsupplemented feed (3.33 ± 0.14), (p<0.05).

The two probiotic treatments resulted in significant daily increase in shell length greater than that obtained from the unsupplemented feed (p<0.05). However, there was no significant difference between the effect of 3-P treatment and that of 2-P treatment on the shell length.

Although quantitative measurement of the colour intensity of the shells was not carried out, apparent brighter blue colour was observed on the shells of abalone fed with either 2-P or 3-P feed than those with unsupplemented feed. **Fig. 5-8** shows a crude evidence of the apparent colour of the shells.

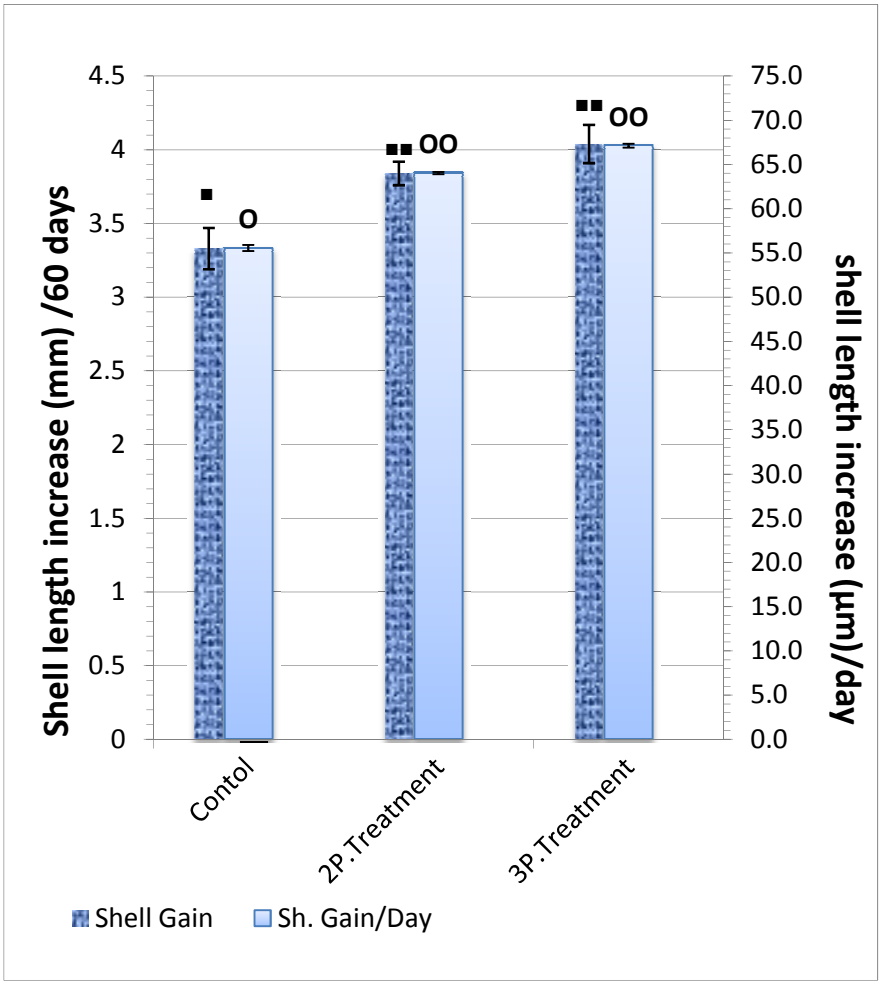

Figure 5-7 Shell length increase and daily shell length increase, different numbers of (■) and (**O**) indicate a significant difference (ANOVA, Tukey post-hoc, $p < 0.05$)

Diet (mm)	Shell length gain (µm)	(g)	Shell length/day (mg)	Weight gain	Weight gain/day	Mortality %
Control	3.33 ±0.14		55.57 ±2.41	0.36 ± 0.25	5.98 ± 0.4	16.67± 3.33
2-Probiotics	3.84 ± 0.8		64.05± 1.29	0.39 ± 0.03	6.55 ± 0.06	3.33 ±0.33
3-Probiotics	4.03 ± 0.13		67.14 ± 2.16	0.43 ± 0.01	7.12 ± 0.15	3.33 ± 0.66

Table 5-2 Growth data and mortality for three groups; means ± SE differences (95% Confidence Interval)

Figure 5-8 Improvement of shell coloring associated with probiotic diet; (Left) 2, 3-probiotic group of abalone, (right) control abalone group

5.3.2.2 Changes in weight

The trend of weight change was similar to the shell length. Results show that the use of probiotic-supplemented feed increased the wet weight in abalone.
H. iris fed with Abfeed supplemented with 3-P exhibited a significant increase in wet weight after 60 days of feeding trial, **Fig. 5-9** ($p<0.05$) compared to those grown on unsupplemented feed.

The 3-P feeding resulted in wet weight gain (g) of 0.43 ± 0.04. This meant a significant increase in total weight equivalent to 19.8% ($p<0.05$) compared with the control. The 2-P treatment produced higher wet weight gain equivalent to 9.47% than did the unsupplemented feed. However, there was no significant increase based on the total wet weight after 60 days.

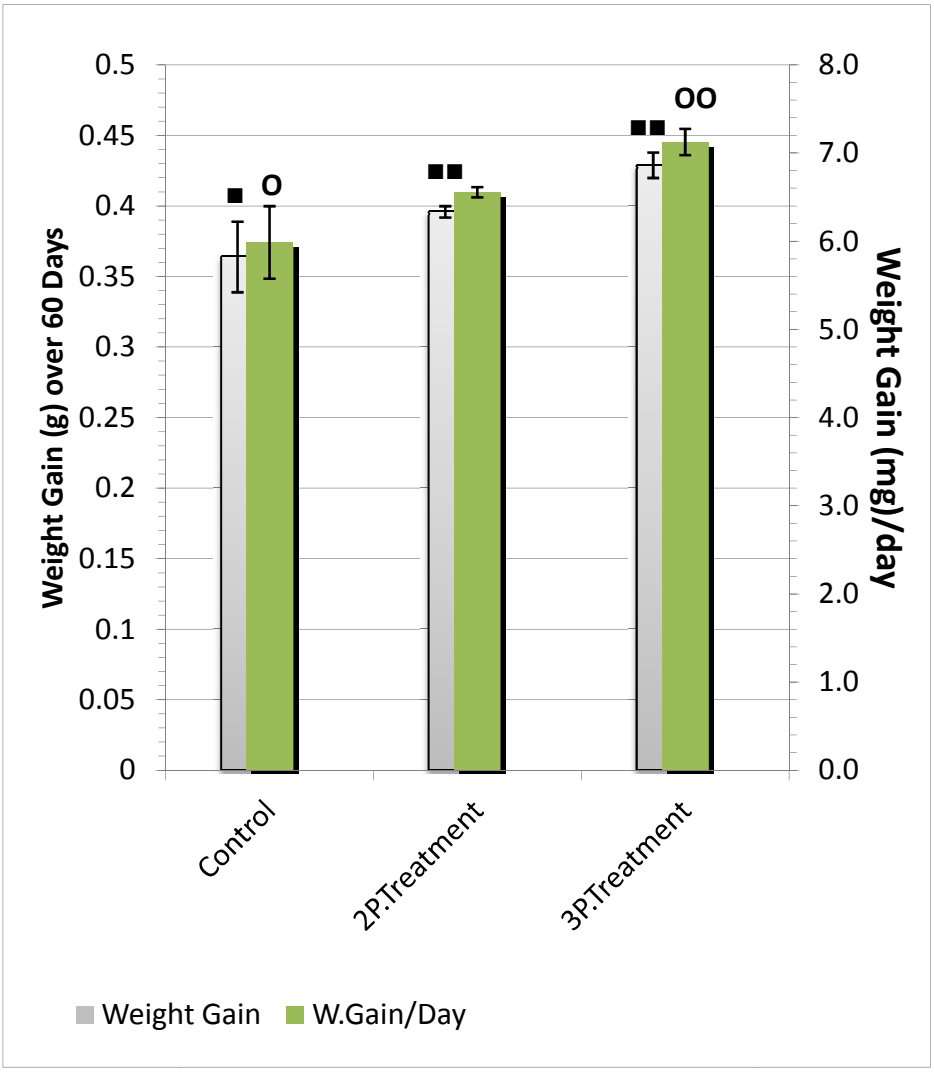

Figure 5-9 Weight gain and daily weight gain, Different numbers of (■) and (O) indicate a significant difference (ANOVA, Tukey post-hoc, $p < 0.05$)

5.3.3 Mortality

The effects on mortality are shown in **Fig. 5-10** & **Table 5-2.** Mortality rates observed in three replicate tank populations of juvenile abalone fed with the two probiotic regimes (2-P and 3-P) were significantly lower than that in the un-supplemented populations (p<0.05). The low mortality rates obtained from 2-P and 3-P treatments were very similar 3.33% ± 0.33 and 3.33% ± 0.66 respectively, compared with the control 16.67% ± 3.33.

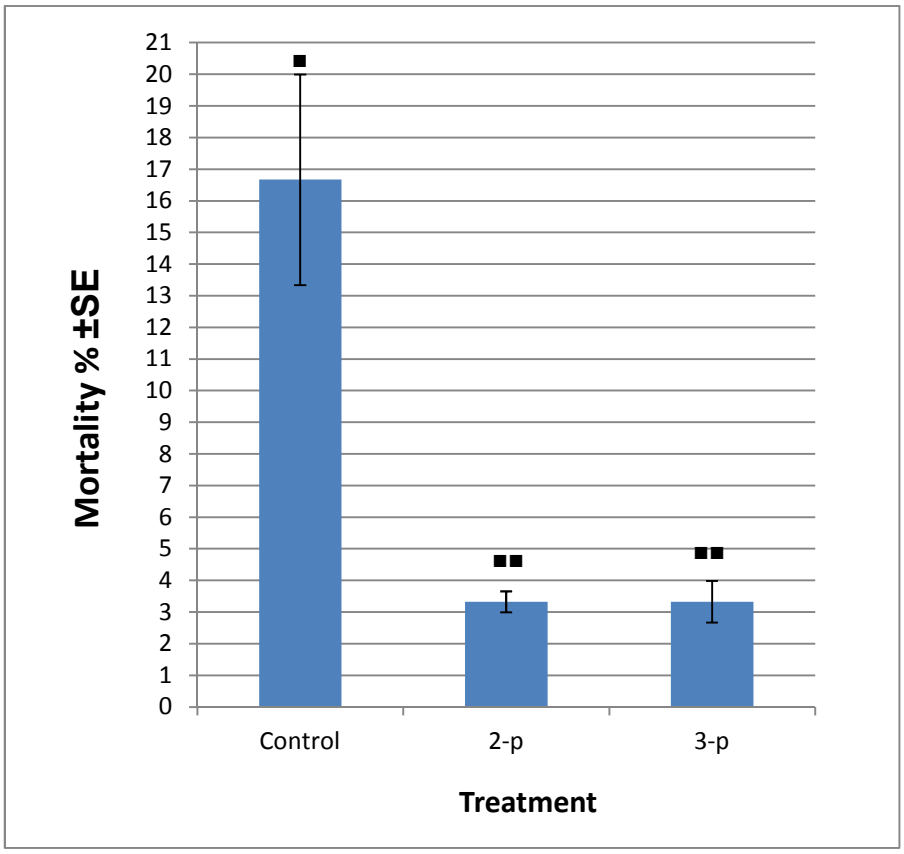

Figure 5-10 Mortality rate percentages for growth trial, Different numbers of (■) indicate a significant difference (ANOVA, Tukey post-hoc, p< 0.05)

5.3.4 Compositions of Growth Trial Samples

Faecal samples taken in the last 10 days of feeding trial showed that there was an apparent decrease in crude protein, crude lipid, and crude carbohydrate from the two probiotic groups (2-P and 3-P) compared to those from the control group (**Fig. 5-11; Table 5-3)**. Further, there was no statistical difference between the faecal components in the 2-P and 3-P treatments. Interestingly, though, the protein level in the faeces produced from both 2-P and 3-P (9.01 ± 0.25 and 7.96 ± 0.26, respectively) were significantly lower than those from unsupplemented feed 11.42 ± 0.83, (p>0.05).

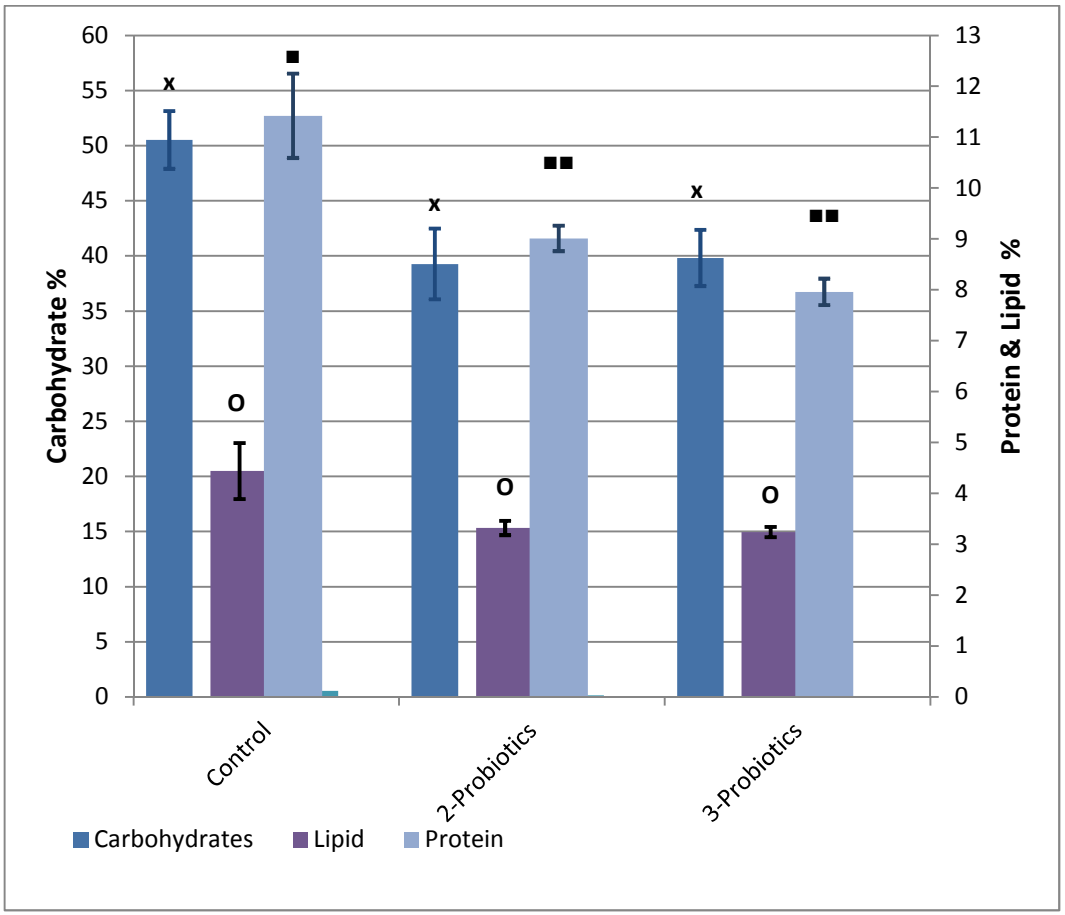

Figure 5-11 Abalone faeces compositions: Crude carbohydrate, Crude protein, and Crude lipid different numbers of (o), (**x**) or (■) indicate a significant difference (ANOVA, Tukey post-hoc, p< 0.05)

Diet	Protein%	Lipid %	Carbohydrate%
Control	11.42 ± 0.83	4.44 ± 0.55	50.52 ± 2.62
2-Probiotics	9.01 ± 0.25	3.32 ± 0.14	39.26 ± 3.21
3-Probiotics	7.96 ± 0.26	3.24 ± 0.1	39.81 ± 2.55
Feed	33.59 ± 0.89	4.47 ± 1.3	42.93 ± 4.46

Table 5-3 Compositions Percentage of faecal matter and commercial feed

5.4 Discussion

A 60-day growth experiment was conducted to identify the optimum probiotic feed developed using a combination of potential probiotic bacteria for the juveniles of *H. iris* (paua).

Results have shown that an increase in growth rate was possible with the supplementation of feed with live, beneficial microorganisms. The highest growth improvement in terms of weight and shell length (19.8% and 20.9%, respectively, at $p<0.05$) was obtained from feeding with 3-P supplemented feed. The 3-P conglomerate consisted of 3 putative probiotic bacteria *Exiguobacterium* JHEb1, *Vibrio* JH1, and *Enterococcus* JHLDc. A lower but significant increase in shell length was observed in juvenile abalone fed with 2-P supplemented feed which contained *Exiguobacterium* JHEb1 and *Vibrio* JH1. There was no significant weight gain obtained with the 2-P supplemented feed suggesting that the more metabolically diverse the probiotic bacteria used, the better the growth performance of abalone. A similar feeding trial in another abalone species, *H. midae* was carried out for a longer period of 163 days (ten Doeschate & Coyne, 2008). In their study, a single probiont was used, *Pseudoalteromonas* strain C4 that was alginolytic and appropriate for the kelp cakes used. After 163 days, strain C4 increased shell length *in H. midae* by 38% and that of weight by 39%. Using 60 days as a basis of comparison, the C4 strain would have produced only a 14% increase in shell length. Results of the present study for the same sized

abalone were higher than those obtained with C4 strain. The 3-P conglomerate produced a 21% increase in shell length, while the 2-P conglomerate produced a 15.4% shell length increase.

Growth improvements obtained in this study are similarly higher than those obtained by Macey & Coyne (2005) in similar sized *H. midae* fed with probiotic feed also consisting of three but different types of microorganisms. *H. midae* fed with a combination of bacteria (SY9) and 2 yeasts strains (SS1 and AY1) exhibited a lower improvement in shell length and weight (7% and 8%, respectively). Macey & Coyne (2005) probiotic combination consisted of proteolytic *Vibrio* and amylolytic yeasts. It can speculated that the lower growth performance in *H. midae* was due to a lower level of digestive enzymes contributed by their selected probionts than those of the 3-P probionts in this study.

The LAB probiont included in the 3-P treatment, *Enterococcus* JHLDc, would have also assisted in the metabolism of carbohydrate by production of lactic acid. El- Shanshoury et al. (1994) and Iehata et al. (2010) suggested that addition of lactic acid bacteria *Pediococcus* spp. as probionts to commercial feed of abalone can alter the gut microflora and increase volatile short chain fatty acids (VSCFAs) and alginate hydrolysis enzymes. Joborn et al. (1997) reported that (LAB) can colonize the gut and improve growth and survival rate in fish and crustaceans. *Enterococcus* JHLDc was selected since it was the highest lactic acid-producing isolate.

Vibrio spp. were used widely as probiotics, for their ability ferment sucrose (Gomez-Gil et al., 2000). Iehata et al. (2010) reported that the dominant species in the GIT of abalone *Haliotis discus hannai* were the non-motile fermenter *Vibrio halioticoli* that could increase the digestive efficiency of abalone. The major role of *Vibrio* spp. could be the provision of additional polysaccharolytic enzymes. In this study, *Vibrio* JH1 was included in two probiotic diets (2 and 3-probiotics) for its efficient alginate hydrolysis, good proteolytic activity, strong polysaccharolytic activity on starch and a ‘bonus’ property of sucrose fermentation (Chapter 3).

Exiguobacterium JHEb1 was included in both probiotic diets for its high proteolytic, amylolytic, and lipolytic properties. *Exiguobacterium* spp. were used in aquaculture to enhance the growth and survival rate of fish and shrimps when used in single or mixed strains treatment (Orozco-Medina et al., 2009).
This study is the first to report the application of this genus to enhance the growth of abalone. This study has provided evidence that *Exiguobacterium* could be a safe probiotic that could be used in abalone rearing environment.

Statistical analysis for faecal protein composition showed that both probiotic feeds were significantly more digestible than the unsupplemented feed. These results support the assumption that the use of probiotic-supplemented feed can help the abalone derive more benefit from the feed more than from the unsupplemented feed. The nutrient digestibility suggested by the faecal composition values obtained in this study is markedly different than those obtained by Macey & Coyne (2005). They reported that the difference between the probiotic group and the control group was not statistically different despite the % protein remaining in the faeces following digestion being higher (28.7%) in *H. midae* fed the basal diet compared to those fed the probiotic diet (20.5%). Macey & Coyne (2005) stated that the digestion and absorption of protein in the GIT of animals fed the probiotic feed was significantly different ($p<0.05$) compared to the control, yet that was not shown in the analysis of faecal protein.

The significant decrease in faecal protein in this study could be attributed to the inclusion of highly proteolytic bacteria in both 2-P and 3-P probiotic conglomerates, *Exiguobacterium* JHEb1 and *Vibrio* JH1. These isolates demonstrated high extracellular proteolytic activity (128.25 IU/ml and 62.4 IU/ml, respectively (Section 4.4.1).

The biochemical analysis for lipid showed no significant difference between the effect of 2-P and 3-P diets. This may be explained by the lack of proteolytic isolates in the probiotic feeds. Only *Exiguobacterium* JHEb1 had lipid-degrading ability according to its phenotypic properties (**Table 3-12**). It should be noted that lipid degradation was not included in the main criteria of probiotic selection.

Both probiotic diets included two highly amylolytic bacteria (*Exiguobacterium* JHEb1 and *Vibrio* JH1). However the percentages of carbohydrate remaining in faeces were not significantly different from the control group. The explanation for this is not certain but this could be due to enzyme regulation such as catabolite repression or substrate inhibition that occurs in each isolate.

The improvement in survival rate (lower mortality rate) observed with the probiotic treatments may be explained by the effect of the bacteria in enhancing the immune system of abalone. Probiotics have been shown to be capable of improving immunity in abalone (Macey & Coyne, 2005; ten Doeschate & Coyne, 2008). Similar increase in survival was observed in brine shrimp *Artemia* administered with *Exiguobacterium* during feeding (Sombatjinda et al., 2011).

Chang & Liu (2002) obtained similar survival rate increase with the use of the LAB *Enterococcus* spp.

Interestingly, an improvement in iridescence of *H. iris* shell color has been perceived (although, not quantified) in abalone fed probiotic supplemented feed compared to control animals. This finding can be related to availability of the components of feed in probiotic diet which are essential to build healthy colourful shell. Similar to findings of Nash (1991) who indicated that shell colour of abalone can be affected by the diet and its components

The increase in shell length and weight of *H. iris* fed probiotic feeds in this has demonstrated the strong potential of 3 probiotic isolates in improving growth of farmed *H. iris* through, most probably, enhancement of the pool of extracellular enzymes such as proteases, amylases, and alginolytic enzymes.

Chapter 6

Conclusions and Recommendations

6.1 Conclusions

The use of probiotics in aquaculture is becoming increasingly popular for a number of reasons. Prevention of infectious diseases and improvement of nutrition are two main objectives of probiotic application. The work presented in this thesis was concerned with improving the growth of New Zealand abalone (paua) *Haliotis iris,* by using probiotic-supplemented feed.

One of the objectives of the study was to isolate bacterial strains from the microflora in the gut of healthy adult abalone (paua) and to evaluate these strains according to well documented criteria for probiotic microorganisms. The potential probiotic bacteria were included in the feed as a multi- strain-supplementation for juvenile paua.

The application of probiotics was aimed at increasing the growth rate of abalone to reduce the cultivation time required to obtain their commercial size. Hence, the isolation and screening of potential probiotic bacteria used criteria or requirements geared towards improvement of the nutritional status of abalone.

These selection criteria involved a) proteolytic ability, b) amylolytic ability, c) alginolytic ability, d) bile tolerance, e) high acid tolerance, and f) acid production. These criteria were designed to obtain probiotic bacteria that could aid the digestion of nutrients present in the artificial feed, AbMax 16, consisting of different carbohydrate sources (e.g. wheat flour, soy flour, tapioca starch, sugar, carrageenan, seaweed) and protein sources (e.g. fishmeal, wheat gluten, yeast).

Enzyme assays were conducted not only to detect presence of particular enzymes but also to quantitatively assess which isolates exhibited the greatest proteolytic activity, amylolytic activity, alginolytic activity.

Findings of Chapter three on isolation and identification were nine isolates that were considered potential probiotic bacteria. These were classified and identified as:

- Gram-positive isolates*:* *Bacillus* JHBa2, *Exiguobacterium* JHEb1, and *Enterococcus* JHLDc.

- Gram-negative: isolates *Vibrio* JH1, *Vibrio* JH2, *Vibrio* JH4, *Vibrio* JH5, *Shewanella* JH3, and *Cobetia (or Halomonas)* JHC.

After evaluation of bacteria by enzyme activity, screening in Chapter four pointed to three most acceptable isolates to use in feeding trial. These were:

- *Exiguobacterium* JHEb1: proteolytic, amylolytic, lipid degrading, bile salts and acid tolerant.
- *Vibrio* JH1: proteolytic, amylolytic, fast alginolytic, and high bile salts and acid tolerant.
- *Enterococcus* JHLDc: high acid producer (LAB) with high bile salts and acid tolerance.

Using these results of the intensive screening for probiotic bacteria, this study designed two novel conglomerates of probiotic bacteria that were supplemented to abalone feed. The two probiotic treatments consisted of:

- a conglomerate of 2 probionts, 2-P, (*Exiguobacterium* JHEb and *Vibrio* JH1)
- a conglomerate of 3 probionts, 3-P, (*Exiguobacterium* JHEb, *Vibrio* JH1, and *Enterococcus* JHLDc)

This study has shown that the two novel probiotic treatments improved growth of *H. iris* juveniles in pilot scale by increasing shell length gain, wet weight gain, and reducing mortality rate. These results confirm growth improvement due to probiotic treatment obtained with the South African abalone, *H. midae*. However, improvement in *H. midae* growth resulted from administration of probiotics consisting of a *Vibrio* sp. and two yeast species (Macey & Coyne, 2005).

Hence, the present study reports for the first time a significant improvement ($p < 0.05$) in the growth rate of *H. iris* by using feed supplemented with three bacterial species. This is also the first time *Exiguobacterium* sp. was used as a probiont in abalone.

Results suggest that by incorporating the probiotic bacteria with specific enzymatic abilities, they add to the pool of digestive enzymes in abalone leading to improvement in digestion and absorption of nutrients. This was demonstrated significantly ($p< 0.05$) in the lower crude protein in the faecal matter present in abalone fed with 2-P, and 3-P diets compared to those fed with unsupplemented diet.

Low levels of crude carbohydrate in faecal matter were also observed in two of the probiotic diets for abalone. However, the results were not significantly different from the control ($p>0.05$). The possible explanation for insignificant carbohydrate uptake might be related to catabolite repression of enzymes, which is known to occur when more than one substrate is present for metabolism. Since in the feed there were several carbohydrate sources (wheat flour, soy flour, tapioca starch, sugar, carrageenan, seaweed), the phenomenon of catabolite repression of bacterial enzymes could have occurred.

The bacterial enzymes and their optimal requirements for activity, such as pH and temperature, were not covered in this study, and more studies are needed to investigate whether or not these are fulfilled in the abalone digestive tract.

Low levels of crude lipid were observed in faecal matter of the two probiotics diets of abalone compared with the control animals, yet the differences were not significant ($p>0.05$). A possible reason for this may relate to the use of the only one host-derived isolate that can produce lipolytic enzyme (*Exiguobacterium* JHEb), which was not sufficient to degrade the lipid composition in the commercial feed.

Extrapolating the increase in growth rate of *H. iris* fed with feed supplemented with probiotics (based on shell length increase over 2 months) juveniles (20mm) could reach the commercial size (80mm) in 29.8 and 31.2 months, when fed on 3-probiotics and 2-probiotics diet respectively, compared with control abalone which need 36.0 months. This would result in high productivity and lower cost of production. These results were obtained from laboratory scale experiments; hence, it is essential to conduct farm growth trials to make projections more accurate.

Further, it would be useful to determine the effect of the probiotic-supplemented feed on both post-larvae and larger abalone.

Mortality observed in three replicates tanks populations of juvenile abalone, fed with the two probiotic regimes (2-P and 3-P) was significantly (p<0.05) lower than that in the un-supplemented (control) populations of abalone. The low mortality can be related to the immune-modulation as possible mechanisms of probiotic microorganisms.

6.2 Significance of the Study

6.2.1 Commercial benefit of the research

Abalone is considered to be a highly valued mollusk delicacy and fetches a high price at the market. Because of their high market value the prospect of aquaculture of abalone continues to be extremely attractive. New Zealand mainly exports paua to China, Japan, USA, Canada, and England. In order to continue to stay competitive, New Zealand must continue to deliver good quality products. Abalone alone is a $62 million /p.a. industry and it is therefore critical to investigate methods to increase the growth of abalone which will result in greater profits.

Abalone aquaculture continues to expand in New Zealand, and the demand for high quality and effective feed for abalone in different stages of cultivation is increasing. Therefore, the present study could help the farm to reach greater potentials by using more effective feed containing probiotic microorganisms.

New Zealand aquaculture operations such as OceaNZ Blue are committed to naturally processed paua of the highest quality (PAUA, 2011). Consequently it is preferable not to use any chemicals or unnatural substances into the feeding, breeding, and protection against diseases. Therefore utilizing probiotic organisms as feeding additives to enhance growth and health is meeting the overall requirements of aquaculture in the country.

6.2.2 Scientific benefit of the research

This study is based on the use of the addition of friendly bacteria (nonpathogenic) like probiotic bacteria to the feed. This is so that the abalone can reach its commercial size in less time by increasing the rate of its growth.

An increasing concern worldwide for the high use of antibiotics in aquaculture has initiated research into less harmful alternative methods of disease control. The use of probiotics can improve resistance against infectious diseases and most importantly may be to avoid severe ecological damages that might caused by use of antimicrobial treatment.

The study was testing the selected potential probiotics for their probiotic criteria, as growth enhancer and approved that three of them to be considered as safe probiotic bacteria to use in aquaculture animals in future. Furthermore the research was employed the genera *Exiguobacterium* for the first time as a probiotic in abalone aquaculture, for there are some studies were utilized this strain in fish and shrimp farms, but not in abalone farms.

6.3 Future Studies and Recommendations

1) Identification of bacterial strains in this study was to the genus level, for comparison with other studies it would be more reliable to be identified to the species level, especially with the most used probiotics like *Vibrio* spp. and *Enterococcus* spp. which already used in this work. For example *Vibrio* JH1in present study was: non-motile Gram-negative, fermentative, and alginolytic strain was showing similarity to *Vibrio halioticoli*, which found in abalone *Haliotis discus hannai* (Sawabe & Sugimura, 1998); (Tanaka & Sugimura, 2003), and to *Vibrio alginolyticus* (Gomez-Gil & Roque, 2000).
2) The use of two and three probiotic bacteria as combination added to the feed would not clarify the role of each individual isolate. It is similar to a team work, but if the feeding trial involved extra tanks for abalone that would be fed the same isolates separately added to the feed, it would give clear confirmation about their role in any changes in growth.

3) In the growth trial, a static water system was employed. However for better results for growth improvement in abalone, a circulating water system is highly recommended. In the abalone farm, circulation (or recirculation) system was shown to give higher growth rates than in the flow-through system in all the time. Usually, the farmed abalone growth rate should be 2mm per month; studies based on the site showed 50% growth increase in recirculation system compared to those in the flow-through system.
4) Tanks that were used in the growth trial were rectangular shaped with 90^{o} corners. From experience, abalone prefer to climb up and cluster in the corners away from the bottom of the tank. Being away from the tank bottom where the feed is placed made it hard for some abalone to reach the feed especially, when they have to creep over each other. Utilizing round tanks with no corners would increase the chances for abalone to be closer to the feed.
5) Future work could involve a histology study for the abalone gastro-intestinal tract epithelia, to study the attachment of probiotic bacteria and the time that is required for each isolate to colonize the epithelia of the gut. Colonization and attachment of probiotic bacteria to the gut epithelium can be correlated with the days of application of probiotic-supplemented feed. It is worth trying if the study involves more than one probiotic to compare which bacteria can colonize the gut in a shorter time and how long they can remain colonized after stopping the probiotic feeding.
6) In this study, protease, amylase, and alginolytic enzymes were studied each as one kind of enzyme during enzyme assay. Nevertheless many bacteria can produce a number of extracellular proteases, and amylases. Therefore, studying each type of enzyme that is produced by an isolate can help with selection of ideal probionts. Isolates that produce more than one protease, for example, are more likely to be selected in feeding trial.
7) Studying enzyme activity in present work was (*in vitro)* for future work, studying of feed -degrading enzymes like protease inside the gut of abalone (*in vivo)* fed probiotic diet, and correlation of the amount of protease with an increase of digested and absorbed protein, could explain the role of such enzyme in growth improvement of the abalone.

8) Evaluation of the ability of selected probiotic bacteria for their ability to challenge pathogenic microorganisms can be great value to aquaculture animals including abalone. A few paua farms experienced disease outbreaks in the summers of 1999/2000 and 2000/2001, and high rate of mortalities of 82.5 and 90% of juvenile paua were reported in one commercial aquaculture operation (Diggles & Oliver, 2005). The use of probiotic strains of bacteria like *Exiguobacterium* spp. that have been approved to produce antibiotics; e.g. bacitracin, gramicidin S, polymyxin, and tyrotricidin, could added to the aquaculture water. Those antimicrobial substances were reported to be active against wide range of bacteria (Sombatjinda et al., 2011).
9) *Exiguobacterium* JHEb1 isolate has shown remarkable ability to produce hydrolytic enzymes for protein, carbohydrate, and lipid. Thus it would be worthy of further investigation, for the possibility of commercial use as probiotic additive to the feed of aquatic and terrestrial animals for growth and survival rate enhancement.

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APPENDICES

APPENDIX 1

Measurements of abalone (Mean) in each tank of three diets:

Abalone Tank	Initial Weight g	Final Weight g	Weight Gain g	Weight Gain/day mg	Initial Shell L. mm	Final Shell L. mm	Shell L. Gain mm	Shell L. gain /Day µm
Control 1	2.06	2.44	0.39	6.4	24.30	27.83	3.53	58.8
Control 2	2.01	2.39	0.38	6.3	24.55	27.97	3.42	57.0
Control 3	2.03	2.34	0.31	5.2	24.44	27.49	3.05	50.8
2-Prob. 1	2.08	2.48	0.40	6.7	24.18	28.18	4.00	66.6
2-Prob. 2	1.99	2.39	0.39	6.5	23.88	27.67	3.79	63.2
2-Prob. 3	2.02	2.40	0.39	6.5	24.84	28.58	3.74	62.4
3-Prob.1	2.07	2.51	0.45	7.4	24.58	28.78	4.20	70.0
3-Prob. 2	1.95	2.37	0.42	7.1	24.01	28.11	4.11	68.5
3-Prob.3	1.94	2.35	0.42	6.9	23.83	27.60	3.78	62.9

APPENDIX 2

Statistical Analysis for Shell Length Changes

Descriptives

Shell length changes

Diet	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1 (Control)	3	3.33	.249	.143	2.71	3.95	3.05	3.53
2 (2-P)	3	3.84	.134	.077	3.50	4.17	3.74	4.00
3 (3-P)	3	4.02	.222	.128	3.47	4.57	3.78	4.20
Total	9	3.73	.359	.119	3.45	4.00	3.05	4.20

ANOVA

Shell length changes

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		.777	2	.388	8.991	.016
	Linear Term	Contrast	.722	1	.722	16.723	.006
		Deviation	.054	1	.054	1.260	.305
Within Groups			.259	6	.043		
Total			1.036	8			

Multiple Comparisons(Tukey HSD)

Dependent Variable: **Shell length changes**

Diet	(Diet	Mean Difference (I-J)	Std. Error	**Sig.**	95% Confidence Interval	
					Lower Bound	Upper Bound
1 (Control)	**2 (2-P)**	-.51	.169	**.053**	-1.0326	.0087
	3 (3-P)	-.69	.169	**.015**	-1.2146	-.1733

*. The mean difference is significant at the 0.05 level.

APPENDIX 3

Statistical Analysis for Weight Changes

Descriptives

Weight Gain

Diet	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1(Control)	3	.359	.043	.025	.25	.47	.31	.39
2 (2-P)	3	.393	.006	.003	.38	.41	.39	.40
3 (3-P)	3	.430	.017	.010	.39	.47	.42	.45
Total	9	.394	.038	.013	.36	.42	.31	.45

ANOVA

Weight Gain

			Sum of Squares	df	Mean Square	F	**Sig.**
Between Groups	(Combined)		.007	2	.004	5.198	**.049**
	Linear Term	Contrast	.007	1	.007	10.391	**.018**
		Deviation	.000	1	.000	.005	**.946**
Within Groups			.004	6	.001		
Total			.012	8			

Multiple Comparisons Tukey HSD

Dependent Variable: **Weight Gain**

(I) Diet	(J) Diet	Mean Difference (I-J)	Std. Error	**Sig.**	95% Confidence Interval	
					Lower Bound	Upper Bound
1(control)	**2 (2-P)**	-.0340	.0219	**.335**	-.1013	.0333
	3 (3-P)	-.0706*	.0219	**.041**	-.1379	-.0034

*. The mean difference is significant at the 0.05 level.

APPENDIX 4

Statistical Analysis for Mortality

Descriptives

Mortality

Diet	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1(control)	3	16.67	5.77	3.33	2.32	31.0	10.00	20.00
2 (2-P)	3	3.33	2.89	1.67	-3.83	10.5	.00	5.00
3 (3-P)	3	3.33	5.77	3.33	-11.00	17.67	.00	10.00
Total	9	7.78	7.94	2.65	1.66	13.88	.00	20.00

Multiple Comparisons Tukey HSD

Dependent Variable: Mortality

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	**Sig.**	95% Confidence Interval	
					Lower Bound	Upper Bound
1(control)	**2 (2-P)**	13.33	4.08	**.039**	.8072	25.8595
	3 (3-P)	13.33	4.08	**.039**	.8072	25.8595

*. The mean difference is significant at the 0.05 level.

APPENDIX 5

Components of feed and faeces samples

Sample	**protein %**	**Lipid %**	**Carbohydrates %**	**Moisture%**	**Ash %**
C1	13.07	3.38	47.36	15.29	20.9
C2	10.44	4.72	48.48	15.06	21.30
C3	10.74	5.23	55.73	11.12	17.18
2P1	8.53	3.57	34.1	27.94	26.27
2P2	9.11	3.09	38.54	29.91	21.0
2P3	9.39	3.30	45.16	20.76	22.48
3P1	8.12	3.19	42.71	24.37	21.2
3P2	7.46	3.42	42.01	21.75	23.71
3P3	8.30	3.11	34.73	22.33	30.44
Feed 1	33.49	5.08	41.69	12.54	7.20
Feed 2	33.30	4.23	44.99	9.93	7.55
Feed 3	33.99	4.12	42.12	10.97	8.80