THE USE OF PROBIOTICS IN THE DIET OF FARMED SOUTH AFRICAN ABALONE *Haliotis midae* L

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ABSTRACT

Physiological stress in farmed abalone can lead to immunosuppression and increase the susceptibility to bacterial, viral and parasitic disease, often followed by mortality. Thus, handling and poor water quality can reduce farm production efficiency. Probiotics in aquaculture have been effective in a wide range of species in enhancing immunity, survival, improving feed utilisation and growth. Three putative probionts identified as a result of *in vitro* screening had been beneficial to laboratory-reared abalone in a previous study.

The aim of this study was to produce an abalone feed that contains a suite of probionts that may promote abalone growth and health under farming conditions. The objectives were to compare growth and physiological responses (i.e., haemocyte and phagocytosis counts) of abalone fed a commercial feed (Abfeed®S 34, Marifeed, Hermanus) supplemented with probiotics (i.e., the probiotic diet) to abalone fed the commercial feed without probiotic supplementation as a control treatment in a factorial design with handling method as an independent variable. This experiment was conducted at HIK Abalone Farm (Pty Ltd) for a period of eight months with initial weight and length 36.1 ± 0.05 g and 58.6 ± 0.06 mm abalone^{-1}. Another experiment was carried out at Roman Bay Sea Farm (Pty) Ltd with initial weight and length 34.7 ± 0.17 g and 62.3 ± 0.18 mm abalone^{-1}, but this experiment included one factor only, i.e. the presence and absence of the probionts in the feed.

At HIK there was no significant interaction between diet and handling on average length and weight gain month^{-1} after four ($p=0.81$ and $p=0.32$) and eight ($p=0.51$ and $p=0.53$) months, respectively. Average length (additional handling = 73.9 ± 0.52 mm, normal farm handling = 75.8 ± 0.57 mm) and weight gain (mean: additional handling = 68.5 ± 1.20 g, normal farm handling = 74.3 ± 1.86 g) increased significantly in animals that were handled under normal farm procedure and were either fed probiotic or control diet after eight months ($p=0.03$ and $p=0.02$, respectively). There was no

difference in length gain or weight gain of abalone fed the probiotic diet and those fed the control diet (ANOVA: $F_{(1,16)}$=0.04, p=0.84; $F_{(1,16)}$=0.14, p=0.71, respectively). After four months phagocytotic count was significantly different between dietary treatments with mean values of 74.50 ± 10.52 and 63.52 ± 14.52 % phagocytosis count per sample for the probionts and control treatment, respectively (p=0.04), there was no difference after eight months at HIK Abalone Farm. There was no effect of stressor application (p=0.14) and no interaction between dietary treatment and stressor application for this variable i.e., phagocytosis count (p=0.61). There was no difference in feed conversion ratio between treatments with values ranging from 2.9 to 3.8.

At Roman Bay Sea farm, there was no significant difference in mean length gain between abalone fed the probiotic and control diet after eight months (repeated measures ANOVA: $F_{(4,28)}$=16.54, p<0.00001). Mean weight gain of abalone fed the probiotic diet was significantly greater than those fed the control diet after eight months (repeated measures ANOVA: $F_{(4,28)}$=39.82, p<0.00001). There was no significant difference in haemocyte counts between animals fed either probiotic or control diet after four and eight months at Roman Bay Sea farm (p>0.05).

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CHAPTER 1

GENERAL INTRODUCTION

Abalone (*Haliotis midae*) is an important commercially farmed gastropod species in South African aquaculture (Britz 1995) and it is farmed in intensive land-based, pump ashore, flow-through facilities (Yearsley 2008). The development of the industry has been stimulated by research and development and by a good market demand for abalone, both live and processed (Sales and Britz 2001). Abalone farming is always at risk due to outbreaks of transmittable diseases (Mialhe *et al*. 1995). In Taiwan, outbreaks of abalone herpesvirus (AbHV) resulted in high mortalities of farmed abalone *Haliotis diversicolor supertexta* (Chang *et al*. 2005). Mortalities due to a virus described as *Haliotis* herpesvirus 1 (AbHV-1) have also occurred in farmed blacklip abalone *Haliotis rubra*, greenlip abalone *Haliotis laevigata*, and to wild abalone stock populations in Victoria coast Australia during December 2005 and January 2006 (Corbeil *et al*. 2012). Symptoms of tubercle mycosis were identified in South African abalone farms in 2006, where a great loss of production was encountered with up to 90% mortality in smaller animals and 30% mortality in adult animals (Greeff *et al*. 2012). In large-scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to diseases and the deterioration of environmental conditions result in economic losses (Balcázar *et al*. 2006).

Antibiotics have been traditionally used to control bacterial disease outbreaks in aquaculture (Defoirdt *et al.* 2007). The use of these drugs for the prevention and control of disease has increased substantially in recent decades (Balcázar *et al*. 2006). Extensive use of a wide range of antimicrobials for controlling diseases in aquaculture has been considered the only option for treatment of diseases to the majority of farmers (Gram *et al*. 2001). Excessive antimicrobial use has lead to the emergence of

bacterial resistance (Verschuere *et al*. 2000). However, "the use of antibiotics in aquaculture also constitutes a threat to human health and to the environment" (Alderman and Hastings 1998; Cabello 2006). In addition, residues of antibiotics in aquaculture products can lead to human health problems and can exacerbate problems of allergy and toxicity by altering gut microflora (Cabello 2006). In many cases, bacteria of the genus *Vibrio* are opportunists, causing disease when the host organism is physiologically stressed, with the frequency of infection often being attributable to environmental conditions (Alderman and Hastings 1998).

Stress has been defined by Barton (1997) as the "response of an organism to any demand placed on it such that it causes an extension of a physiological state beyond its normal resting state to the point that the chances of survival may be reduced". Several studies have demonstrated that stress response alters disease resistance and survival in abalone (Malham *et al*. 2003). In a culture environment, abalone are constantly subjected to a wide range of stressors, which include repeated mechanical disturbances such as sorting, grading and transport. There is a relationship between the magnitude of the stress response and disease, which has been associated with disease outbreaks in abalone and in many other animals (Hooper *et al*. 2007). In abalone the stress response and decreased immune function capacity can lead to bacterial infections and mortality (Cheng *et al*. 2004a). However, this link is based on immune function tests carried out after applying stressors such as salinity fluctuations, handling through shaking, decreased dissolved oxygen concentration, increased concentration of ammonia and increased temperature (Hooper *et al*. 2007).

Aquaculture husbandry processes may induce physiological stress which can inhibit growth in farmed animals by suppressing appetite and feed intake (Schreck *et al*. 1997; McCormick *et al*. 1998). In order to mitigate the mortality associated with infectious diseases in farmed abalone, a better understanding of the abalone's response to bacterial or viral infection is necessary. It has been shown that modification of the gastrointestinal tract of aquatic animals is possible through assimilation of beneficial microorganisms. Therefore, their utilisation has been proposed to form a feasible tool to

eradicate the presence of opportunistic pathogens (Balcázar 2002). In aquatic animals, the intestinal microbiota is assumed to be formed by an autochthonous microbiota together with an unnaturally high level of allochthonous microorganisms from the surrounding environment (Hansen and Olafsen 1989). *Vibrio* and *Pseudomonas* species, which are gram-negative anaerobic bacteria, form the predominant indigenous microbiota of many salt water fish (Onarheim *et al*. 1994). Comparatively, abalone have a relatively long intestine with many folds and grooves which may provide space for microbial colonisation (Harris *et al*. 1998).

The contributions made by probiotics in promoting health of host organisms should be studied considering that the need for alternatives to antibiotics is increasing. Controlling gut microbial balance of abalone by providing probionts may be a potentially important factor (Erasmus *et al*. 1996). Due to an increasing demand for environmentally friendly aquaculture, investment into research and development into the utilisation of probiotics for disease prevention and improved nutrition in aquaculture has increased. Ever since their initial application, several studies have shown that probiotics controlled potential pathogens, increased growth rates and health of captive aquatic organisms (Carnevali *et al*. 2004; Wang *et al*. 2005). As a result, numerous probiotics that are available commercially have been utilised in molluscan, shrimp and fish farming diets or added to the pond water (Wang *et al*. 2005). Probiotics have been defined by Gatesoupe (1999) 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". Possible modes of action for probiotics include (i) production of inhibitory compounds, (ii) competition for nutrients, (iii) competition for adhesion sites in the gastrointestinal tract, (iv) enhancement of the immune response and (v) production of essential nutrients such as vitamins and fatty acids, and enzymatic contribution to digestion (Verschuere *et al*. 2000; Vine *et al*. 2006). Viability of probiotic bacteria, i.e., cell count and survival vary depending on the strain and manufacturer (Schillinger 1999). Large numbers of viable cells have been recommended in probiotics for their high efficacy (Gatesoupe 1999). Doeschate and Coyne (2008) suggested that an increased

growth rate of abalone may be achieved through a number of mechanisms: (1) increasing nutrients available to the abalone for absorption in the gut, (2) increasing the pool of digestive enzymes in the abalone gut, and (3) use of the beneficial bacteria as an additional nutrient source. Most probiotics tested for use in aquaculture have been preparations of gram-negative bacteria such as *Vibrio* (Macey and Coyne 2005*,* 2006) and *Pseudomonas* (Gram *et al*. 1999), gram-positive bacteria such as *Bacillus* (Wang *et al*. 2005) and lactic acid bacteria; yeasts such as *Saccharomyces* (Lara-Flores *et al*. 2003) and *Debaryomyces* (Macey and Coyne 2005) and microalgae such as *Tetraselmis* (Makridis *et al*. 2006).

The most important limitation to the use of probiotics is that in many cases they are not able to maintain themselves in the intestine, and so need to be added regularly (Vine *et al*. 2006). Probiotics selected *in vitro* on the basis of their capability to produce pathogen-restricting compounds may not necessarily produce these compounds *in vivo* (Verschuere *et al*. 2000; Vine *et al*. 2006). To increase the chance of success, selection of probiotics with more than one antagonistic character is advantageous, or a probiont mix coupled with different modes of action may be chosen (Defoirdt *et al*. 2007). It has been shown that three probiotics (*Vibrio midae* SY9, *Cryptococcus* sp. SS1, and *Debaryomyces hansenii* AY1) can colonise the gastrointestinal tract and enhance enzyme activity of abalone *H. midae* (Macey and Coyne 2006). Macey and Coyne (2005) also showed that *H. midae* fed with a diet supplemented with these three probionts had elevated protease and amylase activities in the intestine and stomach, and showed improved growth and survival. Macey and Coyne (2005) tested two diets; the commercially used Abfeed® diet and the same diet supplemented with a mixture of the three putative probionts (SS1, SY9 and AY1).

Vine *et al*. (2009) developed a suite of probiotics that were beneficial to laboratory-reared abalone. From a pool of 200 microorganisms, which were isolated from the gut of cultured abalone (*H. midae*), three bacterial species were suggested to be suitable candidates. These bacterial isolates were selected based on their ability to utilise various carbohydrate and protein sources available in Abfeed® diets

(i.e., alginate, agar, carbocymethycellulose, laminarin, soya, maize and rice flour) and for their ability to inhibit pathogens such as (*Aeromonas salmonicida* and *Vibrio alginolyticus*) known to thrive under aquaculture conditions. Out of 100 bacteria isolates, eight showed evidence of antagonism towards pathogens. The best fifty strains that showed beneficial enzyme activity and antagonism to pathogens were further checked for antagonism towards each other and none of the isolates exhibited such an effect. This suggested that these isolates can grow symbiotically in the abalone gut and offer combined benefits to the host.

Long-term viability of probiont storage was determined and it was above 5 x 10^8 cells g^{-1} of Abfeed® after 28 days (Vine *et al*. 2009). The immunostimulatory effect of diet combinations with bacterial isolates and immunostimulants was, however, tested in the latter study; these additives were included into Abfeed® S34 (Marifeed Pty Ltd, South Africa) and were fed to abalone for 14 days in a multifactorial experiment. Haemolymph was obtained from abalone fed the diets and examined for total haemocyte count, phagocytosis rates and respiratory burst activity (nitroblue tetrazolium reduction assay). Higher phagocytosis counts were attained in animals fed a mixture of three probionts and 0.1% Spirulina suggesting an immune system stimulating effect (Vine *et al*. 2009).

Silva-Aciares *et al*. (2011) found that *Haliotis rufescens* fed a natural diet composed of fronds of fresh macroalgae *Macrocystis integrifolia* supplemented with a mixture of three bacterial strains (*Vibrio sp.* C21-UMA, *Vibrio sp.* F15-UMA and *Agarivorans albus* F1-UMA) average monthly growth rate and survival of these animals increased over a 210-day period. The study comprised two experiments on recently weaned abalone with an initial average size of 19 ± 0.5 mm and on adult abalone of an initial size of 36 ± 0.4 mm. Three tanks with abalone were fed macroalgae without a probiotic supplement and the other three were fed macroalgae colonised with three probiotic bacteria. Monthly length and weight gain of both recently weaned and adult *Haliotis rufescens* fed probiotic supplemented microalgae improved. Moriarty (1998) found similar results when the addition of a mixture of

Bacillus strains, that had been selected for the production of antibiotics against luminescent *Vibrio,* resulted in healthier prawns and lower numbers of luminescent *Vibrio* in the pond water.

Immune response in molluscs is controlled by cytotoxicity, chemotaxis, phagocytosis and cell motility (Ottaviani 2004). Haemocytes are the main defence cells of molluscs and are capable of chemotaxis, antigen recognition, attachment followed by agglutination, phagocytosis, and elimination of invaders by respiratory burst or exocytosis of antimicrobial factors (Adema *et al*. 1991). They are the central cell type of the immune response. Transportation of nutrients and digestion, shell restoration and waste excretion are other physiological functions of molluscan haemocytes (Sahaphong *et al*. 2001).

Phagocytotic activity can be recorded by measuring the proportion of ingested particles or the proportion of cells that have ingested labelled particles (Chang *et al*. 2000; Malham *et al*. 2003). In molluscs, phagocytosis is considered the primary line of cellular defence and agranular haemocytes (hyalinocytes) and granular haemocytes (granulocytes) are considered to be two distinct cell types (Bachére *et al*. 1995).

They play a role in phagocytosis which is an essential process to eliminate pathogens (Bayne 1990).

The aim of this study was to contribute to the development of abalone feeds with probiotics that may promote abalone health and to demonstrate the advantages or disadvantages of using probionts under commercial farming conditions. Growth trials were conducted on two abalone farms to investigate the effect of a probiotic diet on abalone growth using the probionts developed by Vine *et al*. (2009) (Chapter 2), as these probiotics had not been tested under farming conditions. To determine the health status of abalone, total haemocyte count and phagocytotic activity were examined at the end of each growth trial (Chapter 3).

CHAPTER 2

THE EFFECT OF DIETARY SUPPLEMENTATION WITH PROBIONTS AND HANDLING STRESS ON GROWTH OF ABALONE (*Haliotis midae*) UNDER FARM CONDITIONS

2.1 Introduction

In a farm environment, abalone are frequently subjected to a wide range of stressors which include handling, a practice that frequently happens during stock movement (Mgaya and Mercer 1995). Size grading or splitting, which is done to reduce the density of stock in the baskets, and transport are farm-related procedures used for the movement of stock. Size grading is a normal practice in commercial shellfish farming (Wilson 1981), based on the knowledge that abalone (*Haliotis midae*) growth rate of fast growers improves by culling slow and intermediate growers under farm conditions (Pieterse 2010). Depending on the production system and culture techniques, size-sorting is done by eye using animals of a mass below 50 g abalone^{-1}, while grading involves weighing each animal on abalone greater than 50 g. Abalone are passed through holes of varying sizes so that abalone of similar sizes are stocked together (Hooper *et al*. 2011). During the grow-out phase, baskets with abalone are handled every 7-10 days when tanks are being cleaned. Baskets are frequently moved from one tank to another, usually over a distance of less than 10 m. Animals are also handled every three to four months for splitting, i.e., size-grading (Naylor M., HIK Abalone Farm Pty Ltd, pers. comm.). Trolleys are normally used to transport farm baskets from grow-out tanks to splitting or grading stations. Hooper *et al*. (2011) have shown that the process of stock movement is stressful to abalone.

Physiological stress is one of the fundamental factors that can contribute to diseases and increased mortality in aquaculture (Rollo *et al*. 2006; Ige 2013). Stress response has a complex relationship with

disease and has been implicated in reduced growth, immunosuppression and susceptibility to disease outbreaks in farmed abalone (Cheng *et al*. 2004a, b, c; Hooper *et al*. 2007, 2011; Travers *et al*. 2008b; Wassnig *et al*. 2009). "Stress response is the mechanism by which animals try to maintain homeostasis when exposed to physical or biological changes as a result of natural or anthropogenic perturbations" (Malham *et al*. 2003). However, stress response changes from adaptive to nonadaptive behavioural patterns (Barton and Iwama 1991), subsequently leading to a decline in disease resistance, impaired reproduction and reduction in growth. This could also alter the composition of intestinal microflora, which reduces the number of beneficial microorganisms providing an opportunity for invasion of opportunistic and potentially pathogenic bacteria in the gut, considered as the main cause of mortality in the majority of fish hatcheries (Ringo 2004; Rollo *et al*. 2006; Ige 2013).

The gastrointestinal tract is habitat to a complicated and dynamic ecosystem of microflora and the composition varies between individuals, time and the position within the tract (Ige 2013). The indigenous microbiota has numerous benefits on health and survival of the host. The major role of microflora in the gut entails breaking down dietary compounds, induce nutrient partitioning and lipid metabolism, providing necessary nutrients introduced as a result of microbial metabolism, protection against pathogen invasion and gut morphology stimulation (Mulder *et al*. 2009). Additionally, the indigenous flora acts as a natural barrier against gut pathogens by preventing their colonisation in the gut, a priority step of pathogenicity (Rollo *et al*. 2006). The intestinal microbiota does not survive as a single entity, it co-exists with the environment through constant interaction and activities of the host (El-Haroun *et al*. 2006). However, a balanced intestinal microflora is paramount for the health of an organism (Rollo *et al*. 2006). There is a growing interest in finding complimentary additives that will promote health and growth effects of farmed aquatic organisms. Manipulation of the gut microflora with probiotics may be another viable alternative that can be incorporated into a diet to increase the capacity of health promoting or beneficial bacteria in the gut.

Probiotic use as farm animal feed additives dates back to the 1970's (Sayed *et al*. 2011). The use of probiotics to control potential pathogens has since gained wide acceptance in the aquaculture industry (Gomez-Gill *et al*. 2000). An accepted definition of probiotics for aquaculture application is "a live, dead or component of a microbial cell that when administered via the feed or to the rearing water benefits the host by improving either disease resistance, health status, growth performance, feed utilisation, stress response or general vigour, which is achieved at least in part via improving the hosts microbial balance or the microbial balance of the ambient environment" (Merrifield *et al*. 2010a). Probiotics are also referred to as "bio-proteins containing living microbial cells that optimize the colonisation and composition of the growth and gut microflora in animals and stimulate digestive processes and immunity" (Dhanaraj *et al*. 2010). The capability of microorganisms to attach to the intestinal mucosa is regarded as significantly vital when intended for use as probiotics as this has a bearing on the health benefit associated with the probiotics (El-Haroun *et al*. 2006; Collado *et al*. 2007; Vendrell *et al*. 2008).

Most notable probiotic effects targeted in aquaculture involve enhancement of survival in larval stages, inhibition of pathogen growth, immunological improvement, growth improvement and advancement of stress tolerance (Gatesoupe 1999; Balcázar *et al*. 2006; Merrifield *et al*. 2010a; Nayak 2010; Dimitroglou *et al*. 2011). Their fundamental effects in fish are to increase feed efficiency and / or daily weight or length gain (Sayed *et al*. 2011). Probiotics may influence appetite, enrich nutrition through production of vitamins, detoxify harmful compounds in the diet and break down non-digestible constituents (Abd El-rhman *et al*. 2009). Probiotics in fish might detoxicate unrealized virulent compounds through degradation of possibly nondigestible constituents in the diets by hydrolytic enzymes functioning as amylase and protease (Ige 2013). Metabolism of the microbial ecosystem may also be transformed by probiotics in the digestive tract to maximise production of short chain fatty acids, by increasing sodium and water intake as well as reduced colonic activity (Sakata *et al*. 1999).

The aim of incorporating probiotics in the diet is to stimulate growth of certain bacterial strains to the detriment of less desirable ones (McDonald *et al*. 2002). The benefits acquired through the use of probiotics by the fish farmer or consumer include increased feed uptake, improved growth, improvement of carcass and meat quality, utilisation of feed and a reduction of deformities (Ige 2013). Under intensive abalone farming the existence of stress in animals is common. Therefore, a diet which includes probiotics is thought to promote the capability of abalone to cope with stressful conditions. In this study abalone were subjected to a simulated stress which is commonly experienced in a culture environment. The aim was to investigate the effect of three candidate probionts developed by Vine *et al*. (2009) on abalone growth and survival under farm conditions. The objectives were to compare growth of abalone fed three probiotics added to a commercial abalone feed to a treatment where abalone were fed the commercial feed only.

2.2 Material and Methods

2.2.1 Experimental animals

The abalone (*H. midae*) used in the study and the holding facilities were made available by two commercial abalone farms on the west coast of South Africa. These were HIK Abalone Farm (Pty) Ltd and Roman Bay Sea Farm (Pty) Ltd. The experiments at each facility used abalone from that respective facility only. The approximate age of the animals was 26 months for both farms.

HIK Abalone Farm (Pty) Ltd

The initial mean length and wet weight of abalone were 58.6 ± 0.06 mm and 36.1 ± 0.05 g abalone^{-1} (mean ± standard error, SE; n = 1566). These animals had been spawned by different females in a single batch, i.e., they were from one spawning. They were maintained in five canvas tanks each with a volume of 4.1 m^3 each holding 12 oyster mesh baskets under farm conditions with aerated and continuously flowing natural seawater at temperatures between 14 and 20 °C, pH 7.12-8.29, DO 7.14-

9.80 (mg/l) at an exchange rate of 2.5 exchanges h^{-1}. At the start of the experiment each basket was stocked according to farm practice, with 7.0 kg $basket^{-1}$ of abalone in the first four months and 7.40 kg $basket^{-1}$ in the remaining four months.

Roman Bay Sea Farm (Pty) Ltd

Initial length and wet weight of abalone were 62.3 ± 0.18 mm and 34.7 ± 0.17 g $abalone^{-1}$ (n = 600). These animals were from one batch, i.e. they were spawned from males and females during one spawning. They were maintained in flow-through aerated sea water in two 4.0 m^3 concrete tanks holding 12 baskets per tank at temperatures between 19 and 24 °C, pH 7.30-8.30, DO 6.41-8.05 (mg/l). The water volume of the tanks was exchanged

at a rate of 2.7 exchanges h^{-1}. At the start of the experiment, average biomass per basket was 8.73 kg, i.e.,

approximately 268 abalone per basket. At the start of the second four-month growth period, basket biomass averaged 10.20 kg with approximately 196 abalone $basket^{-1}$. At the start of each growth trial, all animals in the baskets were graded before the first data were collected and very large and very small abalone were removed in order to reduce size variation. During the acclimation period of two weeks, abalone were fed the locally produced commercial feed Abfeed® S34 (Marifeed Pty Ltd).

Probiotic bacteria

Candidate probionts were isolated from abalone gut and were checked for probiotic activity as described previously (Vine *et al*. 2009). The probiotic cultures were grown individually in Tryptic Soy Broth (TSB), they were adjusted to approximately 1 x 10^9 cells ml^{-1} in 20 ppt NaCl solution at 25°C. Optical density for each probiont was determined at 600 nm to enable correct dilution of the cultures to the diets.

2.2.2 Diet preparation

Two diets were manufactured according to the proprietary commercial formulation at Marifeed (Pty) Ltd. A commercial feed (Abfeed® S34, Marifeed Pty Ltd, Hermanus, South Africa) was used as the control diet, with 34 % total protein and fishmeal and soya meal comprising the main protein sources. The probiotic-supplemented diet used the same commercial feed but with the probionts included during the manufacturing process. The probiotic diet included 5 x 10^8 g^{-1} of Abfeed® for each probiont for the first 120 days, after which inclusion was doubled for each probiont. To ensure even distribution of probionts in the feed, they were mixed together with the volume of water needed to make 10 kg of feed. Cold water was used to ensure survival of bacteria in the feed during the manufacturing process. Probiotic feed was made using a cold extrusion method. To maintain probiotic viability, new batches of feed were manufactured every four weeks. Dry feed pellets were cut to approximately 10 mm x 10 mm x 1.2 mm thick and stored in covered plastic containers until needed within a temperature range of 5 – 35 $^{\circ}$C to ensure the survival of bacteria and their viability. Post-pelleting viability trials had been conducted by Vine *et al*. (2009), who showed that feed can be manufactured and stored for up to four weeks without compromising probiotic viability. The trials by Vine *et al*. (2009) showed that viability of probionts at ambient temperature 5 – 35 $^{\circ}$C was still higher than 5 x 10^8 cells g Abfeed®$^{-1}$ after 28 days. Food samples were taken after 7 and 28 days to determine long-term viability.

2.2.3 HIK Abalone Farm: Determining the combined effect of probiotic diet and handling stress on abalone growth

The experiment was designed to test two independent variables in a factorial design. The first variable was the use of probionts in the abalone diet, i.e., one treatment was fed a probiont-enriched diet and a control treatment with abalone fed the same diet but without the probionts. The second variable was handling stress. To test this, treatments were exposed to either additional handling or the control

equivalents were not exposed to additional handling. In the context of this thesis, additional handling refers to the normal farm handling activities which include transport, size-grading, size sorting and splitting which was simulated in the growth trial. Thus, the experiment included a 2 x 2 factorial design. Each of the four treatment combinations were randomly allocated to one of 20 baskets of abalone, distributed among five tanks so that each treatment was represented once in each tank, i.e. five independent replicated baskets per treatment. The simulation of handling represented the normal farm handling of abalone during stock movement. Animals subject to conditions similar to those under normal farm conditions were removed from the tank and placed into a clean tank. They were out of the water for approximately seven seconds. Abalone were handled during day time by first closing the inflow and air diffusers to avoid sludge activation at the bottom of the tank. Abalone exposed to additional handling stress were subjected to the same procedure, only their baskets were lifted out of the tank with a winch for five minutes and shaken for one minute before returning them. Immediately after this, these baskets were moved to a clean tank. This was applied twice every week for the duration of the growth trial i.e., with either two or three days between handling events each week. Baskets were randomly moved to different positions in their respective tank once a week after tank cleaning.

2.2.4 Roman Bay Sea Farm: To determine the effect of probiotic diet on abalone growth

One tank holding 12 randomly placed baskets was used for the experiment. There were two treatments, each with six replicated baskets of abalone. These treatments were randomly assigned to the baskets in the tank. In this experiment, abalone growth was compared between animals fed Abfeed® S34 and probiotics (treatment; probiotic diet) to abalone fed Abfeed® S34 (control).

2.2.5 Feeding

Abalone were reared according to farm procedures. At HIK Abalone Farm (Pty) Ltd, feeding was performed restrictedly using a conical cup with 64 g of feed to each basket per day. At Roman Bay Sea farm (Pty Ltd), there were six baskets/treatment. The feed was given by hand. Food weights were recorded at the start and at the end of the experiments for each diet fed and each basket. Abalone were fed daily at 16h00. Uneaten food was removed every morning.

2.2.6 Growth

Prior to each growth period all animals in a basket were size-graded. The next grading was done after 120 days and the experiment continued with the same experimental animals maintained at similar stocking densities. At the beginning and at the end of each experiment, 50 animals from each basket were weighed (g) and measured (mm). Individual abalone weight was recorded to the nearest 0.01 g using an electronic balance (Snowrex BBA-600, Snowrex International, Taipei, Taiwan, R.O.C) and shell length was measured to the nearest 0.1 mm from photographs using computer software (SIGMA SCAN PRO 5, Systat Software Inc., San Jose, California). To obtain the photographs, immediately after recording the weight of each abalone, the animal was placed onto a laminated A3 size paper with grid lines and a photograph of 10 animals at a time on the grid was taken using a camera positioned vertically above the paper. Vernier callipers were included in the image as reference showing shell length measurement of one randomly selected animal from the experiment and they were used for calibrating photographs on the computer software. All length measurements of abalone were taken along the long shell axis. Change in the abalone condition was determined according to Britz (1996) using the equation:

$$\text{Condition factor} = (\text{weight}/\text{length})^{2.99} \times 5575 \quad (1)$$

where weight is individual abalone mass in g and length is in mm. Feed conversion ratio (FCR) was calculated as:

$$\text{FCR} = \text{dry feed consumed/wet weight gain.} \quad (2)$$

Specific growth rate (*SGR*) of abalone was compared between treatments using the equation:

$$SGR = ((\ln(W_f) - \ln(W_i)) / t)100 \quad (3)$$

where *SGR* is the specific growth rate (% body weight d^{-1}), ln(*Wf*) is the log of the mean final weight of abalone, ln(*Wi*) is the log of the mean initial weight of abalone, and *t* is the number of days. Water quality from the header tank, including pH, temperature and dissolved oxygen concentration were measured everyday between 08h00 and 09h00. Portable electronic meters were used to measure dissolved oxygen (YSI Model #55D, USA) and water temperature and pH (YSIModel#60/10FT, Yellow Springs, USA).

2.2.7 Statistical analysis

Data were checked for normality of residuals and assumptions for equality of variance using the Shapiro-Wilk test (Shapiro and Wilk 1965) and Levene's test (Levene 1960), respectively. Weight and length data from the abalone of each basket were averaged and this value was used for the statistical analysis. Thus, the basket was considered as the experimental unit, at HIK there were five tanks that contained each treatment group per basket randomly distributed in each tank. At Roman Bay there was one experimental tank containing 12 baskets (i.e., six probiotic and six control). Multifactor analysis of variance (ANOVA) was used to determine if there was a significant interaction between the factors "diet" and "handling" for mean weight gain; if there was no interaction, means of each factor were then compared separately, at an error level of 5% ($p < 0.05$). Repeated Measures ANOVA was used to compare weight and length values over time between treatments at Roman Bay Sea Farm (Pty). The analysis was performed using a computer software package (STATISTICA V7.0, StatSoft, Inc., Tulsa, OK, 1984-2004). All values are expressed as mean ± standard error unless stated otherwise.

2.3 Results

At HIK Abalone Farm, there was no significant interaction between dietary treatment (probiotic or control) and handling on average length and weight gain month^{-1} after both four and eight months (multifactorial ANOVA: $F_{(1,36)}$=0.06, p=0.81; $F_{(1,14)}$=1.05, p=0.32; Fig. 2.3.1); (multifactorial ANOVA: $F_{(1,34)}$=0.45, p=0.50; $F_{(1,34)}$=0.39, p=0.53), respectively. There was no significant difference in average length and weight of abalone either subjected to additional handling or normal farm handling after four months (ANOVA: $F_{(1,36)}$=0.94, p=0.34; $F_{(1,36)}$=0.87, p=0.36, respectively, Table 2.1). Average length and weight were significantly reduced in handled abalone after eight months when compared to abalone that were not subjected to additional handling (ANOVA: $F_{(1,16)}$=5.93, p=0.03; $F_{(1,16)}$=6.12, p=0.02, Fig. 2.3.2). There was no significant effect of probiotic diet on abalone length or weight gain month^{-1} after four months (ANOVA: $F_{(1,36)}$=0.38, p=0.54; $F_{(1,36)}$=0.14, p=0.71, Table 2.2), and no effect on abalone length or weight gain month^{-1} after eight months (ANOVA: $F_{(1,14)}$=0.02, p=0.96; $F_{(1,14)}$=0.07, p=0.78, Table 2.2). There was no significant difference in feed conversion ratio between dietary treatments in the first four months and between four and eight months (ANOVA: $F_{(1,18)}$=0.09, p=0.76; $F_{(1,18)}$=2.85, p=0.11 respectively, Table 2.2). There was no significant difference in condition factor between dietary treatments after four and between four and eight months (ANOVA: $F_{(1,18)}$=0.02, p=0.89; $F_{(1,18)}$=0.01, p=0.98, respectively, Table 2.2). Length and weight values between treatments were significantly different only after four months (ANOVA: $F_{(3,16)}$=9.42, p=0.01; $F_{(3,16)}$=4.08, p=0.02), and there was no longer a significant difference after eight months. There was no significant difference in SGR after four and between four and eight months (ANOVA: $F_{(1,18)}$=1.13, p=0.30; $F_{(1,18)}$=0.04, p=0.85, respectively, Table 2.2).

At Roman Bay Sea Farm, there was a significant increase over time in abalone length and weight gain between month-4 and month-8 ($p \leq 0.00001$). There was a significant difference in mean length gain between the two growth periods (repeated measures ANOVA: $F_{(4,28)}$ =16.54, $p \leq 0.00001$, Table 2.3). After eight months mean weight gain of abalone fed the probiotic diet was significantly greater than

those fed the control diet (repeated measures ANOVA: $F_{(4,28)}$=39.82, p≤0.00001, Fig. 2.3.3). Abalone fed the probiotic diet had a significantly higher condition factor at the end of the experiment than those fed the control diet ($F_{(1,10)}$ =13.51, p=0.004, Fig. 2.3.4). Normal size distributions were observed at the start, after four months and at the end of the experiment (Kolmogorov-Smirnov d =0.10). However, the specific growth rate was significantly higher between month-four to month-eight on abalone fed probiotic diet (0.33 ± 0.01 % body weight d^{-1}) when compared to those that were fed the control diet (0.28 ± 0.01% body weight d^{-1}), ($F_{(1,10)}$=8.01, p=0.02, Fig. 2.3.5).

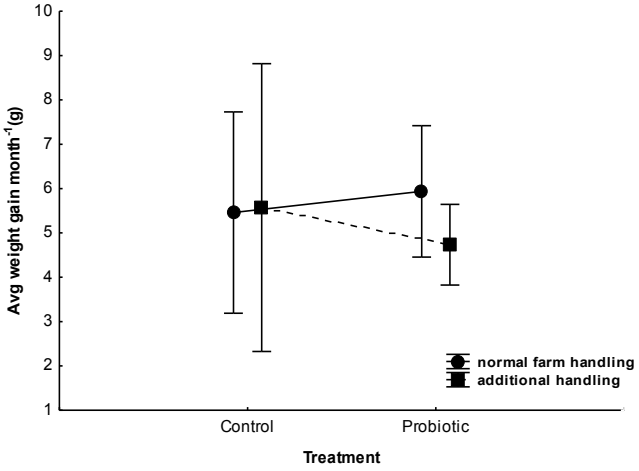

Figure 2.3.1: Weight gain per month (± 95 % confidence intervals) of animals fed probiotic or control diet at HIK Abalone Farm (multifactorial ANOVA; $F_{(1,14)}$=1.05, p=0.32).

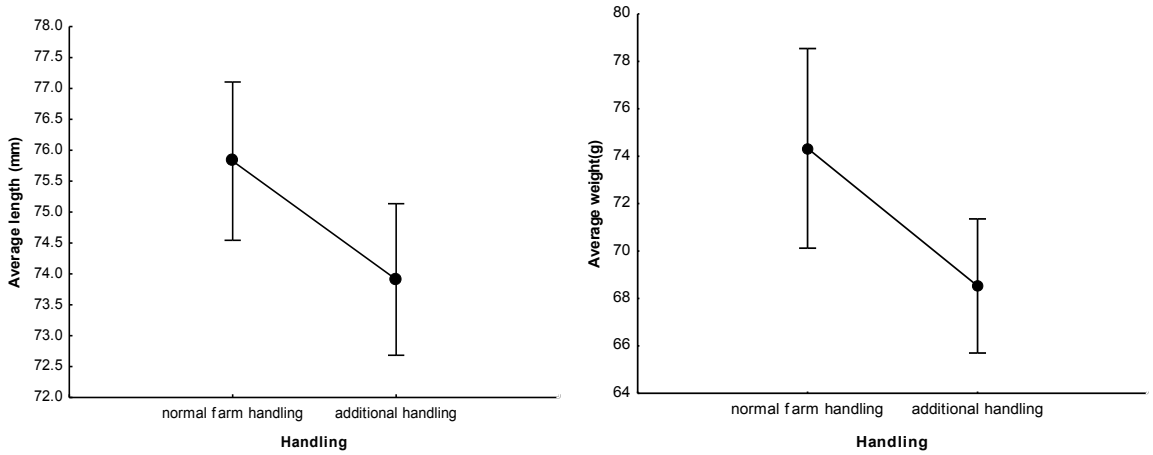

Figure 2.3.2: The mean length and weight (± 95 % confidence intervals) of abalone either subjected to additional handling or normal farm production conditions at HIK Abalone Farm at the end of the second part of the experiment, i.e., after eight months (ANOVA: $F_{(1,16)}$=5.93, p=0.03; $F_{(1,16)}$=6.12, p=0.02, respectively).

Table 2.1: Mean ± standard error of abalone weight (g abalone^{-1}) and length (mm) after the animals had been subjected to additional or normal farm handling.

	Four months			Eight months		
Length Weight	Additional handling	Normal farm handling	p-value	Additional handling	Normal farm handling	p-value
Initial length (mm)	58.69 ± 0.26	59.31 ± 0.85	0.47	61.70 ± 0.83	62.84 ± 0.83	0.34
Initial weight (g)	36.13 ± 0.24	37.36 ± 1.53	0.44	42.24 ± 1.44	44.45 ± 1.81	0.36
Final length (mm)	61.70 ± 0.83	62.84 ± 0.83	0.34	73.91 ± 0.52	75.82 ± 0.51	0.02
Final weight (g)	42.24 ± 1.44	44.45 ± 1.81	0.36	68.53 ± 1.197	74.33±1.87 g	0.02

Table 2.2: Mean ± standard error of abalone fed probiotic or a control diet at HIK Abalone Farm.

	Four months			Eight months		
Length/Weight	Probiotic diet	Control diet	p-value	Probiotic diet	Control diet	p-value
Initial length (mm)	58.20 ± 0.22	59.70 ± 0.81	0.12	61.93 ± 0.85	62.61 ± 0.71	0.57
Initial weight (g)	35.88 ± 0.22	37.61 ± 1.51	0.28	42.92 ± 1.66	43.77 ± 1.64	0.72
Final length (mm)	61.93 ± 0.85	62.61 ± 0.71	0.57	70.22 ± 1.12	69.77 ± 1.21	0.71
Final weight (g)	42.92 ± 1.66	43.77 ± 1.64	0.72	60.62 ± 2.61	59.88 ± 2.89	0.78
FCR	1.50 ± 0.10	1.56 ± 0.15	0.76	1.63 ± 1.64	5.55 ± 1.64	0.11
CF	1.03 ± 0.01	1.03 ± 0.01	0.89	0.99 ± 0.02	0.99 ± 0.02	0.98
SGR %	0.27 ± 0.02	0.24 ± 0.02	0.30	0.29 ± 0.02	0.29 ± 0.02	0.85

Table 2.3: Mean ± standard error of abalone length (L, mm) and weight (W, g abalone^{-1}) fed probiotic or control diet at Roman Bay Sea Farm. Length gain values are in mm month^{-1}.

	Four months		Eight months	
	Probiotic diet	Control diet	Probiotic diet	Control diet
Initial length (mm)	62.79 ± 1.06	62.09 ± 1.59	65.68 ± 0.73	65.57 ± 0.31
Initial weight (g)	34.24 ± 0.53	35.24 ± 0.32	50.51 ± 1.71	50.77 ± 0.82
Final length (mm)	65.68 ± 0.73	65.57 ± 0.31	76.08 ± 0.74	75.91 ± 0.39
Final weight (g)	50.51 ± 1.71	50.77 ± 0.82	75.92 ± 2.54	71.34 ± 1.19
Length gain (mm)	0.72 ± 0.41	0.87 ± 0.42	2.60 ± 0.14	2.59 ± 0.1

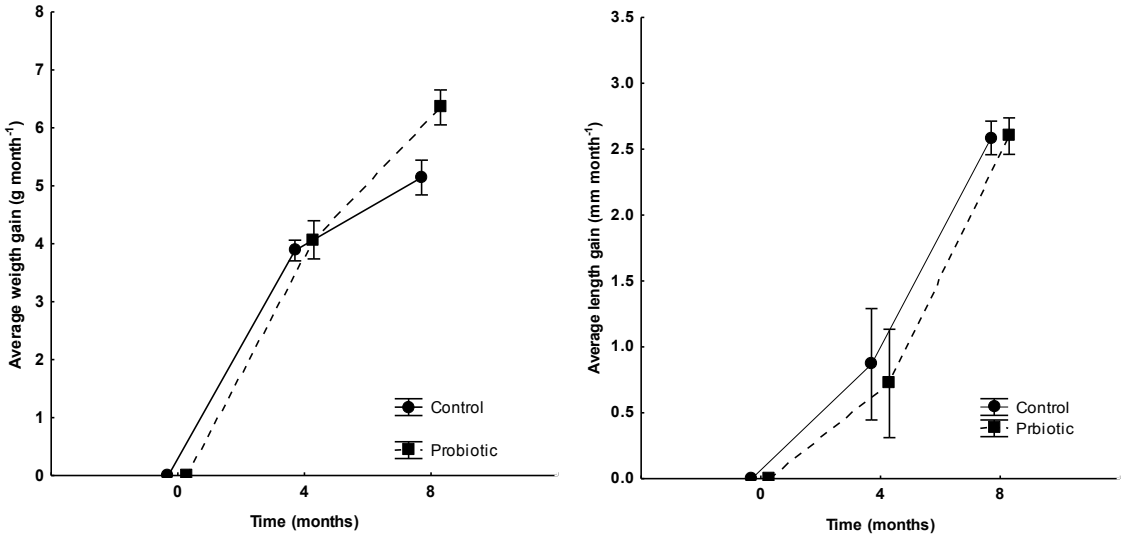

Figure 2.3.3: Length (mm) and weight (g) gain per month (± 95 % confidence intervals) of abalone fed probiotic or control diet after four and four to eight months at Roman Bay Sea Farm (repeated measures ANOVA: $F_{(4,28)}$=16.54, p≤0.00001; $F_{(4, 28)}$=39.82, p≤0.00001, respectively).

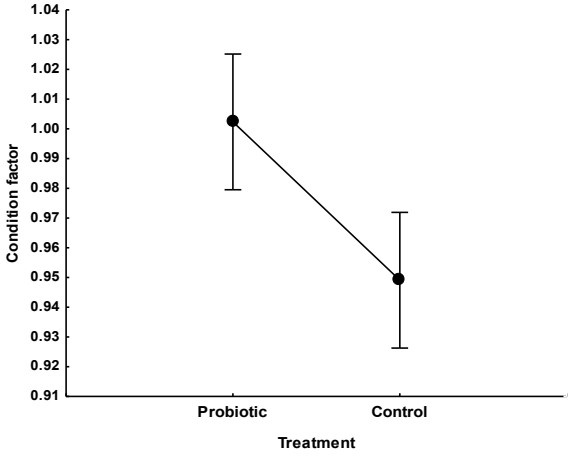

Figure 2.3.4: Final condition factor (± 95 % confidence interval) of abalone fed probiotic or control diet (ANOVA: $F_{(1, 10)}$=13.51, p=0.004) at Roman Bay Sea Farm. The condition factor at the start of the trial was 0.84 ± 0.04.

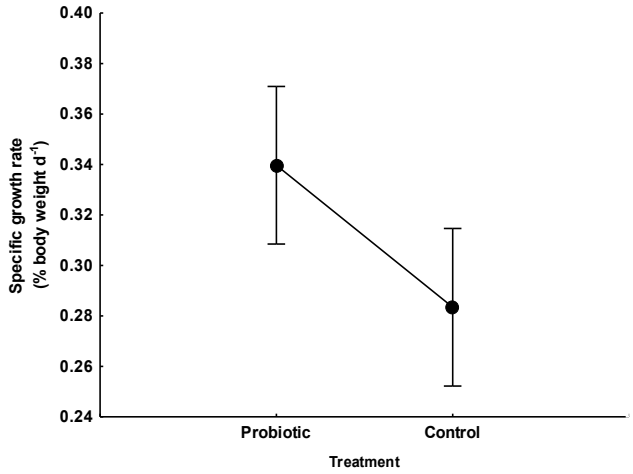

Figure 2.3.5: Specific growth rate (± 95 % confidence intervals) of abalone fed probiotic and control diet between four to eight months (ANOVA: $F_{(1,10)}$=8.01, p=0.02) at Roman Bay Sea Farm.

2.4 Discussion

Relationship between level of handling on the organism and the efficacy of the probiotic

Under the experimental conditions in which this study was conducted, there was no significant interaction between the factors dietary treatment (i.e. treatments with and without the probiotic) and level of handling (additional handling vs no additional handling) on abalone growth at HIK Abalone Farm. Handling during aquaculture processes, which includes grading, capturing and transporting fish has been reported as inherently stressful (Hahn 1989; Barton and Iwama 1991). To alleviate the effect of these stress factors, probiotic supplemented diets have been fed to cultured animals (Mohapatra *et al*. 2013). The primary role of dietary probiotic supplementation has been to promote growth and enhance animal health, although new findings have suggested positive effects on reproduction and stress alleviation, however, this requires further research (Martínez *et al*. 2012). Mohapatra *et al*. (unpublished) reported the positive effect of feeding a multi-species probiotic diet to *Labeo rohita* fingerlings in tolerating the stress caused by the insecticide Fenvalerate, which is generally used as a synthetic pyrethroid. Varela *et al*. (2010) reported that the administration of the probiotic strain,

Pdp11, in the diet of *Sparus auratus*, improved growth and tolerance to high stocking density. Similarly, Liu *et al*. (2010) reported significant acceleration of shrimp (*Litopenaeus vannamei*) larval development, metamorphosis, immuno-stimulation and stress response after adding probiotics (*Bacillus subtilis* E20) to the larval rearing water at a level of 109 $cfuL^{-1}$ (colony forming units per L). Taoka *et al*. (2006a), showed that a commercial probiotic, Alchem Poseidon (a mixture of *B. subtilis*, *Lactobacillus acidophilus*, *Clostridium butyricum* and *Saccharomyces cerevisiae*), increased stress tolerance in *Paralichthys olivaceus*, cultured in a closed recirculation system. Gilthead seabream (*S. auratus*) fed with *Lactobacillus fructivorans* and *L. plantarum*, had increased cortisol levels when subjected to acute stress (Varela *et al*. 2010). Cortisol is used as a measure of the stress response.

Variability exists in results achieved in some studies on the effect of probiotics on the stress response. For example Makridis *et al*. (2000) demonstrated that turbot larvae treated with probionts have shown an increased resistance towards stress or to pathogenic infections, but no improvements in growth and survival through probiotic treatment. Similarly, there were no significant differences on survival or weight gain in turbot larvae treated with probiotic *Pediococcus acidilactici*, compared to control groups (Villamil *et al*. 2010). Hoskonen and Pirhonen (2006) found that by repeatedly handling juvenile rainbow trout, *Oncorhynchus mykiss* without anaesthetics had significantly decreased feed intake and weight gain compared with an unhandled control group during an eight-week experiment. Pickering *et al*. (1982) demonstrated how an acute handling stress on brown trout (*Salmo trutta*) generated physiological changes, although this did not affect growth rate. Physiological stress as a result of husbandry practices in aquaculture can affect growth of cultured animals through suppression of appetite and feeding behaviour (Schreck *et al*. 1997; McCormick *et al*. 1998).

Differences among studies are possibly based on methods and choice of probiont, dietary concentration, type of species strain, dosage, age/size of animal, feeding and duration period, environmental conditions, handling process and stocking densities, which may influence results (Welker and Lim 2011). Differences on the effectiveness may be dependent on the severity or

mildness of the stressor applied. However, the success of the probiotic effect relies on bacterial population growth (Nayak 2010).

Based on the results obtained in this study and in relation to the available literature, the findings in this study may be attributed to the severance of the stressor applied. More research is needed to determine the efficacy of probiotics in mitigating stress response on the severity of the stressor applied on abalone under farming conditions.

Abalone growth subjected to additional handling

At HIK Abalone Farm abalone *H. midae*, which were handled under normal farm production conditions had a significantly higher length and weight gain compared to abalone subjected to additional handling. The reduction in growth when abalone are handled more frequently could be due to the nature of the stressor applied. Arrangements for an optimal environment with minimal stress exposure is fundamental for the success of abalone mariculture (Hooper *et al*. 2011), although under farm conditions, vital routine operations can result in stress (van Schalkwyk 2011). One such routine operation is grading or sorting and research showed that abalone are easily stressed by handling (Malham *et al*. 2003; Hahn 1989). Generally it has been acknowledged that growth and metabolism are affected by chronic stress as a result of cortisol activities (van Weerd and Komen 1998). Results in the current study are similar to findings in other studies involving stressor application. For example, McCormick *et al*. (1998) found that handling stress decreased growth of Atlantic salmon (*Salmo salar*) parr.

The observed effect of additional handling in this study is consistent with the results from similar studies. Reduced growth in handled abalone, suggest that energy demand surpasses the energy available from metabolism and feed intake (Goncalves *et al*. 2011).

Growth of abalone fed probiotic or control diet

The addition of three candidate probionts into a commercial diet (i.e. Abfeed®) significantly improved growth of abalone (*H. midae*) compared to those fed the control diet at Roman Bay Sea Farm. The improvement of growth rate in the current study supported the findings of some other probiotic-based nutritional studies. Macey and Coyne (2005) reported improved monthly growth under standard farming conditions in abalone *H. midae* fed Abfeed® supplemented with a mixture of probiotics for eight months, especially in larger abalone. Macey and Coyne (2005) showed a 33% (basal diet: 0.86 ± 0.05 mm; probiotic diet: 1.14 ± 0.07 mm) and 35% (basal diet: 3.44 ± 0.22 g abalone^{-1}; probiotic diet: 4.66 ± 0.17 g abalone^{-1}) improvement in length and weight compared to smaller abalone with 7% (basal diet: 2.31 ± 0.04 mm; probiotic diet: 2.48 ± 0.069 mm) and 8% (basal diet: 1.61 ± 0.04 g abalone^{-1}; probiotic diet: 1.74 ± 0.0814 g abalone^{-1}) improvement, respectively. Silva-Aciares *et al*. (2011) showed improved monthly growth rates of recently weaned abalone *Haliotis rufescens* of 16.5% (control diet: 1.63 ± 0.24 g abalone^{-1}; probiotic diet: 1.95 ± 0.20 g abalone^{-1}) and adult 15.94% (control diet: 2.90 ± 0.15 g abalone^{-1}; probiotic diet: 3.45 ± 0.13 g abalone^{-1}) when they were fed macroalgae supplemented with three probiotic strains compared to a control diet for 210 days. Doeschate and Coyne (2008) suggested that growth rate increase may be attained through numerous coherent mechanisms: (1) increasing the amount of nutrients available to the abalone for absorption in the gut, (2) increasing the pool of digestive enzymes in the abalone gut, and (3) use of the bacterial supplements as an additional nutrient source. The mixture of candidate probiotic cultures used in this study has shown potential for use in commercial abalone farming, possibly reducing the time abalone take to reach market size.

The results from the study at Roman Bay Sea Farm showed that length of abalone fed probiotic or control diet increased over time at the same rate. Probiotics diets are known to significantly improve abalone shell length when compared to a control diet (Macey and Coyne 2005; Doeschate and Coyne 2008; Hadi 2012). The difference between those results and the ones presented here may be attributed

to the efficacy of probionts and experimental conditions. However, more research is needed to better understand the effect of probiotic on abalone shell length.

An improvement in fish growth when probiotics are included in the diet has been found to be a result of improved dietary digestion (El-Haroun *et al*. 2006; Dhanaraj *et al*. 2010). Thus, abalone fed the probiotic diet at Roman Bay Sea Farm had a higher condition factor and specific growth rate. These results are similar to trends observed by other authors. For example, Marzouk *et al*. (2008) showed that *Oreochromis niloticus* fed probiotic-supplemented diets showed a significant increase in body weight gain, specific growth rate, protein efficiency ratio, feed conversion ratio and condition factor compared to a control treatment. Bagheri *et al*. (2008) demonstrated that the application of *Bacillus subtilis* and *B. licheniformis* could significantly improve the FCR, specific growth rate (SGR), weight gain and protein efficiency ratio (PER) after two months in rainbow trout fry.

The results obtained at Roman Bay Sea Farm were not the same at HIK Abalone Farm. Such variability in results can be attributed to experimental conditions hence, the use of probiotics as stimulus for growth may provide differing results under different culture conditions (Gullian *et al*. 2004). For example, Ziaei-Nejad *et al*. (2006) reported that when probiotic was administered both in hatchery and farming stages growth and survival parameters of *Fenneropenaeus indicus* were significantly higher ($p<0.05$) than in controls but when probiotic was administered to the farming stage only, there were no significant differences between treatment and controls in each of the parameters. Taoka *et al*. (2006a) reported that significant difference in growth rate was not observed between the control and the probiotic diet group in Japanese flounder *Paralichthys olivaceus*.

2.5 Conclusion

Abalone fed the probiotic diet had a better growth compared to abalone fed the control diet on one of the farms. These abalone had a higher biomass, but without an increase in length which accounted for a higher condition factor.

In the present study, growth improvements induced by probiotics to abalone handled under normal farm production conditions over the grow-out phase at Roman Bay Sea Farm suggest the possibility of reducing the time required for these animals to reach market size, thereby assuming that extra food costs are not higher than the gain from faster growth and increasing productivity. Candidate probionts used in this study have shown some potential to enhance abalone growth. This study underscores the necessity for more commercial farm-based research, which will assist in developing better management strategies on abalone growth and survival. The aim is to raise hypotheses that will help address problems in abalone farming. This will give greater advantage and cost benefits to commercial abalone farmers.

CHAPTER 3

THE EFFECT OF PROBIOTIC DIET AND HANDLING ON ABALONE (*Haliotis midae*) IMMUNE RESPONSE

3.1 Introduction

Many intensive aquaculture production facilities experience a high number of animal diseases and pathogens, posing a threat to animal health and welfare and ultimately the profitability of the operation. In these production facilities, economic losses are possible as a result of animals being exposed to stressful conditions, degenerating conditions in the environment and diseases (FAO 2004; Subasinghe 2005). Presently with the rapid growth in the aquaculture production food sector, diseases, in particular bacterial or viral infections of aquatic animals, have become a limiting factor to the sustainable existence of the industry (El-Haroun *et al*. 2006; Pieters *et al*. 2008; Abd El-rhman *et al*. 2009). Conventional treatment procedures for diseases, for example the use of drugs and vaccines are regulated or require cumbersome ways of delivery. Comprehensive wide-spread use of chemotherapeutants resulted in drug resistance complications which may constitute a threat to human health (Marzouk *et al*. 2008) depending on the types of antibiotics used on farms.

Antibiotics have been used successfully to treat bacterial infections and to limit fish mortalities in rearing systems (Taoka *et al*. 2006b). The negative effects due to antibiotic use have led to problems (Ige 2013). These include accumulation of antibiotics in the tissue and immune system suppression (El-Haroun *et al*. 2006; Tukmechi *et al*. 2007; Nayak *et al*. 2007). More of a concern, antibacterial use due to the rise in antibiotic resistance of human microbiota has prompted European Union (EU) countries in 2006 to implement severe restrictions (Angelis *et al*. 2006). The autochthonous micro-biota of the host is often modified as a result of antibiotic treatment, resulting in changes in viable numbers and population heterogeneity (Ige 2013). Owing to the constraints and predicaments of

hormonal and antibiotic use for animals and consumers, probiotics are an ideal alternative for improvement of overall health, disease resistance, nutrient digestion and growth (Irianto and Austin 2002; Lara-Flores *et al*. 2003). Thus, there is a mounting interest in research and development of suitable alternatives to chemotherapeutants to combat bacterial diseases (Delbert *et al*. 2012).

Probiotic utilisation in aquaculture, especially in the culturing of molluscs, has mainly focused on controlling diseases (Kesarcodi-Watson *et al*. 2008). Prevalence of disease may be decreased by using probiotics in aquaculture feeds or rearing systems (Ige 2013). Strains of probiotics have been demonstrated with an ability to prevent infections either *in vitro* or *in vivo* through several processes (Balcázar *et al*. 2006). Generally probiotics have been used as dietary supplements in preventing virulent gastrointestinal diseases by secreting microtoxins which prevent proliferation of pathogenic bacteria e.g. *Escherichia coli* and *Salmonella* in the intestinal lumen (Barth *et al*. 2009). In fish they are known to inhibit invasion of pathogenic bacteria in the intestinal tract by virtue of nutrients and site adhesion competition, production of metabolites such as organic acids, hydrogen peroxide and bacteriocins (Ringo *et al*. 2010; Ige 2013). Attachment of probiotics to intestinal mucous may prevent colonization of opportunistic pathogens (Gatesoupe 1999; Vine *et al*. 2004; Ringo *et al*. 2010). However, the inhibitory capacity for pathogen attachment seems to be reliant on the mucosal site, and on particular probiotic strains and pathogens (Young-Hyo *et al*. 2001; Collado *et al*. 2007). Immune system stimulation is a well documented effect of probiotic microorganisms (Nayak 2010), enhancing production of antibodies and elevating phagocytotic activity (Ige 2013). Colonisation of mucosal surfaces by probionts may prevent colonisation of opportunistic bacteria or pathogens by competing for binding sites, release of bacteriocins and other antimicrobial compounds in the mucous layer and stimulate the immune system (Merrifield *et al*. 2010b). Taoka *et al*. (2006b) showed that viable probiotics administered to tilapia (*O. niloticus*) increased the nonspecific immune response by enhancing lysozyme activity, neutrophile migration, and bactericidal activity, as well as improving fish resistance to infection by *Edwardsiella tarda*. Robertson *et al*. (2000) administered live isolates

of a *Carnobacterium* strain isolated from salmon intestines and showed *in vitro* antagonism against known fish pathogens: *Aeromonas hydrophila, Aeromonas salmonicida, Flavobacterium psychrophilum, Photobacterium damselae,* and *Vibrio* species.

Thus, beneficial bacteria have opened new strategies to manage health from humans to aquatic animals (Nayak 2010). In the absence of an adaptive immune response in abalone, they are reliant on an innate immune response for protection against pathogenic infections (Xue *et al*. 2008). Their immune defence system uses haemocytes and humoral mechanisms to eradicate infectious bacteria (Sminia and van der Knaap 1987).

Haemocytes are free buoyant cells found in circulation in the haemolymph of abalone and they penetrate into the tissues (Cheng 1975, 1981). They play a fundamental role for the innate immune defence (Cheng 1981) as the primary immune effector cells (Travers *et al*. 2008a). The immune defence system mainly by phagocytosis is intermediated with haemocytes. Hence invertebrates lack an adaptive immune response (Auffret 1988). Phagocytosis is an essential part of the cellular immune response in molluscs (Fryer and Byne 1989), and consists of distinct phases involving recognition, chemotaxis, adhesion, ingestion and elimination of pathogens (Adema *et al*. 1991, 1994; Bayne 1990, 2001; van der Knaap *et al*. 1993; Yakovleva *et al*. 2001). The possibility of determining the immune status in molluscs through haemocyte counts is dependent on the quantification of haemocyte counts and phagocytosis assays and they may be useful immune system parameters, although more research is needed (Hooper *et al.* 2007). A reduction in phagocytotic rate indicates a decrease in immunity. This has been demonstrated following exposure to various stressors (Cheng *et al*. 2004a; Chen *et al*. 2005; Travers *et al*. 2008a). A number of research studies on abalone immunosuppression have been done which include changes in phagocytotic rate and haemocyte counts as a result of different stressors applied (Malham *et al*. 2003; Cheng *et al*. 2004a; Chen *et al*. 2005). Lacoste *et al*. (2002) demonstrated that the number and phagocytotic activity of circulating haemocytes was significantly reduced in *Crassostrea gigas* exposed to mechanical stress. Malham *et al*. (2003) initially found a

reduction in haemocyte counts and phagocytosis activities which was followed by a significant simultaneous increase, and eventually return to near basal levels (Hooper *et al*. 2011) after a 15-minute application of mechanical shaking to abalone. Hooper (2011) also demonstrated similar results on phagocytotic rate and total haemocyte counts when determining detachment effects by anaesthesia, with or without movement i.e., handling.

The aim of this study was to examine the effect of a probiotic-supplemented diet on immunological parameters, i.e. haemocyte count and phagocytosis assay of abalone (*Haliotis midae*) which were subjected to handling as described in the material and methods section of Chapter 2.

3.2 Material and Methods

3.2.1 Animals and sample collection

Abalone (*H. midae*) were made available by two commercial abalone farms on the west coast of South Africa; HIK Abalone Farm (Pty) Ltd and Roman Bay Sea Farm (Pty) Ltd. They were kept under the conditions and fed the same diets described in Chapter 2. The approximate age of the animals was 26 months for both farms and the animals were spawned from a single batch on each farm. At HIK Abalone Farm (Pty) Ltd, they were maintained under farm conditions with aerated and continuously flowing natural seawater in five canvas tanks holding 12 oyster mesh baskets at temperatures between 14 and 20 °C. At Roman Bay Sea Farm (Pty) Ltd they were maintained at temperatures between 19 and 24 °C in flow-through aerated sea water in two concrete tanks with 12 baskets per tank. Two animals were randomly sampled from each basket of abalone in both experiments after four and eight months of the trial. These samples were used for haemolymph analysis, haemocyte counts and a phagocytosis assay.

3.2.2 Haemolymph collection and total haemocyte count

The haemolymph (0.2 ml abalone^{-1}) was collected from the adductor muscle using 2-ml syringes and

26 G x 1/2 inch needles. An equal volume of haemolymph (100 µl) from the two animals taken from the same basket was withdrawn at each time and added into 200 µl of Alsevers buffer solution in an eppendorf vial, which was immediately placed on ice to prevent clotting. The total number of circulating haemocytes was counted with a Neubauer haemocytometer and a light microscope (100x magnification) within approximately five minutes after extraction from the abalone. The Neubauer haemocytometer was cleaned prior to each count with ethanol to avoid any external contamination. On both sides of the chamber, cells on every square were counted three times and the mean also was determined with the total multiplied by 5 x 10^4 to give the cell count ml^{-1}. A smear of haemolymph was added at the centre groove line of the Neubauer Haemocytometer Counting Chamber with a pipette so as to cover both sides.

3.2.3 Phagocytosis assay

Phagocytosis testing was conducted as described by Malham *et al.* (2003) and Macey and Coyne (2005) with minor modifications. The bacterium *Vibrio anguillarum* was grown at 22 $^{\circ}$C for 24 hour in tryptone soya broth (TSB, Bio lab) supplemented with 2.5% (w/v) NaCl (Macey and Coyen 2005). Formalin (10%) was added to kill the bacteria and cells were pelleted by centrifugation at 12 000 x g for 10 minutes. Cells were harvested and washed twice in sterile phosphate buffered saline (PBS) solution before they were re-suspended in 0.1 M $NaHCO_3$ pH 9.0, which contained 0.1 mg ml^{-1} fluorescein 5-isothiocyanate, isomer 1 (FITC, Sigma). Labelling of cells was done in the absence of light for one hour at 25 $^{\circ}$C. Labelled bacteria were diluted to 1 x 10^8 bacteria ml^{-1} after centrifugation by re-suspending cells in PBS. The labelled bacteria were stored at -20 $^{\circ}$C until needed for the phagocytosis assay. One hundred µl of haemolymph containing haemocytes at a concentration of 10^6 haemocytes ml^{-1} in Modified Hank's Balance Salt Solution (MHBSS), were placed onto a glass slide with a well. The slides were kept in a dark incubation chamber for 20 minutes to allow the haemocytes to adhere to the glass (Macey and Coyne 2005). One hundred µl of FITC-labelled *V.*

anguillarum were immediately added to the cells. Slides were then returned to the incubation chamber and incubated for another 30 min. Modified Hank's Balance Salt Solution was used to rinse the slides three times before adding 100 µl of ethidium bromide (Sigma) solution (50 Ag ml^{-1} in PBS). After one minute the ethidium bromide solution was removed by rinsing the slides with MHBSS removing remaining liquid with a pipette and placing a glass cover slip on top of each slide. All slides were prepared in duplicate, they were counted using a 488 nm emission filter on an Olympus fluorescent microscope. Phagocytotic cells were distinguishable from non-phagocytotic cells as they contained green fluorescent bacteria (Malham *et al.* 2003). The percentage phagocytosis was calculated for each slide as the number of red cells/green cells x 100.

3.2.4 Statistical analysis

A mean value was calculated for each replicated basket using the data collected from the individual abalone in that basket, and this mean value was used in all further analyses. The assumptions of normality and the equality of variance were assessed using Shapiro-Wilk test (Shapiro and Wilk 1965) and Levene's test (Levene 1960). Where necessary, data were transformed (log_{10}) prior to the statistical analysis. Results are presented as means ± standard error (SE) unless stated otherwise. Student's t-test was used to test for differences between two means. The interaction between dietary treatment and handling was analysed by factorial analysis of variance (ANOVA) using computer software (STATISTICA V7.0, StatSoft, Inc., Tulsa, OK,1984-2004)

3.3 Results

On both farms there were no significant differences in total haemocyte count of abalone fed either a probiotic or control diet after four or eight months: HIK abalone farm (Student's *t*-test: *df* =18; p=0.24 *t* =0.63; *df* =18; p=0.53, respectively, Figure 3.1); Roman Bay Sea farm (Student's *t*-test: *t*-value =1.41; *df* =10; p=0.21 and *df* =10; p=0.60, respectively, Figure 3.2).

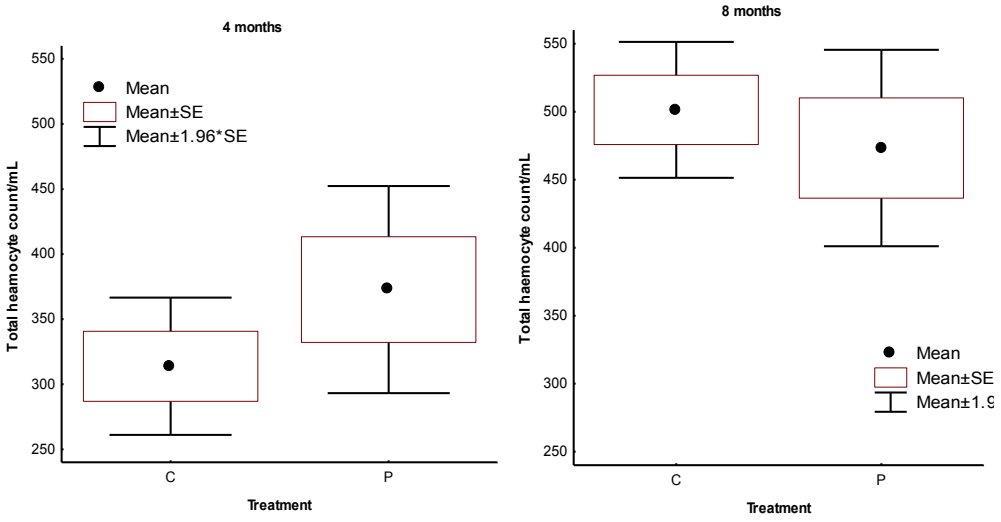

Figure 3.1: Haemocyte counts of abalone haemolymph (± 95 % confidence intervals) from animals fed probiotic (P) or control (C) diets at HIK Abalone Farm (*t*-test; p=0.24) and (*t*-test; p=0.51).

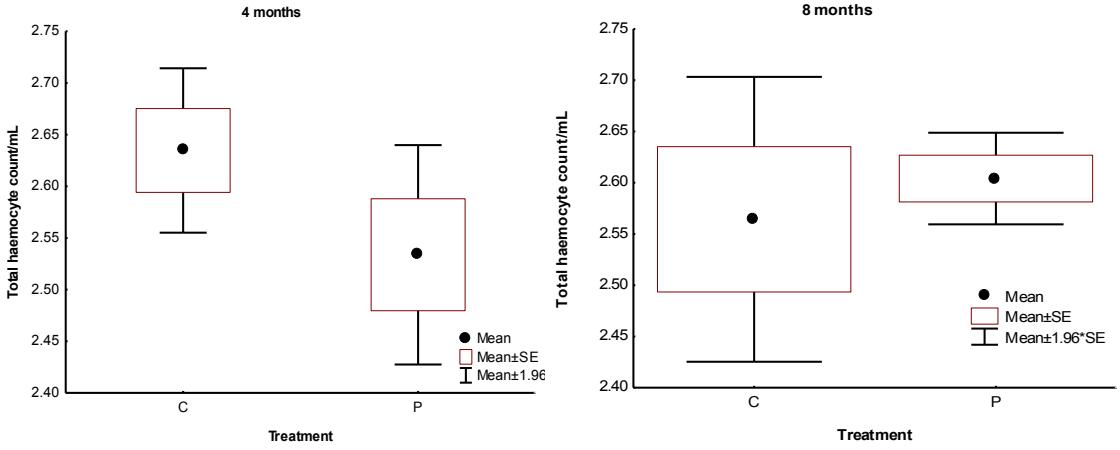

Figure 3.2: Haemocyte counts (expressed as logged values) in abalone haemolymph (± 95 % confidence intervals) from animals fed probiotic (P) or control (C) diets at Roman Bay Sea Farm (*t*-test; p=0.21) and (*t*-test; p=0.60).

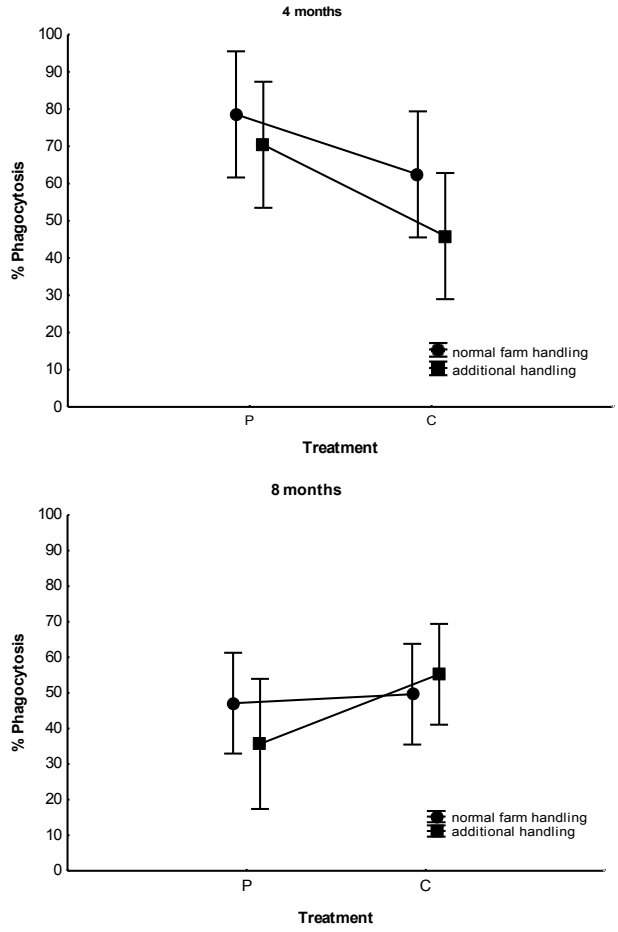

Figure 3.3: Interaction between diet and handling (± 95 % confidence intervals) on immune parameter i.e., phagocytosis (logged values) fed either probiotic (P) or control (C) after four and eight months (multifactorial ANOVA:$F_{(1,16)}$=0.27, p=0.61; $F_{(1,14)}$=1.42, p=0.25) respectively) at HIK Abalone Farm.

There was no significant interaction between dietary treatment and handling on phagocytosis counts after four and eight months (multifactorial ANOVA: $F_{(1,16)}$=0.27, p=0.61; $F_{(1,14)}$=1.42, p=0.25, Figure 3.3) at HIK abalone farm. However, when the factor dietary treatment was analysed separately, phagocytosis counts were significantly different between the probiotic and control treatments after four months (*t*-test: t =2.26; df =18; p=0.03, Figure 3.4).

There was a significant interaction between dietary treatment and handling on the immune parameter

at HIK Abalone Farm, i.e., haemocyte, after four months (multifactorial ANOVA; $F_{(1,16)}$=11.1, p=0.003), but no significant interaction between these variables was observed after eight months (multifactorial ANOVA: $F_{(1,16)}$=0.14, p=0.71, Figure 3.5).

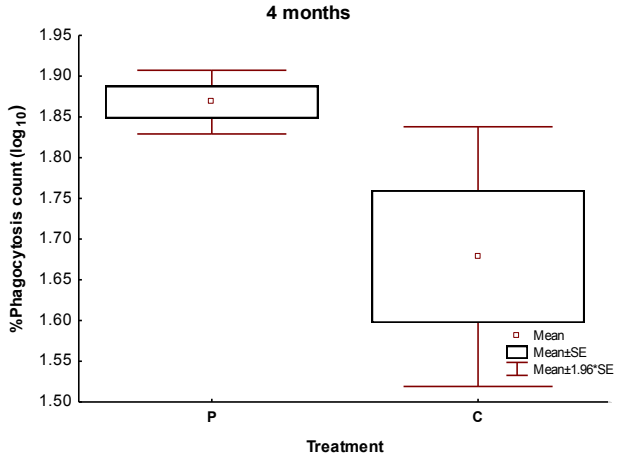

Figure 3.4: Phagocytosis counts (expressed as logged values) in abalone haemolymph (± 95 % confidence intervals) from animals fed probiotic (P) or control (C) diets at HIK Abalone Farm (*t*-test; p=0.03).

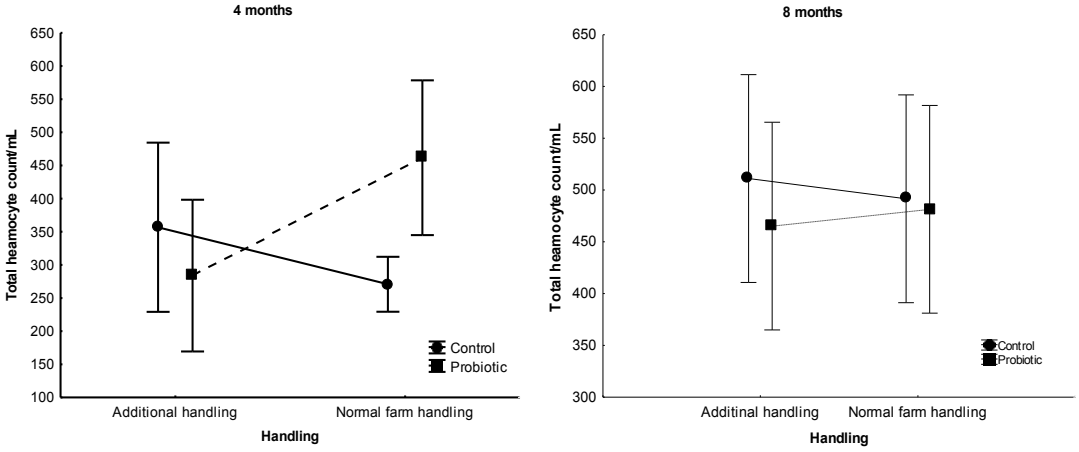

Figure 3.5: Interaction between diet and handling (± 95 % confidence intervals) on immune parameter i.e., haemocyte after four and eight months (multifactorial ANOVA: $F_{(1,16)}$=11.1, p=0.003; $F_{(1,16)}$=0.14, p=0.71 respectively) at HIK Abalone Farm.

3.4 Discussion

Haemocyte counts of abalone fed probiotic or control diet

Haemocyte numbers in abalone fed either probiotic or control diet in this study showed no significant difference between treatments on both farms after four and eight months. The immune defence of abalone, including that of other invertebrates, is centred on haemocytes (Day *et al*. 2010). In molluscs haemocytes are commonly recognized by their effective phagocytotic ability towards invading pathogenic bacteria, i.e. protozoans (Travers *et al*. 2008a). Haemocytes form an integral part of the first line of defence against invading microorganisms, and an elevated number of circulating haemocytes may help to better withstand invasion of pathogenic bacteria (Peraza-Gómez *et al*. 2009). The results in this study are consistent with other studies. For example, before infection with *V. anguillarum* Macey and Coyne (2005) observed no significant difference in total haemocyte count amongst abalone (*H. midae*) either fed the basal or probiotic diet. Similarly, there was no significant difference in total haemocyte count ($4.26 \pm 0.34 \times 10^6$ $5.64 \pm 1.23 \times 10^6$ cells ml^{-1}) of shrimp between all treatments after they were fed experimental diets containing probiotic *B. subtilis* E20 (Tseng *et al*. 2009). Conversely, based on literature, Jiang *et al*. (2013) found that total haemocyte count of diets supplemented with probiotics WA64 or WA65 at 109 cells g^{-1} showed a distinct increase which eventually decreased to baseline levels. However, in the studies conducted by Hai *et al*. (2009) and Hai and Fotedar (2009), a reduction in haemocyte count as a result of probiotic application was observed. The results in this study suggest that the probiotic may have been ineffective at inducing floating haemocytes in the open circulatory system of abalone.

Relationship between haemocyte count and handling on abalone fed probiotic or control diet

At HIK abalone farm there was a significant interaction between the two factors dietary treatment and handling on haemocyte count, at four months, but this was not significant at the end of the experiment. Intermediaries of stress response and main immune responses are produced by

haemocytes (Ottaviani and Franceschi 1996; Ottaviani *et al*. 1997; Ottaviani and Franceschi 1997). Few research studies have been conducted on abalone stressors and stress responses, although in abalone and other molluscs, haemocytes are central to stress and immune responses (van Schalkwyk 2011). Similar results to this study were reported by other authors although animals were not treated with probiotics. For example, in a laboratory experiment, Day *et al*. (2010) found a significant difference in haemocyte count between treatments within three hours after heat was applied, but abalone recovered the following day. Variability in the haemocyte count depended on the applied stressor on abalone and the time gap prior to sampling (Malham *et al*. 2003; Travers *et al*. 2008a). However, Malham *et al*. (2003) showed a direct link between handling stress and immunity in *Haliotis tuberculata.* Thus, a transient drop in haemocyte count has been shown with mild stressors in abalone (Malham *et al*. 2003; Cheng *et al*. 2004a, 2004e). The observed decrease in haemocyte counts of stressed abalone has not been fully understood, since they neither go through lysis nor an integrated response (Hooper *et al*. 2007). Further studies are needed to investigate the interaction betweenhaemocyte counts and probiotics on different size classes of abalone subjected to a mild or severe stressor.

Relationship between phagocytosis count and handling on abalone fed probiotic or control diet

In the present study, no significant interaction was found between dietary treatment and handling stressor on phagocytosis counts after four and eight months. Phagocytosis counts were however significantly different between dietary treatments after four months. Phagocytosis is a crucial process in the invertebrate immune system (Day *et al*. 2010). A number of studies have been conducted on how stress affects the immune function of abalone (Malham *et al*. 2003; Cheng *et al*. 2004a, b, c, d, e; Shuhong *et al*. 2004).

Probiotic combinations can elicit a non-specific immune response of the host through production of immunostimulants (Hadi 2012). However, Macey and Coyne (2005) have shown significant

differences in percentage phagocytotic haemocytes of animals fed probiotic diet compared to animals fed the basal diet except on days 0 and 18 before challenge with the pathogen, *V. anguillarum*. Similarly, black tiger shrimp fed with probiotic diet showed stimulation of phagocytosis, phagocytotic activity in the haemolymph and increased resistance to *V. harveyi* (Rengpipat *et al*. 1998, 2000). On the other hand, some authors have reported on abalone immunosuppression, altered phagocytotic rate and haemocyte counts when different stressors were applied (Malham *et al*. 2003; Cheng *et al*. 2004c; Chen *et al*. 2005). Literature has thus shown that various stress applications stimulate responses which may or may not have an effect on immunity (Day *et al*. 2010).

Candidate probiotic cultures used in this study did not mitigate the negative effects of handling stressor on phagocytotic counts. These results further suggest the influence of severity of additional handling over prolonged time, although longer periods are less likely on the farms (Day *et al*. 2010). Furthermore, doubling the dose of probiotics might have had an effect on phagocytotic counts at end of the experiment. Magda *et al*. (2011) reported a significant decrease in immunological parameters such as phagocytic activity and immunoglobulin level through increased probiotic dose, which might account for the drop in immune response between months four and eight that was seen in this trial.

More research is necessary to determine the potency of probiotic on abalone phagocytosis counts under different stress levels. The observed significant increase in phagocytosis count of abalone fed probiotic-supplemented diet after four months suggests that the level of stress imposed had a remarkable effect and that abalone were able to acclimatize to the adjusted environmental conditions (Day *et al*. 2010). Further studies are needed to confirm these findings.

3.5 Conclusion

The addition of three candidate probiotics in Abfeed® showed no significant improvement on abalone immune parameters examined at the end of this study, although there was evidence of an immune response after four month in abalone fed the probiotic diet. A doubling of the dose of probiotics may

have led to a lack of significant differences in immune parameters measured at the end of the experiment. More studies are required to explain the interrelationship between handling and probiotic effect on the abalone immune system.

CHAPTER 4

GENERAL DISCUSSION

Probiotic use in aquaculture has increased steadily over the years for beneficial purposes. Considerable activities of probiotic strains have been suggested, which include antagonism towards infectious bacteria (Rengpipat *et al*. 2000; Li *et al*. 2006), excretion of substances which inhibit the proliferation of bacterial pathogens (Chythanya *et al*. 2002; Longeon *et al*. 2004), supply of vital nutrients (Tovar *et al*. 2002; Tovar-Ramírez *et al*. 2004) and/or digestive enzymes (Macey and Coyne 2005) for growth improvement of the host animal (Gatesoupe 2002), immune stimulation (Verschuere *et al*. 2000; Panigrahi *et al*. 2005) and improved stress tolerance (Rollo *et al.* 2006).

The work presented in this study reports on some probiotic effects of abalone feed supplemented with three candidate probionts. These bacterial isolates were able to inhibit growth of pathogenic bacteria, utilise various carbohydrate and protein sources in Abfeed® and enhance growth in laboratory-reared abalone (Vine *et al.* 2009). Verschuere *et al*. (2000) suggested that the efficacy of probiotics is greater in the host species from where they were derived. The aim of this work was to investigate the possible effects of three candidate probionts which were isolated from the host gut, on growth and health of abalone under farming conditions. This was attained by determining the efficacy of probiotics on abalone growth with animals either subjected to additional handling or normal farm handling and by measuring some immune parameters, i.e., phagocytosis and haemocyte counts.

The results have shown no significant interaction between diets supplemented with or without probiotic microorganisms and the magnitude of handling on abalone growth (Chapter 2, Figure 2.3.1). There was no significant effect on feed conversion ratio, specific growth rate and condition factor. Conversely, in most studies it appeared that there were positive gains from probiotics to alleviate stress. Some studies have shown improved stress tolerance in fish and other species fed a probiotic-

supplemented diet (Taoka *et al.* 2006a; Hernandez *et al.* 2010; Liu *et al.* 2010; Varela *et al.* 2010). Results in this study may be due to the severity of the stressor applied, which might have compromised the efficacy of the probiotics. Probiotic mode of action normally depends on host and strain-specific attributes (Ibnou-Zekri *et al*. 2003; Madsen 2006). Additionally, probiotic viability (Gill *et al*. 2001), dose (Donnet-Hughes *et al*. 1999) and supplementation period (Vollstad *et al*. 2006) can also affect their efficacy (Nayak 2010). It would be interesting to examine the effect of probiotics in alleviating stress response on abalone under farming conditions.

Here, length and weight gain of abalone fed a diet supplemented with or without probiotics while subjected to additional handling stress was lower when compared to abalone, which were handled under normal farm production conditions (Chapter 2, Figure 2.3.2, Table 1). Generally, handling stress is known to affect abalone growth. For example, changes in growth may be due to the adverse effects of stress on appetite (Iwama *et al*. 2006; Portz *et al*. 2006). Hoskonen and Pirhonen (2006) discovered in juvenile rainbow trout (*Oncorhynchus mykiss*) that the average feed intake and weight gain during the experiment were lowest in a handled control group. During stress exposure abalone deplete their energy reserves as they strive to adapt to the stressful condition (Vandepeer 2006), consequently this leads to growth reduction. The findings of the present study on the effect of additional handling agree with earlier studies on similar work. These results indicate that handling negatively affected abalone production.

In this study, under some conditions, growth of abalone fed three candidate probiotics supplemented in the diet showed significantly improved growth compared to those fed Abfeed® without probionts (Chapter 2, Figure 2.3.3). Thus, this result was not consistent since dietary probiotic had no effect on growth at the end of the trial. Improvements in growth may be due to more efficient use of nutrients conferred by probionts. A number of studies have reported that effective utilisation of probiotics is dependent upon their successful establishment in the host and the secretion of growth-promoting nutrients (Bagheri *et al*. 2008). Diets consisting of extracts from *Ecklonia maxima* and *Gracilaria*

gracilis with probiotic bacterial isolates added appeared to promote nutritional benefit and improved abalone *H. midae* growth (Macey and Coyne 2005; Troell *et al.* 2006). It has been demonstrated by Maeda and Liao (1992) that the bacterial strain, PM-4, used as a dietary source stimulated growth of *P. monodon* nauplii. Beneficial lactic acid bacteria have shown to inhabit gastrointestinal tracts of fish and crustacean, improving growth and survival of the host organism (Balcázar *et al*. 2008; Iehata *et al.* 2009). However, enzyme activity acquired from diets supplemented with probiotics enhanced the assimilation of protein, starch, fat and cellulose which may justify the growth observed from probiotic-supplemented diets (Wang 2007). This study support the promotion of abalone growth through probiotic treatment isolated from the host gut only under some circumstances.

In the current study, haemocyte count of abalone fed diets supplemented with probiotic or control on both farms, showed no significant difference after four and eight months (Chapter 3, Figure 3.3.1). Haemocyte activities are the most important part of cellular immunity and they include phagocytosis which subsequently fights infectious bacteria (Hooper *et al*. 2007). A high number of circulating haemocytes may support immune defence (Day *et al*. 2010). A greater number of haemocyte counts can enhance the immune system at times of high pathogen loads (Jiang *et al*. 2013). Literature reveals that animals fed probiotic-supplemented diets attained a significant increase in haemoctye counts when compared to those fed a control diet and variability of results occur due to age, environmental conditions and type of species. Peraza-Gómez *et al*. (2009) found that total haemocyte count was significantly higher in *Litopenaeus vannamei* fed daily with probiotics during 20 days when compared to controls fed a commercial diet. Studies conducted by Hai *et al*. (2009) and Hai and Fotedar (2009), found a reduction in haemocyte count as a result of probiotic application. Similar to the current study, Macey and Coyne (2005) and Tseng *et al*. (2009) observed no significant difference in total haemocyte count of animals fed diets containing probiotics. Zhao *et al*. (2012) reported no significant differences in haemocyte counts of sea cucumber after 30 days of probiotic feeding. The findings in this study can be attributed to the ineffectiveness of the candidate probiotics to influence the

production of circulation haemocytes in the abalone immune system.

In this study, significant interactions were observed between the factors handling and dietary treatment on immune parameter i.e., haemocyte count after four month at HIK abalone farm (Chapter 3, Figure 3.4). A relationship has been found between increased stress and reduced immune competency in abalone which leads to periods of bacterial infections and mortality (Martello and Tjeerdema 2001; Malham *et al*. 2003; Cheng *et al*. 2004a, b, c, d, e). Thus, there is a benefit in studying the combined effect of probiotics and a wide range of stressors on the haemocyte count.

There was no significant interaction observed in this study, between dietary treatment and handling stressor on phagocytosis counts after four and eight months at HIK abalone farm (Chapter 3, Figure 3.3). Phagocytosis is mainly instrumental in the immune defence of invertebrates and vertebrates (Day *et al*. 2010). A decrease in phagocytotic rate is an indication of deteriorating cellular immunity, and in abalone it is a typical response to a considerable amount of stress (Cheng *et al*. 2004a; Chen *et al*. 2005; Travers *et al*. 2008a).

In this study, significant differences between dietary treatment and handling stressor on haemocyte counts showed simultaneously with no significant interaction between these two factors on phagocytosis count after four months, which may mean that circulating haemocytes might be a supportive evolutionary adaptation (Day *et al*. 2010) to compensate the relationship of phagocytotic count under handling stress. Phagocytotic actions by probionts have been reported in many species of fish (Irianto and Austin 2002; Panigrahi *et al.* 2004; Brunt *et al.* 2007; Pieters *et al.* 2008). For example, Pirarat *et al.* (2006) significantly stimulated phagocytotic activity of tilapia (*Oreochromis niloticus*) in two weeks of feeding *Lactobacillus rhamnosus*. Based on the immune parameters measured in this study, doubling the dose of probiotics showed no improvement of these aspects of the abalone immune response. Magda *et al*. (2011) reported an increase in phagocytotic activity with increased probiotic dose from 10^5 to 10^7 CFU g^{-1}, although increased probiotic dose to 10^9 CFUg^{-1} reduced phagocytotic activity but remained significantly high compared to fish fed control diet

Conclusion

Abalone fed a combination of probiotics showed improved growth compared to those fed a control diet under the conditions of one of the two farms. Thus, severity of stress imposed on the abalone in this study might have covered up the efficacy of probiotics as probiotics microorganisms are also known to alleviate stress. Thus, probiotic used in this study appeared not to resist negative effects generated from the application of handling stress on abalone. Growth improvements prompted by probiotics on abalone which were not subjected to additional handling on both farms in the current study, implies possible reduction in time needed for abalone to achieve market size. In the present study, probiotic-supplemented diets were not consistent to significantly influence abalone immunity based on immune parameters measured on both farms. Further studies are needed to demonstrate the effect of different measures of stress and the possible interaction with dietary probiotics.

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