PROBIOTIC EFFECT OF *VIBRIO MIDAE* SY9, *CRYPTOCOCCUS* SP. SS1 AND *DEBARYOMYCES HANSENII* AY1 ON THE GROWTH AND DISEASE RESISTANCE OF FARMED *HALIOTIS MIDAE*

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town, South Africa.

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ABSTRACT

Although the South African abalone, *Haliotis midae*, has been commercially harvested since 1949, successful cultivation of this species only began in the 1980s. Since then, the abalone mariculture industry has expanded dramatically and currently produces between 500 and 800 tons of abalone per year with a net farm gate value of approximately R125 million. However, disease has had a severe impact on the international aquaculture industry and is anticipated to become an increasingly important factor, together with the slow growth rate of *H. midae*, that will negatively impact on the further development and success of the local abalone mariculture industry. Thus, the future of *H. midae* mariculture in South Africa depends in part on the development of methods to enhance the growth rate and disease resistance of farmed *H. midae*. Erasmus *et al.* (1997) showed that abalone enteric bacteria enhanced digestive efficiency by secreting polysaccharolytic enzymes and it was suggested from these results that these bacterial enzymes could affect the growth rate of abalone. Furthermore, an overwhelming body of evidence has shown that probiotic microorganisms can significantly improve the growth rate and disease resistance of aquacultured animals. The aim of this study was to isolate enteric microorganisms from *H. midae* that are capable of hydrolyzing the various protein and starch substrates included in formulated abalone feeds. Upon identification, the selected microbes would be tested for their ability to colonize the digestive tract, improve digestion, growth and immunity of farmed *H. midae*.

A number of different microorganisms were isolated from the digestive tract of *H. midae* that are capable of utilizing the protein and starch substrates included in commercial abalone feeds. Isolates SY9 and SS1 were selected for further characterization as they exhibited enhanced protease and amylase activity respectively on all of the protein and starch substrates tested. Furthermore, a second yeast (isolate AY1), previously isolated from *H. midae* in our laboratory, also hydrolyzed all of the protein and starch substrates tested. Based on these findings and the knowledge that yeasts and bacteria have immunostimulatory properties, these three strains were selected for further investigation.

The 16S rRNA gene sequence analysis, together with a comparison of the physical and phenotypic characteristics of bacterium SY9 to that of related bacteria, suggested that it was a new species of the genus *Vibrio*. The bacterium was designated *Vibrio midae* SY9. The 18S rRNA gene sequence analysis, together with a comparison of the physical and phenotypic characteristics of yeast strains SS1 and AY1 to that of related yeasts, suggested that yeast isolate SS1 belonged to the genus *Cryptococcus* and yeast isolate AY1 belonged to the genus *Debaryomyces*.

A growth curve analysis established that all three strains could be successfully cultivated to high cell densities. A mixture of the three putative probionts was added to feed so as to achieve a final culturable cell concentration, of each probiont, of approximately 1.0×10^7 cfu g^{-1} of dried feed. We showed that *H. midae* continuously fed the probiotic-supplemented diet have an improved growth rate compared to animals not fed probiotics. The growth rate of small (20 mm) and large (67 mm) abalone was improved by 8% and 34% respectively in two separate eight-month farm trials. *In situ* protease and amylase assays showed that probiotic treatment significantly increased ($P<0.05$) both protease and amylase activity in the intestinal region of the digestive tract of animals fed the probiotic-supplemented feed. This correlated with a significant increase ($P<0.05$) in the amount of protein digestion and absorption measured in this region of the abalone gut.

An assessment of different immune parameters clearly demonstrated the immunostimulatory effect of the three putative probionts included in the diet fed to farmed *H. midae*. Furthermore, enhancement of the immune response in *H. midae* resulted in increased survival following

challenge with the pathogenic bacterium, *Vibrio anguillarum*. Seven days after challenging with *V. anguillarum*, the probiotic-fed animals had a 62% survival rate compared to a 25% survival rate for non-treated animals. Furthermore, the number, phagocytic rate and respiratory burst activity of circulating haemocytes was significantly higher ($P<0.05$) in probiotic-treated animals compared to non-treated animals following challenge with *V. anguillarum*. Histological analysis showed that the digestive glands of animals receiving probiotics were bacteria-free, whereas the digestive glands of 70% of the animals receiving the non-supplemented feed had a high bacterial load.

Viable cell counts and/or *in situ* hybridization was used to evaluate the colonization potential of each probiont in the digestive tract of *H. midae*. After feeding *H. midae* with the probiotic-supplemented feed for three weeks, the number of culturable probiotic cells ranged from 10^6 to 10^7 cfu g^{-1} of crop/stomach and intestinal tissue. The 16S and 18S rRNA gene sequencing confirmed the identity of the presumptive probiotic strains isolated from the digestive tract. The mini-Tn10-*gfp-kan* transposon, delivered from pLOFKm*gfp*, transposed at a high frequency (2.4 x 10^5 cfu ml^{-1}) in *V. midae* SY9, resulting in single random chromosomal insertions. A direct comparison of viable cell-counts and *in situ* hybridization using the *gfp-kan* probe, showed that plate counts of *V. midae* SY9 were lower in the digestive tract of *H. midae* compared to estimates from *in situ* hybridization. There was a significant decrease ($P<0.05$) in the cell numbers of each probiont two days after cessation of feeding with the probiotic-supplemented feed. This correlated with a significant decrease ($P<0.05$) in intestinal protease and amylase activity in abalone previously fed probiotics. A Pearson product movement correlation analysis revealed a significant positive correlation between *Cryptococcus* sp. SS1 and amylase activity ($r^2 = 0.681$) and *V. midae* SY9.8 and protease activity ($r^2 = 0.711$) in the intestine of *H. midae*.

ABBREVIATIONS

α	alpha
β	beta
γ	gamma
λ	lambda
π	PI
µ	micro
µCi	micro Curie(s)
µg	micro gram(s)
µl	microliter(s)
µg	microgram(s)
µm	micrometer(s)
µM	micromolar
x g	gravity acceleration
$	US dollars
R	SA rand(s)
°E	degrees east
°S	degrees south
°C	degrees Celsius
%	percentage
ASW	artificial sea water
bp	base pairs
C	cytosine
Ci	Curie
cm	centimeters
cpm	counts per minute
CTAB	cetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dCTP	deoxy-cytosine 5'-triphosphate
dH_2O	distilled water
dNTP	deoxy-ribonucleoside triphosphates (dATP, dCTP, dTTP or dGTP)
dsDNA	double stranded DNA
EDTA	ethylenediaminetetra-acetic acid
g	grams
G	guanine
h	hour(s)
hrs	hour(s)

k	kilo
kb	kilobase(s)
kDa	kilodalton(s)
kg	kilogram(s)
L	liter(s)
LA	Luria agar
LB	Luria broth
m	meter(s)
M	molar
MA	marine agar
MB	marine broth
Mb	mega base pairs
mg	milligram(s)
min	minutes
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
mol	mole(s)
ng	nanogram(s)
nm	nanometer(s)
O/N	overnight
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
rDNA	ribosomal DNA
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
s	second(s)
SDS	sodium dodecyl sulphate
S.E.	standard error
sp.	species
SSC	sodium chloride tri-sodium citrate buffer
STE	sodium chloride tris-EDTA buffer
T	time
^{T}	type strain
TAE	tris-acetate-EDTA buffer

TE	tris-EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
U	units
UV	ultra violet
v	volume
w	weight
WBA(B)	wash buffer A or B
YPD	yeast peptone D-glucose

CHAPTER 1

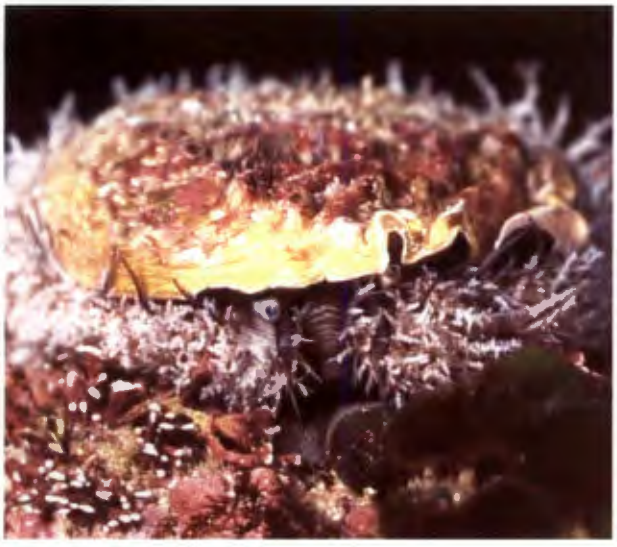
GENERAL INTRODUCTION

CONTENTS

1.1 The Biology of *Haliotis midae*

1.1.1 Classification

Abalone are marine molluscs belonging to the phylum Mollusca, a diverse group of organisms that includes, amongst others, the chitons, clams, snails, squids and octopuses (Bevelander, 1998). The molluscs are non-segmented, soft-bodied invertebrates with a mantle cavity that typically contains the gills, an anterior head with a radula and a large muscular foot. Abalone, together with the snails, slugs and nautibranchs, are members of the class Gastropoda. Members of this class either have a one-piece shell or no shell at all, as is in the nautibranchs, and move by means of a broad muscular foot.



(a)

(b)

Figure 1.1. *Haliotis midae* (a) and various Haliotid shells (b) showing the characteristically flat, ear-shape with the characteristic row of respiratory pores on the left of each shell. The cephalic tentacles and the eyes of *Haliotis midae* (a) are seen protruding at the anterior end of the shell. Protruding around the rest of the shell margin is the epipodium, edged by small sensory lobes and tentacles. Photograph of *H. midae* courtesy of Robb Tarr © Marine and Coastal Management.

Abalone belong to the family Haliotidae and the genus *Haliotis*, which means "sea ear" and refers to the flattened shape of the shell. Indeed, the most conspicuous part of the abalone is the shell, which has a greatly reduced spire and an enormously enlarged aperture that almost covers the entire body of the animal. The flattened shell also has a distinctive row of respiratory pores along the left side (Bevelander, 1998; Branch *et al.*, 1994) (Fig. 1.1).

1.1.2 Distribution

There are approximately 100 species of abalone worldwide that are found in tropical, temperate and cold waters, ranging in depth from the low tide line to depths exceeding 30 meters (Bevelander, 1998). However, of all these species, only fifteen are commercially exploited (Britz, 1990). In South Africa, there are five endemic species of *Haliotis* (*H. midae, H. parvum* L., *H. spadicea*, *H. queketti, H. speciosa*) that occur along specific regions of the coastline (Branch *et al.*, 1994) (Fig. 1.2). Of these, only *Haliotis midae*, known locally as 'perlemoen', is of commercial significance and has been commercially exploited since 1949 (Tarr, 1992). This species is distributed from St Helena Bay (32° 45'S; 18° 10'E) on the west coast to just north of Port St Johns (31° 40'S; 29° 35'E) on the East coast of Southern Africa (Simpson, 1994) (Fig. 1.2).

1.1.3 Habitat

Juvenile abalone are commonly found under intertidal boulders or under the spines of sea urchins where they feed mainly on a diet of red crustose algae and/or *Ulva* (Sales and Britz, 2001). Sea urchins play a vital role in the ecology of juvenile abalone by providing them with protection from predators and assisting them with the recruitment of food. Adult abalone are commonly found living in rock crevices or on exposed positions of shallow reefs where there is strong wave action (Branch *et al.*, 1994). They attach themselves firmly to rock surfaces with their strong muscular foot and feed on pieces of drifting seaweed, which they frequently trap by clamping down their foot. Seaweeds are the major food source for adult wild abalone, with *Plocamium*, *Laminaria* and *Ecklonia* being the preferred seaweed species (Erasmus, 1996). However, *Ecklonia maxima*, known locally as "sea bamboo" or kelp, is the largest and the most abundant

seaweed species on the Southern West Coast of South Africa (Branch *et al.*, 1994) and therefore forms the bulk of the adult wild abalone diet. This fast-growing seaweed forms extensive underwater forests, known as 'kelp forests', that provide a sheltered habitat for many marine organisms, including *Haliotis midae,* which occur in dense populations amongst these kelp beds.

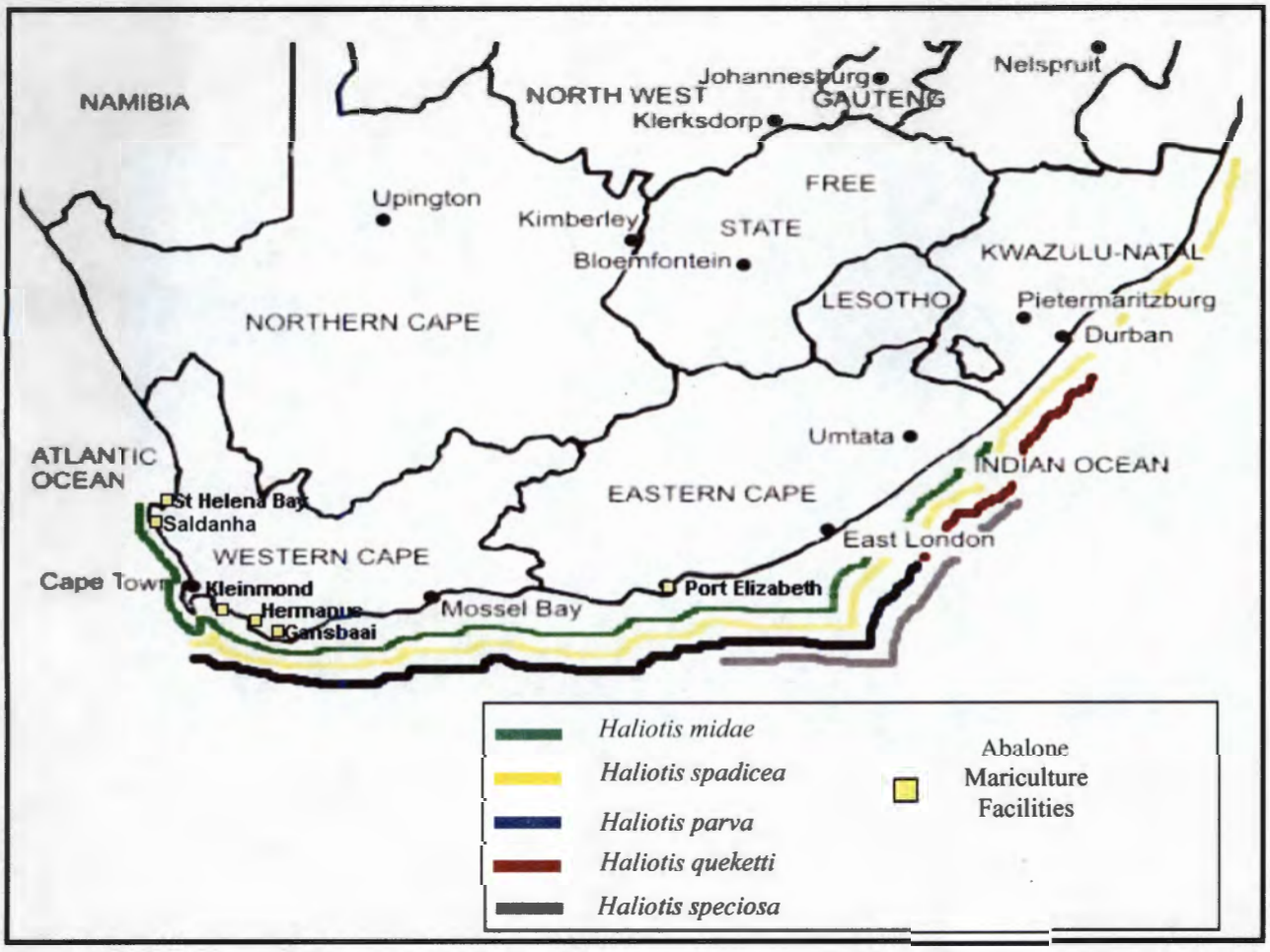


Figure 1.2. Map showing the distribution of endemic Haliotid species along the coastline of Southern Africa and the location of the various commercial abalone farms. There are approximately 12 commercial farms in production, with the majority occurring in the Hermanus and Gansbaai area (figure adapted from: http://web.uct.ac.za/depts/zoology/abnet/safrica.html).

1.2 Commercial abalone fishery

Abalone have been a highly prized food source since ancient times and as a result, resources have been greatly reduced due to over-fishing and poaching (Bevelander, 1998). Currently, the major suppliers of wild abalone are Mexico, California, Australia, New Zealand, South Africa, China, Taiwan and Japan. Conversely, the primary market for this seafood delicacy is the Far East, where high demand for this "caviar in a shell" is said to be due to the subtly flavored meat and its presumed aphrodisiac properties.

The high demand for abalone has led to the virtual collapse of the commercial industry in many countries, with some countries, such as the USA, closing down the commercial fishery completely (Gordon and Cook, 2001). According to recent statistics from the Food and Agricultural Organization (FAO) of the United Nations, abalone fisheries of the world have declined by as much as 35 percent over the last ten years. Worldwide, abalone landings were estimated to be approximately 10 150 metric tons in 1999 compared to 14 830 metric tons in 1989 (Gordon and Cook, 2001).

In South Africa, the annual commercial landings of *Haliotis midae* reached a peak of approximately 3 000 tons around 1965, then declined rapidly to approximately 700 tons in 1970. This resulted in the implementation of a quota system, total allowable catch (TAC), which limited the amount of abalone that could be collected each year by commercial fishermen (Tarr, 1992). Following the implementation of these quotas, the annual commercial catch remained relatively constant, around 700 tons per year, up until 1995. Subsequently, the TAC has been drastically reduced due to a steady decline in the abundance of natural stocks. This rapid decline in natural stocks has partly been due to ecological changes that have occurred along the South African coastline, but the main reason for the decline is due to a marked increase in poaching. As a result, local authorities in South Africa were forced to closed down the recreational abalone fishery in 2003 and are threatening to close down the commercial industry.

Worldwide, the decline of natural stocks, together with an increase in demand for abalone meat, particularly in the Far East, has resulted in the investigation of alternative strategies to increase

the supply of abalone. However, of all of the strategies that have been investigated, aquaculture seems to be the most feasible option for significantly increasing abalone supplies (Britz, 1990).

1.3 Global status of aquaculture

Aquaculture has been defined as "the cultivation or rearing of aquatic animals and/or plants in a controlled environment for all or part of their lifecycle" (http://www.siu.edu/~readi). Similarly, marine aquaculture, 'mariculture', has been defined as "an effective means for intensive seafood production under 'controllable' conditions" (Olafsen, 2001).

Aquaculture is a practice that has been performed for centuries, with oysters being cultured in Rome and Gaul, and carp reared in ponds in China as early as the fifth century BC (Bardach *et al.*, 1972). However, the world aquaculture industry has expanded dramatically since these times, especially when looking at the cultivation of fish, shrimps and bivalves, and today it is considered as one of the fastest-growing food production sectors in the world (http://www.siu.edu/~readi). This rapid expansion has been closely related to population growth; diminishing wild-stocks due to habitat loss, over-fishing and poor stock management; and an increase in consumer demand for selected species.

FAO statistics indicate that approximately three quarters of the major marine fish stocks are presently harvested to their maximum limit (http://www.siu.edu/~readi). Furthermore, it has been estimated that approximately 33% of the US fish stocks are over-fished or depleted (Hilborn *et al.*, 2003). In contrast, global aquaculture production has increased by almost 40% over the same period and in 1998 contributed more than 31% to the total annual global seafood supply compared to 19% in 1990. Consequently, many countries regard aquaculture as the only real solution to help sustain a growing demand for both freshwater and marine organisms; to help supplement wild stocks; and to help serve as a producer of low-cost, high-protein food. Furthermore, this industry is growing approximately six-times faster in developing countries compared to developed countries, demonstrating the potential that this industry has for supplying low-cost, high-quality protein.

1.3.1 Global status of abalone aquaculture

Although aquaculture practices have existed since ancient times, intensive abalone aquaculture is still a fairly young enterprise (Britz, 1990). Japan was one of the first countries to develop and pioneer abalone aquaculture. This practice began in the 1960s and started mainly with the aim of restocking wild stocks due to over-exploitation (Erasmus, 1996; Simpson, 1994). Today Japan has a very successful abalone aquaculture industry, producing approximately 200 metric tons of cultured abalone per year. Overall, world abalone culture has expanded dramatically (over 600%), with a production figure of 8 696 metric tons produced in 2002 compared to only 689 metric tons produced in 1987 (Gordon and Cook, 2001).

The main countries producing cultured abalone are California (USA), Japan, China, Taiwan, Australia/Tasmania, Chile, and South Africa, with over 15 species of abalone being cultured. The major consumer of both cultured and wild-harvested abalone is the Far East, with current supplies well below the growing demand for this product. Figures estimated for 2004 indicate that the supply of abalone, for both wild harvests and cultured abalone, will be about 15 000 metric tons, but the potential demand is likely to remain over 20 000 metric tons, resulting in a substantial shortfall (Fig. 1.3) (Gordon and Cook, 2001). In the Far East, this highly prized food can fetch prices of between $34 – 36 per kilogram (in shell weight) (Stanford, 2004). These high market prices and the growing demand for this product are therefore a great incentive for entrepreneurs to become involved in this industry.

1.3.2 Status of abalone aquaculture in South Africa

Contrary to some of the major abalone producing countries, such as China and Japan, South Africa has a very young abalone aquaculture industry. Initially, South Africans were very reluctant to get involved in the industry. The main reasons for this initial reluctance were twofold. Firstly, wild populations of *H. midae* have very slow growth rates, taking between 8 and 12 years to reach a legal harvestable size. Secondly, wild populations feed mainly on a diet of kelp, a diet which prospective abalone farmers thought would be insufficient for a full-scale production process if it was to be freshly harvested (Britz, 1990). However, interest in this

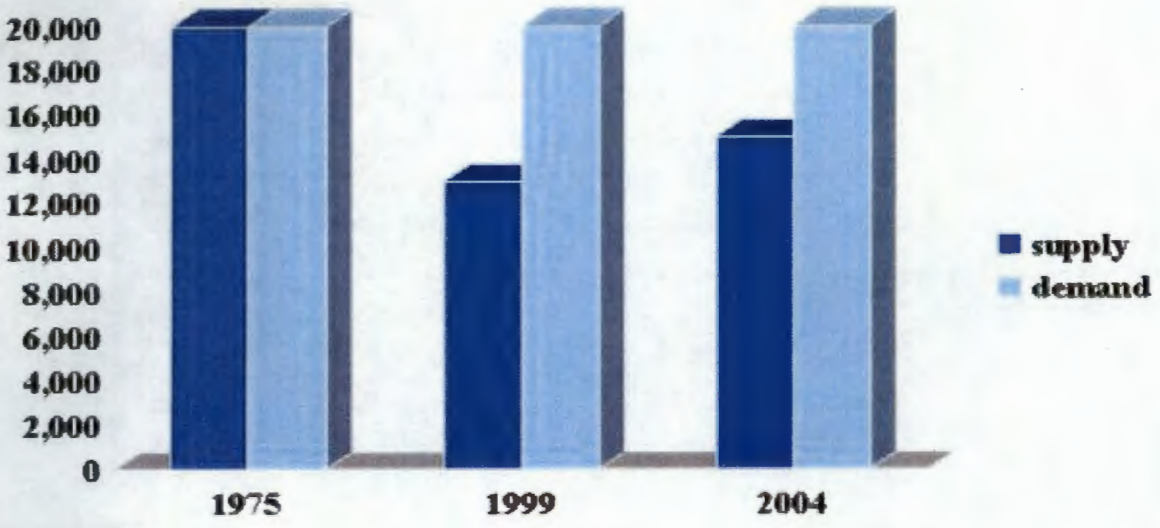


Figure 1.3. Abalone supply and demand in 1975, 1999 and 2004 (estimated figures). In 1975 there was no major shortage of product and both supply and demand balanced at around 20 000 metric tons. In 1999 there was a shortfall of 7 000 metric tones and it is estimated that this shortfall will be approximately 5 000 metric tons in 2004 (Gordon and Cook, 2001).

industry was renewed when it was shown in 1981 that captured specimens of *H. midae* could be successfully spawned to produce spat and juveniles (Sales and Britz, 2001). Furthermore, research showed that the growth rate of *H. midae*, when kept in captivity, was faster than in the wild (Cook, 1990). Coupled to these findings was the development of a market for small "cocktail" sized abalone (approximately 50 mm shell length) in the Far East. These research findings, together with market developments in the Far East and the success of the abalone aquaculture industry in other countries, led to the development of the industry in South Africa.

The commercial abalone farming industry in South Africa essentially began in the 1990s and has grown rapidly over the last few years. Rapid expansion of this industry was assisted by a close relationship established between local abalone farmers and research institutions in South Africa (Sales and Britz, 2001). As a result of this close interaction, approximately 14 abalone farms have since been established along the South African coastline, stretching from Port Nolloth on the Atlantic coast to East London on the Indian Ocean (Fig. 1.2). These land-based farms are

situated very close to the shoreline where they have access to large quantities of good-quality sea water. Once the abalone are large enough, they are grown-out in large flow-through raceways (Fig. 1.4) or recirculation systems. It typically takes 4 - 5 years for abalone to grow from spat to a market size of approximately 100 grams.

Figure 1.4. Typical commercial abalone farm raceway system used for growing-out abalone before being sent to market. Abalone are housed in baskets which are suspended in raceways and fed a natural seaweed diet such as kelp (as seen in the close-up) or artificial feeds. Fresh seawater is continuously pumped through the raceways and the water is continuously aerated (http://www.abnet.co.za).

It has been estimated that approximately R150 million has been invested in the industry (excluding the cost of land) since its establishment in South Africa and that the industry is producing approximately 500 – 800 tons of abalone (in shell weight) per year (Sales and Britz, 2001). Thus, although the industry is still young, farmed abalone production already exceeds wild harvests, which were estimated to be around 314 tons in 2002 (Statistics from a report given at the 6th Conference of the Aquaculture Association of Southern Africa in 2002). Furthermore,

the farm gate value in 2003 was estimated to be approximately R125 million (http://www.developtechnology.com), indicating the substantial economic value of this industry and its potential for further growth and investment.

1.4 Aquaculture and its associated problems

Abalone farming, as with any other intensive animal production practice, involves the rearing of large numbers of animals at densities that far exceed that commonly found in nature. These high stocking densities, together with other farming practices, can have an adverse effect on an animal's health if incorrectly managed. The types of stress that farmed animals are exposed to can be classified as either physical (water temperature and oxygen availability, the presence of chemical pollutants, and handling of animals) or biological (food quality and quantity, competition for space and the presence of opportunistic pathogens) (Malham *et al.*, 2003). The majority of these stresses can be controlled if aquaculture facilities are stringently managed. However, this is often very difficult to achieve on large farm operations and as a result, animals are periodically subjected to stress, resulting in decreased growth rates and outbreaks of disease.

1.4.1 Physical stress

The effects of physical stress (such as pH, high ammonia and nitrite, temperature and low dissolved oxygen) on marine organisms and more specifically, farmed abalone, has been clearly documented (Cheng *et al.*, 2004ab; Harris *et al.*, 1998ab; Martello *et* al., 2000; Martello and Tjeerdema, 2001). Studies on both abalone and oysters have also clearly shown that mechanical stresses such as sorting, grading and transport, all of which are common farming practices, can lead to a reduction in an animal's ability to respond to infection (Lactose *et al.*, 2002; Malham *et al.*, 2003). Furthermore, the effects of hypoxia (low dissolved oxygen) and high ammonia on the giant freshwater prawn, *Macrobrachium rosenbergii* (Cheng *et al.*, 2002; Cheng and Chen, 2002), the effects of hypercapnic hypoxia (low dissolved oxygen and high carbon dioxide) in the Atlantic blue crab, *Callinectes sapidus* (Holman *et al.*, 2004), and the effects of environmental contaminants such as fluoranthene and cadmium on the common mussel, *Mytilus edulis* (Coles *et al.*, 1994; Coles *et al.*, 1995), have also been documented. All of these studies have clearly

shown how physical stress can suppress the immune system of an animal, making it more susceptible to disease.

Opportunistic pathogenic bacteria are commonly found in seawater and these bacteria rapidly take advantage of poor water conditions (Skjermo and Vadstein, 1999) and immune-compromised animals. Cheng *et al.* (2002) showed that low dissolved oxygen in the tank water of the giant freshwater prawn, *M. rosenbergii*, depressed the immune system of this animal and consequently increased its susceptibility to infection by the pathogenic bacterium, *Enterococcus*. Furthermore, Cheng and Chen (2002) showed a similar effect on *M. rosenbergii* subjected to high ammonia conditions and Holman *et al.* (2004) demonstrated that hypercapnic hypoxia reduced the ability of *C. sapidus* to clear *Vibrio campbellii* from its haemolymph following infection. Lactose *et al.* (2001) also showed that the juvenile oyster, *Crassostrea gigas*, was more susceptible to infection by the pathogen, *Vibrio splendidus*, when subjected to mechanical stress. Cheng *et al.* (2004ab) showed that high water temperatures and high ammonia levels led to a reduction in the innate immunity of the Taiwan abalone *Haliotis diversicolor* supertaxa making it more susceptible to *Vibrio parahaemolyticus* infection. Therefore, physical stress, and ultimately, the susceptibility of farmed animals to opportunistic pathogenic bacteria, are of paramount importance to aquaculturists.

1.4.2 Biological stress

Biological stresses are caused by such factors as the quality and quantity of feed added to the water, competition between animals for space and/or sexual partners and the presence of opportunistic pathogens (Malham *et al.*, 2003).

On most aquaculture facilities, animals are reared at densities that far exceed that commonly found in nature. As a result of these high stocking densities, feed needs to be added to tanks at a high concentration in order to sustain and/or enhance the growth of the animals being cultured. Consequently, water quality needs to be carefully monitored as it can quickly deteriorate under these conditions. Furthermore, most of the feeds utilized are artificial feeds that are high in protein, resulting in an excellent medium for the growth of opportunistic pathogens once added

to the pond or tank water (Olafsen, 2001). Rapid colonization of artificial feeds by bacteria has been shown to accelerate their breakdown, thus exacerbating water quality problems (Bissett *et al.,* 1998). Furthermore, artificial feeds can act as reservoirs for pathogenic bacteria that can then be introduced into the culture system and possibly infect susceptible animals (Ringø and Birkbeck, 1999). Another disadvantage of the use of artificial feeds is the loss of the normal, beneficial bacteria that are often found associated with natural feeds. These beneficial bacteria can play an important role in the establishment of a normal non-opportunistic microbial community in the gut of farmed animals as well as in the water. However, natural feeds can also be a source of entry for pathogenic bacteria. Furthermore, the continual use of large volumes of natural feed is not always possible, whereas the use of artificial feed allows for a consistent supply of a cost-effective, nutritionally complete diet (Bissett *et al.,* 1998) that can be strictly formulated to meet the physiological needs of the animal being cultured (Ringø and Birkbeck, 1999). Consequently, the successful development and continuation of most aquaculture practices is believed to be dependent on the development of high quality, nutritionally complete feeds, promoting research in this field.

Physical and biological stresses are therefore intimately linked, with deterioration in either physical or biological factors adversely affecting animal growth and resulting in the possible onset of disease as a result of opportunistic bacteria. Bacteria play a vital role in aquaculture systems and have therefore been the subject of extensive research.

1.4.3 The role of microorganisms in aquaculture

As previously mentioned, the global aquaculture industry has expanded dramatically over the last 20 years. However, this rapid expansion has not occurred without the general problems associated with intensive rearing systems. As pointed out by Olafsen (2001), "disease and disease control is an inherent part of any intensive animal production system". However in an aquatic environment, where open production systems are commonly utilized, problems with disease are intensified due to the close relationship shared between bacteria and the host animal (Olafsen, 2001). This close relationship ensures that bacteria are continually in contact with all surfaces of the host animal, including the gills, skin and gastrointestinal tract, resulting in the

development of intimate relationships in which host and bacteria are often uniquely adapted (Ringø and Birkbeck, 1999).

In aquatic environments, microorganisms are assumed to be either autochthonous, belonging to the normal bacterial flora, whereas others are regarded as being allochthonous, possibly leading to the development of disease in the host animal (Ringø and Birkbeck, 1999). The type of relationships that exist between bacteria and their invertebrate hosts can be described as either commensalism, mutualism, predation or parasitism (Erasmus, 1996).

In a commensal relationship, the invertebrate host is not harmed by the presence of the bacteria and does not benefit from its presence. This type of relationship has been demonstrated in a number of invertebrates, such as prawns and molluscs, where microorganisms associated with the digestive tract of the host have no effect on digestion in the host (Erasmus, 1996). In a mutualistic relationship, both the invertebrate host and the resident bacteria benefit from the association. Bacteria could benefit the host by assisting in the digestion of ingested food, achieved by adding to the pool of enzymes within the digestive tract (Erasmus, 1996; Vitalis *et al.*, 1998). In turn, the bacteria benefit from residing in a nutrient rich environment. However, bacteria can also be utilized as a protein source. Such a relationship is termed predation. Lastly, there is a parasitic relationship, where the bacteria have an adverse effect on the host organism. Bacterial pathogenesis of marine invertebrates has been well documented and is caused by a wide variety of bacterial species. Bacteria often involved in epizootics of fish, molluscs and crustaceans belong to the genera *Enterococcus, Pseudomonas, Aeromonas, Flavobacteria* and *Vibrio* (Cheng *et al.,* 2002; Chythanya *et al.,* 2002; Nikoskelainen *et al.,* 2001).

1.4.4 Documented outbreaks of disease in aquaculture

In an aquatic environment, animals are continually exposed to a wide variety of microorganisms, which include both beneficial and/or pathogenic microorganisms. Disease outbreaks are an inherent part of aquatic environments, as in terrestrial environments, and occur naturally in the wild as well as in aquaculture facilities. However, in the wild the occurrence of disease often goes largely unnoticed, but due to the intensive nature of aquaculture facilities and their

economic importance, disease outbreaks are far more pronounced. In fact, disease outbreaks are considered as one of the major obstacles preventing the successful development and continuation of aquaculture production in many countries (Verschuere *et al.*, 2000). Since the establishment of intensive aquaculture, disease outbreaks have been recorded that have had catastrophic effects on aquaculture production, and in some instances, the organism being cultured has been almost completely wiped out, having a severe impact on the local economy.

The giant freshwater prawn, *M. rosenbergii*, is a commercially important cultured species in many parts of the world, particularly in Taiwan, with farmed production of this species reaching a peak of 16 196 tons in 1991. However, production of this species was dramatically reduced in subsequent years and in 1999 only 7 223 tons were produced (Cheng *et al.*, 2002). This dramatic decline in production was due to disease outbreaks caused by yeast infections in the cooler seasons and bacterial infections in the warmer seasons of the year. One of the bacteria implicated in these mass mortalities was *Enterococcus*, which was isolated from diseased prawns (Cheng *et al.*, 2002).

Similar disease outbreaks have also occurred in the shrimp culture industry, resulting in a dramatic reduction in production figures over the last decade (Bachère, 2000; Gullian *et al.*, 2004; Vershuere *et al.*, 2000). Shrimp culture has existed since the 1970s and accounts for roughly 30% of the total shrimp supplied to world markets. Total production in earlier years reached peaks of approximately 712 000 metric tons (Bachère, 2000), indicating the economic value of this industry. However, this industry has been severely plagued by disease outbreaks, caused mainly by viruses and bacteria. In Taiwan for example, production decreased by 60% over the period of 1987 to 1988 due to both bacterial and viral infections and in Ecuador, shrimp production decreased by 65% in the year 2000 due to a white spot syndrome virus (WSSV) infection (Gullian *et al.*, 2004). Some of the bacterial species that were implicated in these disease outbreaks include *Vibrio harveyi, Vibrio anguillarum, Vibrio parahaemolyticus* and *Vibrio vulnificus*.

Molluscan aquaculture has also been severely affected by infectious diseases, with viral, bacterial, rickettsial, chlamydial and protozoan pathogens implicated in mass mortalities (Mialhe

et al., 1995). The oyster farming industry, which has existed since ancient times, has been dramatically affected by mass mortalities for many years. In the 1970s, European stocks of the cupped oyster, *Crassostrea angulata,* originally imported from Portugal, were almost completely wiped out due to a suspected Iridovirus infection (Bachère *et al.,* 1995; Roch, 1999). Following the decimation of these stocks, the Pacific oyster, *Crassostrea gigas,* was introduced into Europe. However, this species is presently suffering from several diseases, with mass mortalities of oyster larvae caused by a Herpes-like virus reported in both France and New Zealand (Bachère *et al.,* 1995).

Bacterial and rickettsial infections have also had a severe impact on the abalone mariculture industry. Withering Syndrome (WS), caused by an intracellular Rickettsiales-like prokaryote, is a chronic wasting disease responsible for mass mortality of the black abalone, *Haliotis cracherodii,* found on the Channel Islands of Southern and Central California (Moore *et al.,* 2001). This disease caused a virtual collapse of black abalone populations, with mortalities greater than 90% recorded in some areas. Bacterial disease was also responsible for mass mortalities of the Taiwanese abalone, *Haliotis diversicolor* supertaxa, in 1998 (Liu *et al.,* 2001). Diseases in aquatic environments are therefore highly prevalent and are further complicated due to the use of intensive rearing systems, poor feed quality and overfeeding, and movement of spat between different geographical regions or countries. It can have catastrophic effects on cultured organisms, their trade and hence the economy. Consequently, methods for disease prevention and cure are of paramount importance to aquaculturists and several methods for disease prevention are presently being investigated.

1.5 Probiotics

Disease is a major problem in the aquaculture industry and several strategies have been proposed for its control. Broad-spectrum antimicrobials have been extensively used as a means of disease control on many aquaculture facilities and unfortunately still remain the method of choice for many farmers (Gram *et al.,* 2001). However, excessive antimicrobial use, in both aquaculture and agriculture, can lead to the emergence of bacterial resistance (Verschuere *et al.,* 2000), as has already occurred in the aquaculture industry (Ringø and Birkbeck, 1999). Furthermore, fear

that this resistance could spread to human pathogens has recently led to the European Union banning the use of several antibiotics in animal husbandry (Gram *et al.,* 2001). Consequently, greater emphasis has been placed on improved husbandry through better nutrition, improved water quality and lower stocking densities, and the use of vaccines and non-specific immunostimulants, such as β-glucans or laminarin, and/or the use of non-pathogenic bacteria as probiotic control agents, including the use of prebiotics to select for beneficial microorganisms (Gram *et al.,* 1999; Gullian *et al.*, 2004; Ringø and Birkbeck, 1999; Robertson *et al.*, 2000; Roch, 1999).

Improved water quality through microbial maturation is well known for its ability to regulate the composition of bacterial communities by selecting for desirable bacteria (Skjermo and Vadstein, 1999), a concept that has been used by ornamental fish culturists for many years. This concept was proposed by Vadstein *et al.* (1993) (reviewed in Skjermo and Vadstein, 1999) and is based on the principle of the 'r/K-concept'. Essentially, it considers microorganisms as either r-strategists (opportunistic organisms that are capable of rapid growth, resulting in the formation of unstable 'pioneer' communities) or K-strategists (non-opportunistic organisms that grow slowly and form stable, mature communities) (Ringø and Birkbeck, 1999). The above authors proposed that r-strategists are favored by high nutrient conditions as apposed to K-strategists, which have a high substrate affinity and are more competitive when substrate availability is low. This concept has been tested in a number of trials with fish and has proved successful when the formation of stable 'beneficial' bacterial communities are desired. Trials conducted with Atlantic halibut larvae, *Hippoglossus hippoglossus,* using matured water, showed a 76% improvement in larval survival. Similar trials with turbot larvae resulted in a 51% increase in weight over a 14 to 16 day experimental period, compared to larvae reared in membrane-filtered water (Skjermo and Vadstein, 1999). These results emphasize the advantage of introducing 'beneficial' bacteria into a system. Furthermore, the above authors speculated that these benefits could be enhanced in combination with other strategies, such as the use of vaccines, non-specific immunostimulants and probiotics.

Vaccines are currently being used to good effect for disease control in fish (Gram *et al.,* 1999; Nikoskelainen *et al.,* 2001). Their use in Norway over the period 1987 to 1997 resulted in a 50

metric ton per year reduction in the use of antimicrobial drugs (measured as active components) and an increase in production of farmed fish from 50 000 to 350 000 metric tons (Verschuere *et al.,* 2000). However, their use is limited to vertebrates, which have an acquired (memory-based) immune response as opposed to the innate (non-memory based) immune response of invertebrates. Effectiveness of vaccine use has also been found to be variable for juvenile fish, as they are not yet fully immunocompetent and do not always respond to being vaccinated (Gram *et al.,* 1999). Additionally, the administration of vaccines to large numbers of fish is impractical, as injection is sometimes the only effective route of administration.

Consequently, the use of probiotics for disease prevention and improved nutrition in aquaculture is becoming increasingly popular and has received a great deal of attention due to an increase in demand for environment-friendly aquaculture.

1.5.1 Definition of probiotics

An initial defense mechanism of an animal against infection is the ability of certain indigenous microorganisms to prevent the colonization and proliferation of opportunistic bacteria (Gomez-Gil *et al.*, 2000; Skjermo and Vadstein, 1999). Consequently, this concept has been extended to the introduction of beneficial or 'probiotic' bacteria to help prevent infection. Traditionally, the term 'probiotic' referred to Gram-positive bacteria belonging to the genera *Lactobacillus* or *Bifidobacterium* (Verschuere *et al.*, 2000). Dietary inclusion of these organisms has been shown to improve lactose digestion in people lacking lactase and has also been shown to have some immune enhancing effects (http://www.usprobiotics.org/). Elie Metchnikoff is regarded as one of the pioneers of probiotic research and first described probiotics as "microbes ingested with the aim of promoting good health" (Gomez-Gil *et al.*, 2000). In particular, he was referring to the inclusion of lactic acid bacteria in the human diet, with the aim of suppressing the detrimental effects of other deleterious organisms (Gatesoupe, 1999). Later, Fuller (1989) gave a more precise definition of probiotics as, "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Gomez-Gil *et al.*, 2000). However, Gatesoupe (1999) proposed that the above mentioned definitions are better suited to

humans and terrestrial animals, where the resident microbes exist in a more stable and constant habitat that is fairly independent of the external environment.

In the aquatic environment, there is a direct and constant interaction between the host and the immediate environment, leading to a direct interaction between microorganisms from the environment with both internal and external surfaces of the host animal (Ringø and Birkbeck, 1999). Furthermore, the host organism continually ingests microorganisms and it is believed that the types of microorganisms colonizing the digestive tract of the host are obtained directly from live and/or artificial feeds and from the surrounding water. Therefore, host-microbe interactions are far more complex and transient in aquatic environments and as such, the definition of probiotics has been amended.

Gatesoupe (1999) proposed an alternative definition of 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". Later, this definition was broadened by Gram *et al.* (1999) to: "a live microbial supplement which beneficially affects the host animal by improving its microbial balance", in order to include the skin and the gill microflora of the host, which they assumed to be equally important in disease prevention, together with the gut microflora. However, this definition was modified further by Verschuere *et al.* (2000) to encompass all aspects of modern aquaculture. Thus, an aquatic probiotic has been more specifically defined as "a live microbial adjunct, which has a beneficial effect on the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". According to this definition, the authors proposed that a probiont must be able to: prevent the proliferation of pathogens; aid in the digestion of feed; improve water quality; and/or stimulate the immune system of the host organism (Verschuere *et al.*, 2000).

1.5.2 Possible modes of action of aquatic probiotics

Several papers have been published in the literature documenting the successful use of probiotics in aquaculture. The majority of this research has shown that exogenous addition of specific

microorganisms can enhance growth and/or immunity of various target organisms. However, most of this research has failed to look at the exact mode of action of the probiotic being tested, with arguments regarding the specific mode of action often circumstantial (Verschuere *et al.*, 2000), as many of these arguments are based on results that have been obtained from *in vitro* assays, which have been extrapolated to *in vivo* results.

There are several modes of action which a potential aquatic probiotic could have. The possible modes of action of aquatic probionts as reviewed by Verschuere *at al.* (2000) are as follows: "production of inhibitory compounds; competition for chemicals or available energy; competition for adhesion sites; enhancement of the immune response; improvement of water quality; interaction with phytoplankton; source of macro- and micronutrients; and enzymatic contribution to digestion". All of these proposed modes of action require that the potential probiont is able to reach the location where the probiotic effect is required and is able to successfully colonize this region. Alternatively, the probiont must be continually added in sufficient quantities in order to exert the desired effect (Verschuere *et al.*, 2000).

1.5.3 Selection criteria of putative probionts for aquaculture

Selection criteria for putative probionts for aquaculture have largely been based on an organism's ability to produce antimicrobial metabolites, an experimental process which is often based on limited scientific evidence due to the lack of *in vivo* experiments (Gomez-Gil *et al.*, 2000; Vine *et al.*, 2004). This can easily result in the selection of inappropriate microbes that do not function optimally in the chosen environment and/or host organism. Verschuere *et al.* (2000) and Gomez-Gil *et al.* (2000) proposed that the following selection criteria should be used for selecting putative probionts for aquaculture:

(1) Background information on culture practices for the particular aquaculture farm/s should be extensively researched in order to identify possible problems or bottlenecks in the farming process, paying particular attention to the role of microorganisms in the environment. Possible problems could include slow

growth rates of the cultured organism, a problem often associated with the cultured abalone *Haliotis midae*, and/or the occurrence of disease on the farm.

(2) Suitable putative probionts need to be identified. Ideally, these strains should be isolated from a healthy host organism or from the environment in which the putative probionts are supposed to function.

(3) Putative probionts should be evaluated for their ability to out-compete potential pathogens or produce beneficial compounds, such as extracellular enzymes. This sort of screening process would involve doing *in vitro* antagonism tests, where known pathogens are exposed to putative probionts and the ability of the putative probiont to prevent growth of pathogens is evaluated. Candidate probionts can also be screened for their ability to break down complex compounds, such as polysaccharides and proteins. Additionally, the ability of the putative probiont to colonize and persist in the host organism should be evaluated.

(4) Pathogenicity of the putative probionts needs to be evaluated in order to ensure that no pathogenic effects can occur in the host organism.

(5) The effect of the putative probiont on the host organism needs to be evaluated. If the probiotic effect is presumed to be nutritional (candidate probiont produces extracellular enzymes which could assist the host organism with digestion of ingested food), then their effect on growth of the host organism needs to be evaluated. However, if the putative probiont has been selected for its ability to exclude potential pathogens, then *in vivo* challenge tests need to be conducted to detect probiotic effects, such as increased survival of the host organism following challenge with a known pathogen.

(6) Lastly, an economic cost benefit analysis needs to be conducted to determine the economic effects of administering probiotics.

1.5.4 Microorganisms effectively used as probiotics in aquaculture

The first documented use of probiotics appeared in the late 1980s (Verschuere *et al.*, 2000) and since then a great deal of effort has been devoted to this area of research. Initially, most of the probiotics tried and tested for aquaculture purposes were based on commercial preparations

designed for terrestrial animals (Gatesoupe, 1999). Strains that were tested included *Bacillus* sp., *Streptococcus faecium*, as well as various lactic acid bacteria, which have been successfully used as probiotics for humans and terrestrial animals. Although the use of these organisms appeared to improve growth and enhance resistance of the host organisms tested in an aquaculture environment, these studies were limited and the fate of the putative probionts in the gastrointestinal tract of these organisms was uncertain. However, these initial studies were very important as they highlighted the potential use of probiotics in aquaculture. Consequently, researchers have focused their attention on the isolation of autochthonous microorganisms with probiotic properties, as they are proposed to have a greater potential for effectively colonizing the gastrointestinal tract of the host organism (Gatesoupe, 1999). As a result, a wide variety of microorganisms have been isolated from a number of marine species and many of these microorganisms have been shown to be effective probionts. Some of the main observations that have been observed in the host organisms, following probiotic treatment, include enhanced growth, increased survival and/or decreased mortality following challenge with known pathogens of the host in question.

1.5.4.1 Probiotic use in fish

In fish mariculture, as with many other aquaculture processes, the hatchery is considered as the most vulnerable stage in the production process and is often plagued by high mortality rates. It is assumed that most of these mortalities are a result of uncontrolled growth of opportunistic and/or pathogenic bacteria (Verschuere *et al.*, 2000). Traditionally, antibiotics were extensively used to control the growth of opportunistic pathogens during this vulnerable stage of production, but due to the adverse effects of antibiotics on microbial communities and new legislation preventing antibiotic use, alternative means of disease prevention are now being employed (Vershuere *et al.*, 2000; Gram *et al.*, 2001). Consequently, the establishment of a stable, non-pathogenic bacterial flora at this early stage of development, through the use of probiotics, is considered as the best way to overcome this problem by acting as an effective barrier against colonization of opportunistic bacteria. A study carried out by Gatesoupe (1994) (reviewed in Verschuere *et al.*, 2000) clearly showed that the addition of lactic acid bacteria to the enrichment medium of rotifers, which are used as a live feed for the turbot larvae *Scophthalmus maximus*, reduced

mortality of the larvae and enhanced larval survival following challenge with a pathogenic *Vibrio* species. Additionally, the authors isolated large amounts of the putative probiont after feeding and suggested that the positive probiotic effect was due to the ability of the probiont to exclude the pathogen.

In adult fish, there is a significantly higher bacterial load in the digestive tract compared to the surrounding medium, with as many as 10^8 cells g^{-1} (Ringø *et al.*, 1995). The composition of the intestinal microflora of a number of fish species, including salmon, artic char and turbot has been investigated to gain a better understanding of the types of microorganisms colonizing the digestive tract. Several factors, including the effect of diet and other microorganisms on the intestinal microflora of these organisms has also been investigated (Ringø *et al.*, 1995; Ringø *et al.*, 1996; Ringø and Gatesoupe, 1998; Ringø *et al.*, 1998; Ringø and Olsen, 1999). Additionally, it has been shown that some of the indigenous bacteria are capable of suppressing the growth of known bacterial pathogens and may therefore act as potential probionts (Gomez-Gil *et al.*, 2000). In particular, lactic acid bacteria such as *Lactobacillus* and *Carnobacterium* have been identified as potential probionts.

Probiotic microorganisms can also play an important role in the nutrition of the host organism. De Schrijver and Ollevier (2000) showed that inclusion of the probiont *Vibrio proteolyticus* in the diet of juvenile *S. maximus* enhanced protein digestion, especially in the distal segments of the gastrointestinal tract. However, these authors failed to show the effects that this could have on nitrogen retention and growth. Similarly, Tovar *et al.* (2002) showed that addition of the yeast *Debaryomyces hansenii* HF1 to the diet of the sea bass *Dicentrarchus labrax* was able to enhance amylase and trypsin activity in 27 day old larvae. Furthermore, inclusion of the probiont in the diet enhanced survival of *D. labrax* larvae.

Pseudomonas fluorescens (AH2) was shown to be strongly inhibitory against *Vibrio anguillarum* in model systems and it was found that this effect could be transferred to an *in vivo* situation where the mortality in rainbow trout, following infection with *V. anguillarum*, was reduced significantly by the addition of the probiotic bacterium to the tank water (Gram *et al.*, 1999). An improvement in disease resistance in cod (*G. morhua)* fry given a dry feed containing

Carnobacterium divergens, three weeks before challenge with *Vibrio anguillarum*, has also been observed (Olafsen, 2001), and recently, the use of a *Carnobacterium* sp. as a probiotic for Atlantic salmon and rainbow trout has been reported (Robertson *et al.*, 2000).

1.5.4.2 Probiotic use in crustaceans

Shrimp culture accounts for approximately 30% of the total shrimp supplied to world markets (Bachère, 2000). In countries such as Taiwan and Ecuador this industry contributes significantly to the local economy, with losses due to bacterial and viral infections having a detrimental affect on the economy. As an alternative to the use of antibiotics, probiotics are being successfully used to promote shrimp disease resistance (Meunopol *et al.*, 2003).

Nogami and Maeda (1992) and Maeda & Liao (1992) (reviewed in Gomez–Gil *et al.*, 2000 and Verschuere *et al.*, 2000) isolated a bacterial strain from seawater, originally coded PM-4 and later identified as *Thalassobacter utilis*, and found that the addition of this strain to tank rearing water promoted the growth of both shrimp (*Penaeus monodon*) and crab (*Portunus trituberculatus*). Furthermore, this bacterial strain showed an *in vitro* inhibitory effect against the pathogen *Vibrio anguillarum* and when added to larval rearing water it was able to enhance survival of both *P. monodon* and *P. trituberculatus*. Others (Rengpipat *et al.*, 1998) showed that the survival and growth of the black tiger shrimp (*P. monodon*), fed the probiont *Bacillus* S11, was increased compared with non-treated shrimp. Additionally, after challenging shrimp with the shrimp pathogen, *Vibrio harveyi*, those shrimp fed a *Bacillus* S11 supplemented feed had a 100% survival rate compared to a 26% survival rate for shrimp fed a non-supplemented feed. It was also shown that the administration of this strain enhanced immunity in shrimp, as measured by the percentage phagocytosis and the phagocytic index (PI) of the haemolymph (Rengpipat *et al.*, 2000). Garriques and Arevalo (1995) (reviewed in Gomez-Gil *et al.*, 2000) isolated a non-pathogenic strain of *Vibrio alginolyticus* from seawater and found that it improved survival of *Litopenaeus vannamei* larvae following challenge with pathogenic *Vibrio parahaemolyticus*. Furthermore, this putative probiont was found associated with healthy rotifers, which is used as a feed for larvae, and it outgrew potential pathogenic vibrios isolated from the haemolymph of diseased shrimp (Gomez-Gil *et al.*, 2002). Gullian *et al.* (2004) isolated two putative probiotic

strains from the hepatopancreas of healthy wild shrimp that were identified as *Vibrio* P62 and *Bacillus* P64. Both strains showed good probiotic qualities as they were able to effectively colonize the hepatopancreas of *P. vannamei* and prevent the growth of the shrimp pathogen, *V. harveyi,* in the hepatopancreas. In addition, *Bacillus* P64 was able to stimulate the immune system of *P. vannamei* as indicated by a significantly greater ($P<0.05$) immune index value compared to the non-stimulated control group, indicating that this organism has a potential application for the control of shrimp disease in aquaculture systems. In a similar study, a *Pseudomonas* I-2 strain was identified as a potential probiont for controlling shrimp pathogenic vibrios as it showed good inhibition against *V. harveyi, V. fluvialis, V. parahaemolyticus, V. damsela* and *V. vulnificus* (Chythanya *et al.*, 2002).

1.5.4.3 Probiotic use in molluscs

As with most aquatic animals, shellfish are susceptible to disease and as a result, the use of probiotics to prevent disease outbreaks has been investigated.

Douillet and Langdon (1994) found that they could consistently enhance growth of the oyster larvae, *Crassostrea gigas*, regardless of the seasons of the year, if they supplemented the larval algal diet with a bacterium designated CA2. The authors suggested that the bacterium was either supplying essential nutrients to the larvae, which were absent in the feed, or were improving digestion in the host by contributing to the pool of digestive enzymes (reviewed in Gomez-Gil *et al.*, 2000). Furthermore, when CA2 was added to axenic, bacteria-free, oyster larvae cultures, survival of larvae was increased by approximately 22%. Erasmus *et al.* (1997) showed that enteric bacteria of the South African abalone *H. midae* made a significant contribution towards the pool of digestive enzymes, suggesting a possible probiotic effect by gut bacteria. Later, Sawabe *et al.* (2003) isolated a strain of *Vibrio halioticoli* from several species of Japanese abalone, as well as the South African abalone, which produces acetic acid from the fermentation of alginate, an algal polysaccharide, in the digestive tract of these abalone. Acetic acid, a volatile short-chain fatty acid (VSCFA), can be metabolized as an oxidative energy source and be used as a precursor for protein, sugar and lipid synthesis in the host animal, as is the case in ruminants

and termites. The authors therefore suggested that this strain could have an important probiotic role in abalone by contributing towards the metabolism of the host.

Riquelme *et al.* (1997) isolated a *Vibrio* species from seawater that produced inhibitory substances against a *Vibrio anguillarum*–related strain, which is a known pathogen of the Chilean scallop larvae, *Argopecten purpuratus*. Pre-treatment of scallop larvae with the probiotic enhanced larval survival following experimental infection with *V. anguillarum*. Furthermore, ingestion of putative probionts was shown to be strain dependent as some species of bacteria were ingested significantly better than others (Riquelme *et al.*, 2000). Gibson *et al.* (1998) isolated the bacterium *Aeromonas media* A199 which produced bacteriocin-like inhibitory substances (BLIS) against a wide range of fish and shellfish pathogens *in vitro*. Strain A199 improved survival of the oyster larvae, *Crassostrea gigas*, when challenged with the pathogen *Vibrio tubiashii*, demonstrating its potential as a probiotic for the oyster aquaculture industry. Thus, the administration of probiotics has been shown to be effective in a wide range of species for the promotion of growth, enhanced nutrition, immunity and survival.

1.6 Overview of invertebrate immunity

Due to exponential growth of the aquaculture industry and the associated increase of disease, current research efforts are being directed towards improving methods of disease prevention and control (Bachère *et al.*, 2000). Obtaining disease resistant animals through the use of probiotics, non-specific immunostimulants and selective breeding is one of the primary focuses of current research. However, in order to achieve this goal, an improved understanding of marine invertebrate immunology is required.

Vertebrates have a well developed and highly effective acquired immune system, consisting of lymphocytes and immunoglobulins, capable of adapting to foreign particles and invading microorganisms (Roche, 1999). Additionally, vertebrates also possess an innate immune system that functions in a similar manner to the innate immune system of the more primitive invertebrates. Invertebrates do not possess a memory-based immune system (Johansson *et al.*, 1994; Pipe *et al.*, 1997). Instead, they possess an innate, non-adaptive immune system. However,

this 'basic' immune system is capable of employing a wide variety of circulating molecules in response to foreign particles and infection that are capable of phagocytic, cytotoxic and inflammatory responses. Additionally, these molecules produce a number of hydrolytic enzymes and highly reactive, microbicidal metabolites associated with the partial reduction of molecular oxygen (Pipe, 1992; Bachère *et al.*, 1995; Roche, 1999). Due to the evolutionary success of the invertebrates, one can only assume that this 'basic' immune system posses efficient mechanisms for disease prevention that has allowed for the evolutionary success of this group.

1.6.1 Cellular effectors in invertebrate immunity

1.6.1.1 Haemocytes

Circulating haemocytes play a key role in the initial defense of invertebrates against infection, performing functions such as phagocytosis, encapsulation, oxidative killing and lysis of foreign cells (Johansson *et al.*, 2000). This has therefore prompted researchers to characterize the different haemocytes found in invertebrates, in terms of structure and function, as an understanding of these mechanisms could play a vital role in disease prevention and control.

Initial research on the classification of circulating haemocytes focused largely on differential staining, ultra-structure morphology, lectin binding and antigenic characterization. More recently, Pipe *et al.* (1997) separated the circulating haemocytes from the mussel *Mytilus edulis* using continuous Percoll gradients and found they separated into three distinct layers consisting of two distinct cell types, basophilic cells and eosinophilic cells. The basophilic cells contain small granules as opposed to the large granules found in eosinophilic cells. The large granules of the eosinophilic cells contain a range of hydrolytic enzymes, including proteinases, glycosidases and sulphatases. These cells are capable of cell proliferation and appear to be more active than the basophilic cells during phagocytosis and the release of reactive oxygen intermediates (ROIs). Data from this study indicated that the eosinophilic cells have a greater ability to destroy potential pathogens compared to the basophilic cells (Pipe *et al.*, 1997).

In crustaceans, three distinct types of circulating haemocytes have also been identified (reviewed in Bachère *et al.*, 1995: Johansson *et al.*, 2000). These include the hyaline cells, which are small phagocytic cells; semi-granular cells, which contain small granules and display phagocytic and cytotoxic activity; and granular cells, which are cytotoxic and are involved in the storage and release of the pro-phenoloxidase (proPO) system, which together with the semi-granular cells, is an important component of the host cellular defense system of many invertebrates.

1.6.1.2 Phagocytosis

Phagocytosis of non-self particles is a universal phenomenon that occurs throughout the entire animal kingdom, from the most primitive unicellular organisms to complex multi-cellular organisms (Bayne, 1990). Phagocytosis has been defined as "the ingestion by a cell of food particles, microorganisms, other cells, or foreign material by endocytosis" (Withers, 1992). In dealing with phagocytosis by circulating haemocytes, Cheng and Coombs (1990) (reviewed in Pipe *et al.*, 1997) redefined phagocytosis to include bacteria or foreign material attached to the surface of haemocytes. They reasoned that if bacteria are attached to the surface of haemocytes, then they are effectively removed from circulation and may be destroyed by the release of degradative enzymes, ROIs or any other anti-bacterial molecules released from circulating blood cells (Pipe *et al.*, 1997).

The process of phagocytosis can be subdivided into several stages. These include chemotaxis, recognition and internalization of foreign particles (Bayne, 1990). Chemotaxis by definition is the "direct movement in response to a gradient in chemical concentration" (Withers, 1992). During an inflammatory response in vertebrates, leukocytes will adhere and squeeze through blood capillary walls to the site of wounding in a chemotaxic process known as diapedesis (Prescott *et al.*, 1993). Chemotaxis is not very well understood in invertebrates. However, it has been shown that invertebrate haemocytes accumulate around wounds in injured tissue in a similar manner to leukocyte accumulation in injured vertebrate tissue (Mah *et al.*, 2004). Furthermore, as invertebrates have an open blood system, where the heart distributes haemolymph into sinuses that constitute the body cavity, the process of chemotaxis is possibly simplified because the probability of haemocyte molecules encountering foreign particles is

increased (Bayne, 1990). Furthermore, soluble blood factors, such as opsonizing lectins, play an important role in mediating this response (discussed in section 1.6.2.2).

Phagocytic recognition of foreign particles is achieved either directly by means of integral membrane recognition molecules, such as lectins, or indirectly through soluble blood factors (opsonic factors) which recognize and bind to the surfaces of foreign particles, effectively marking them for phagocytosis (Bayne, 1990). These molecules play an important role in the invertebrate humoral response and will be discussed in more detail in section 1.6.2.2.

Lastly, there is the internalization of foreign particles. Generally, when a phagocyte encounters a foreign particle it will send out finger-like pseudopods to engulf the foreign object (Bayne, 1990). If the foreign particle is too large, then several phagocytic haemocytes will collectively bind to the particle and engulf it. This process is associated with the release of lysosomal enzymes, such as acid phosphatases, proteases, lysozymes and β-glucuronidases. These enzymes can either act internally or externally to destroy and digest the foreign particle. Various techniques exist for assessing the phagocytic capacity of circulating haemocytes and the process of phagocytosis has been demonstrated in a number of marine organisms (Lactose *et al.*, 2002; Malham *et al.*, 2003; Pipe *et al.*, 1995).

1.6.1.3 Oxidative killing

When invertebrate haemocytes encounter and engulf a foreign particle, the process is not only associated with the release of hydrolytic enzymes, but is also associated with the release of ROIs, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen (O_2), hydroxyl radical (OH) and hypochlorous acid (HOCl) (Bachère *et al.*, 1995; Ordás *et al.*, 2000a). The release of these highly reactive, microbicidal metabolites by stimulated haemocytes is caused by an increase in oxygen consumption associated with the activation of the enzymes NAD(P)H-oxidase, a membrane-bound flavoprotein oxidase, and myeloperoxidase (MPO), which is an intracellular enzyme associated with lysosomes. The activity of these two enzymes, known as the myeloperoxidase-peroxide-halide system, transforms molecular oxygen into the ROIs mentioned above (Ordás *et al.*, 2000a). Several methods have been developed and tested for assessing

invertebrate phagocyte activation and phagocytosis (Pipe *et al.*, 1995; Ordás *et al.*, 2000a; Lactose *et al.*, 2002) and these processes have accordingly been well demonstrated in a number of marine invertebrates (Pipe *et al.*, 1992; Ordás *et al.*, 2000ab; Martello and Tjeerdema, 2001; Lactose *et al.*, 2002; Malham *et al.*, 2003).

1.6.1.4 Peroxynitrite anions

In addition to the ROIs that are released by stimulated haemocytes, another molecule, nitric oxide (NO), is also released from the haemocytes of certain gastropods following appropriate stimulation. Nitric oxide on its own is not toxic to cells, but in combination with superoxide anions it generates peroxynitrite anion ($ONOO^-$), an unstable and highly toxic compound with potent antibacterial activity (Roch, 1999). The production of this compound has been observed in the mussel *Mytilus galloprovincialis* and in the freshwater snail *Viviparus ater* (reviewed in Roch, 1999).

1.6.2 Humoral effectors in invertebrate immunity

Humoral immunity is defined as "immunity that is a consequence primarily of blood-borne factors in solution (antibodies and complement proteins) rather than blood cells" (Withers, 1992). Humoral effectors are non-specific soluble defense molecules that include agglutinins, opsonizing lectins, bactericidins, lysozymes and serine proteases (Bachère *et al.*, 1995; Roch, 1999).

1.6.2.1 Lysosomal enzymes

Haemocytes, particularly those containing large granules, such as the eosinophilic cells of molluscs, contain a range of hydrolytic enzymes within lysosomes, including lysozymes and serine proteases. These lysosomal enzymes act on the surface of microorganisms and other foreign particles and in so doing contribute to their recognition and destruction by circulating haemocytes (Bachère *et al.*, 1995; Genthner *et al.*, 1999). These lysosomal enzymes are widely distributed amongst the invertebrate phyla and lysosomal activity has been demonstrated in a

number of marine organisms in response to stress and/or bacterial infection (Nikoskelainen *et al.*, 2003; Rengpipat *et al.*, 2000; Martello and Tjeerdema, 2001). In addition to lysosomal enzymes, invertebrate haemocytes may also produce a variety of proteinase inhibitors in response to bacterial infection. Johansson *et al.* (1994) isolated several proteinase inhibitors from the freshwater crayfish, *Pacifastacus leniusculus*, following injection with heat-killed bacteria. One of the inhibitors had significant sequence similarity to the Kazal family of serine proteinase inhibitors and showed inhibitory activity against a number of serine proteinases. Serine proteinase inhibitors have also been isolated from other invertebrates, including the horseshoe crab and various insects (Johansson *et al.*, 1994).

1.6.2.2 Opsonizing factors

As previously mentioned, phagocytes either directly recognize foreign particles by means of integral membrane recognition molecules, such as lectins, or indirectly through soluble blood factors, the opsonizing factors, which recognize and bind to the surface of foreign particles, thus marking them for phagocytosis (Bayne, 1990).

Agglutinins/lectins are glycoproteins of non-immune origin that have the ability to bind to specific carbohydrates located on the surface of different cells. They occur throughout different phyla, including plants, fungi, bacteria, vertebrates and invertebrates. They are non-catalytic proteins and are generally multivalent, having at least two carbohydrate binding sites, enabling them to bind cells and promote an agglutination reaction (Marques and Barracco, 2000). More specifically, a lectin is an agglutinin whose ability to bind to a specific sugar group has been unequivocally demonstrated. In order for a lectin to be a true opsonic factor, it must be able to bind to the surface of non-self particles and to specific receptors on the surface of phagocytic haemocytes, thus facilitating phagocytosis (Marques and Barracco, 2000).

Lectins have been identified in a number of marine organisms, including the lobster *H. americanus*, the freshwater prawn *M. rosenbergii*, the crab *C. antennarius* and the shrimp *P. monodon* (Bachère *et al.*, 1995). Examples of identified lectins are lipopolysaccharides (LPS) and Concanavalin A (Con A), and their ability to act as opsonins have been demonstrated

(Bayne, 1990; Bachère *et al.*, 1995). Furthermore, multiple plasma lectins have been identified in the cockroach, *Blaberus discoidalis*, and the Japanese horseshoe crab, *Tachypleus tridentatus*, and their ability to recognize different invading pathogens has also been demonstrated, suggesting a certain degree of functional analogy between invertebrate lectins and vertebrate antibodies (Marques and Barracco, 2000).

1.6.2.3 Anti-microbial peptides

Anti-microbial peptides (AMPs) are low molecular weight, predominantly cationic molecules that are classified into five distinct groups based on their amino acid sequences, secondary structures and functional similarities (Roch, 1999). They play an important role in the innate immune response of vertebrates and invertebrates and are active against a broad range of pathogens, including Gram-positive and -negative bacteria, fungi, yeasts, viruses and some protozoa (Bachère, 2003).

AMPs function by acting either directly on microbial membranes, thus disrupting cell permeability, or they interfere with membrane biosynthetic pathways, which leads to cell death (Bachère, 2003). AMPs have been characterized in molluscs, insects, tunicates and crustaceans. In the horseshoe crab, *Limulus polyphemus*, AMPs are released into the blood plasma following suitable stimulation by microbial substances such as LPS and β-glucans (Supungul *et al.*, 2002). Furthermore, an expressed sequence tag (EST) analysis conducted on the black tiger shrimp, *P. monodon*, identified several genes encoding for anti-bacterial peptides, including lysozymes, penaeidins and anti-LPS factor. Similarly, a family of penaeidins was isolated from the haemolymph of the shrimp, *P. vannamei* (Bachère *et al.*, 2000; Supungul *et al.*, 2002). Penaeidins have a broad spectrum of anti-microbial activity, including fungicidal activity and strong anti-bacterial activity against both Gram-positive and -negative bacteria (Supungul *et al.*, 2002). Furthermore, this activity has been shown to increase in response to bacterial challenge (Bachère, 2003). AMPs, such as defensins and mytilins, have also been isolated from the mollusc, *Mytilus edulis,* that have broad spectrum specificity against several Gram-positive and -negative bacteria (Charlet *et al.*, 1996). Additionally, these authors isolated a 6.2 kDa cysteine-rich anti-fungal peptide called mytimycin.

1.7 Concluding remarks and aim of this study

The South African abalone, *Haliotis midae*, is an economically important species that has been commercially harvested since 1949. However, due to ecological changes that have occurred along the South African coastline, high consumer demand and an uncontrollable increase in poaching, wild abalone stocks have been severely depleted. This has led to the closure of the recreational fishery, with further threats to close down the commercial fishery. These factors, together with others discussed in this chapter, have led to the rapid development of the local abalone mariculture industry. At present, there are approximately 14 abalone farms in full production, producing between 500 and 800 tons of abalone per year with a net farm gate value of approximately R125 million (Sales and Britz, 2001; http://www.developtechnology.com).

As outlined in this chapter, the intensive nature of aquaculture farming can negatively impact on animal health and growth. Indeed, one of the factors hampering the success of abalone mariculture in South Africa is the slow growth rate of *H. midae*, requiring up to 5 years to reach market size. Furthermore, disease has had a severe impact on the international aquaculture industry, affecting most species presently under intensive cultivation (Bachère *et al.*, 1995; Bachère, 2000; Cheng *et al.*, 2002; Gullian *et al.*, 2004; Liu *et al.*, 2001; Mialhe *et al.*, 1995; Moore *et al.*, 2001; Roch, 1999; Verschuere *et al.*, 2000). Although the local abalone mariculture industry has not as yet suffered severe setbacks due to disease outbreaks, disease will invariably become an increasingly important factor that will impact on further development and success of this industry.

Erasmus *et al.* (1997) showed that bacteria exist throughout the digestive tract of *H. midae* that are capable of breaking down the complex polysaccharides present in their natural seaweed diets, namely *Ecklonia maxima* and *Gracilaria gracilis*. Furthermore, these studies showed that the enteric bacteria enhanced digestive efficiency by secreting polysaccharolytic enzymes and it was suggested from these results that these bacterial enzymes could affect the growth rate of abalone. The suggestion that exogenous enzymes produced by gut microflora may be of benefit to the host animal (Erasmus *et al.*, 1997; Ringø and Birkbeck, 1999; Sawabe *et al.*, 2003; Vitalis *et al.*, 1988) has prompted researchers to investigate the effect of selected microorganisms on the

growth of various marine species. Indeed, this chapter has summarized several examples of the effective use of probiotic microorganisms for growth enhancement and improved disease resistance in several aquacultured species.

Abalone farmers in South Africa are progressively utilizing formulated feeds in a pellet form, which offer convenience and cost benefits to farm operators (Sales and Britz, 2001). Thus, the aim of this study will be to isolate enteric bacteria from *H. midae* capable of utilizing the components of a local commercial feed and to test the isolates for their ability to improve digestion, growth and the immunity of farmed abalone. Furthermore, the colonization potential of the selected microorganisms will also be investigated to determine whether they are able to colonize and persist in the digestive tract of *H. midae*.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF *VIBRIO MIDAE SY9, CRYPTOCOCCUS* SP. SS1 AND *DEBARYOMYCES HANSENII* AY1

CONTENTS

2.1 Summary

A number of different microorganisms were isolated from the digestive tract of *Haliotis midae* that are capable of utilizing the protein and starch substrates included in commercial abalone feeds produced in South Africa. These isolates were separated according to differences in colony morphology and pigmentation, resulting in 21 morphologically distinct isolates. A comparative analysis of all of these isolates identified two isolates with enhanced enzyme activities. The bacterium SY9 exhibited enhanced proteolytic activity on all of the protein substrates tested and the yeast SS1 exhibited amylase activity at least 7 times greater than any of the other amylase producing isolates. Furthermore, a second yeast (isolate AY1), previously isolated from *H. midae* in our laboratory, hydrolyzed all of the protein and starch substrates tested in this study. Based on these findings and the knowledge that some yeasts and bacteria have immunostimulatory properties, these three strains were selected for further investigation.

The 16S rRNA gene sequence analysis, together with a comparison of the physical and phenotypic characteristics of the bacterium SY9 to that of related bacteria, suggested that it was a new species of the genus *Vibrio*. The bacterium was designated *Vibrio midae* SY9. The 18S rRNA gene sequence analysis, together with a comparison of the physical and phenotypic characteristics of yeast isolates SS1 and AY1 to that of related yeasts, suggested that yeast isolate SS1 belonged to the genus *Cryptococcus* and yeast isolate AY1 belonged to the genus *Debaryomyces*. The yeast SS1 was designated *Cryptococcus* sp. SS1 and the yeast AY1 was designated *Debaryomyces hansenii* AY1.

The growth curve analysis of the three strains identified *Vibrio midae* SY9 as the best candidate probiotic based on its short lag phase (1.56 hrs) and fast growth rate (0.37 h^{-1}). Although the two yeast strains had a longer lag phase and slower growth rate, the maximum biomass yield of these two strains was far greater than the bacterium. Collectively these results mean that the three putative probionts, SY9, SS1 and AY1, can be successfully cultivated to high cell densities for incorporation into commercial abalone feed formulations.

2.2 Introduction

The substantial growth of the marine aquaculture industry has been accompanied over the last few years by an increased prevalence of disease outbreaks (Olafsen, 2001; Verschuere *et al.*, 2000). Due to mounting pressure from the World Health Organization over the use of antimicrobials, several alternative strategies for disease control are being investigated and have already been implemented (Verschuere *et al.*, 2000). One of these strategies includes the use of indigenous bacteria as biological control agents.

Unlike terrestrial animals, where the resident microorganisms benefit from a fairly constant habitat within the gastrointestinal tract, marine animals are continuously exposed to a wide range of microorganisms (Gatesoupe, 1999; Ringø and Birkbeck, 1999). Furthermore, the types of microbes found associated with the gastrointestinal tract may vary with species, water temperature, salinity and/or dietary components (Ringø *et al.*, 1995). In general, the types of bacteria isolated from marine invertebrates include *Aeromonas, Pseudomonas, Vibrio, Bacillus, Acinetobacter, Flavobacteria, Alcaligenes, Moraxella, Enterobacteriaceae* and *Lactobacillus* (Ringø and Birkbeck, 1999). The *Pseudomonas* and *Vibrio* genera are most commonly found in crustaceans, marine fish and bivalves, whereas the *Aeromonas, Plesiomonas* and *Enterobacteriaceae* are more dominant in freshwater fish (Gatesoupe, 1999). Erasmus (1996) isolated several bacterial strains from the digestive tract of *H. midae* and found that the *Vibrio, Alcaligenes, Flavobacteria* and *Pseudomonas* genera were most common in this species. Of these genera, the *Vibrio* and *Pseudomonas* comprised the largest percentage of the resident bacterial community in the gastrointestinal tract. Furthermore, large coccoid bodies were also seen in the gut of *H. midae* under the electron microscope. These bodies were similar in size and shape to yeast cells observed in the digestive tracts of fish and it was therefore proposed that these bodies could be yeast cells (Erasmus, 1996).

The selection of probiotics for aquaculture has predominantly been based on their ability to produce antimicrobial metabolites, resulting in the isolation of a number of potential probiotic microorganisms (Chythanya *et al.*, 2002; Gibson *et al.*, 1998; Gullian *et al.*, 2004; Gram *et al.*, 1999; Gram *et al.*, 2001; Rengpipat *et al.*, 1998; Riquelme *et al.*, 1997; Robertson *et al.*, 2000).

However, another important selection criterion, which is often overlooked, is the production of extracellular enzymes by indigenous microflora and their role in the nutritional processes of the host organism (Ringø and Birkbeck, 1999). Previous studies have reported that indigenous microflora can enhance the growth rate of certain species (Bonar *et al.*, 1986; Vitalis *et al.*, 1988). Furthermore, a study conducted on *H. midae* clearly showed that the indigenous microflora of this species made a significant contribution towards the pool of digestive enzymes and it was hypothesized from this study that enteric bacteria could enhance the growth rate of *H. midae* (Erasmus *et al.*, 1997). Indeed, a number of studies have reported growth enhancement of various marine organisms through dietary supplementation with probiotic bacteria (Douillet and Langdon, 1994; Nogami and Maeda, 1992; Rengpipat *et al.*, 2000). However, in contrast to the importance of extracellular enzyme production by gut microbiota, few people have used this criterion for screening of potential probiotic organisms.

Abalone farmers in South Africa are progressively utilizing formulated feeds in a pelleted form, which offer convenience and cost benefits to farm operators (Sales and Britz, 2001). Although Erasmus (1996) identified several bacterial species capable of degrading the polysaccharides found in *Ecklonia maxima* and *Gracilaria gracilis*, the natural seaweed diets of *H. midae*, the ability of these bacteria to produce protease and amylase enzymes was not investigated. Therefore, the first objective of this study was to isolate and identify potential nutritional probiotic bacteria capable of degrading the various protein and starch substrates included in a local commercial formulated feed. Enteric bacteria exhibiting elevated protease and amylase activities were considered as putative probionts and were investigated further.

The second aim of this chapter was to characterize the potential probiotic organisms identified in the initial enzymatic analysis. Putative probionts were characterized using a polyphasic approach, i.e. the integrated use of both phylogenetic and phenotypic characteristics, as suggested by Murray *et al.* (1990). According to a report of the ad hoc committee on approaches to bacterial systematics (Murray *et al.*, 1990), it was concluded that the optimal methods for determining phylogenetic relationships of bacteria above the species level are DNA-DNA reassociation experiments and 16S rRNA gene sequencing of 1,000 or more bases. Furthermore, the 18S rRNA gene has also been identified as the molecule of choice in developing molecular

phylogenies among the fungi (Li *et al.*, 1996). Due to the remarkable improvements made in sequence analysis of nucleic acids over the last few years (Stackebrandt *et al.*, 1992), a large database of both 16S and 18S rRNA gene sequences exist for studying phylogenetic relationships amongst the Prokaryotes and Eukaryotes. Therefore, 16S and 18S rRNA analysis will be one of the tools used for the classification of putative probionts identified in this study. Furthermore, as mentioned above, a variety of physical and phenotypic characteristics of the putative probionts will also be determined. Murray *et al.* (1990) stated "that phylogenetic data alone is insufficient to provide an adequate description of genera and taxa of any rank above species and that coherent phenotypic characteristics are essential for both description and recognition". Therefore, together with the 16S and 18S rRNA analysis, the physical and phenotypic characteristics will be used to characterize the putative probionts identified in this study.

The third aim of this chapter was to assess the growth profiles of the characterized putative probiotic strains. As the putative probionts are to be added to artificial abalone feeds for future experiments, their ability to grow sufficiently well is important. In a similar study conducted on the isolation of candidate aquaculture probiotics (Vine *et al.*, 2004), the authors emphasized the importance of assessing the *in vitro* growth characteristics of candidate probiotics. Their paper suggested that bacterial growth characteristics such as the lag period and doubling time may influence the ability of a candidate probiotic to effectively colonize the digestive tract of the host organism and hence exert their probiotic effect. However, it was suggested that candidate probiotics which have a long lag period or doubling time should not be discarded, but rather cultured in large numbers before addition to the culture system. Indeed, many aquaculture probiotics are introduced via live or artificial feeds and are added at concentrations ranging from 10^5 to 10^7 culturable cells per gram or ml of feed (Douillet and Langdon, 1994; Rengpipat *et al.*, 1998; Robertson *et al.*, 2000). Therefore, the third aim of this chapter was to determine the relationship between absorbance and viable cell numbers of candidate probiotics during growth in shake flasks. This information will be used for future experiments involving the addition of the candidate probiotics to a commercial formulated feed.

2.3 Materials and methods

All media and solutions used in this study are listed in Appendix A.

2.3.1 Animals

The abalone used in this study were kindly donated by two commercial abalone farms in South Africa, Hermanus Abalone and Sea Plant Products, and kept at the Marine and Coastal Management Research Aquarium in Cape Town, South Africa. Animals were maintained in large polyethylene tanks containing 98 l of aerated and continuously flowing (330 L/h) natural seawater at 15 – 18 °C. Abalone were fed a basal 'Abfeed' diet, formulated and supplied by Sea Plant Products Limited, South Africa, which consisted of fishmeal, starch, vitamins and minerals.

2.3.2 Isolation of enteric bacteria of *Haliotis midae*

Microorganisms capable of degrading the various protein and starch substrates included in different Abfeed formulations, were isolated from the gastrointestinal tract of *Haliotis midae*. The major components included in the different Abfeed formulations are Fishmeal (FM), Sunflower seed (SC), Soya (SY) and two starches designated SS and PS starch. Three abalone (~50 mm shell length) were sacrificed by placing them at 4 °C for approximately 30 min. Under sterile conditions, the entire digestive tract was removed from each animal and individually masticated in 10 ml sterile phosphate buffered saline (PBS, Appendix A.2.4). An aliquot (1 ml) of each homogenate was transferred to 100 ml of minimal media, in a 1 litre conical flask, supplemented with either FM, SC, SY, SS or PS as the sole energy source. The basic minimal media consisted of (w/v): 0.1% yeast extract and 0.5% of the appropriate substrate in sterile artificial seawater (ASW, Appendix A.2.2). Cultures were incubated at room temperature on an orbital shaker at 100 rpm for 48 hrs. Following incubation, two samples from each culture were serially diluted to 10^{-12} from which aliquots of 100 µl were plated onto Petri dishes containing the same media i.e. either FM, SC, SY, SS or PS agar (minimal media supplemented with 2% Bacteriological agar, Biolab). The Petri dishes were incubated at room temperature and scored

for growth on a daily basis. Colonies were isolated and separated according to colony morphology and pigmentation.

2.3.3 Enzyme assays

The colonies isolated on the selective solid media were investigated further using quantitative enzyme assays. Furthermore, a yeast strain (AY1), previously isolated in our laboratory from the digestive tract of *H. midae* (Andlid, T., *unpublished data*), was also included. Cells were grown in minimal media supplemented with either FM, SC, SY, SS or PS as the respective substrate. The cultures were incubated at room temperature for 12 hours at 100 rpm. Subsequently, 500 µl of each culture was transferred into 20 ml of fresh minimal media (in triplicate), containing the appropriate substrate, and incubated for a further 24 hrs. Following incubation, serial dilutions were made of each culture to dilutions of 10^{-12} from which aliquots of 100 µl were spread-plated onto marine agar. Plates were incubated for 18 hrs at room temperature after which colony-forming units (cfu) were counted. Furthermore, an aliquot of cells (2 ml) from each culture was harvested by centrifugation at 12 096 (x g) for 10 min at 4 °C. The culture supernatant was collected in order to assay for the presence of extracellular protease or amylase activity. The results were expressed as units of enzyme activity/10^8 cfu/ml.

2.3.3.1 Azocacein assay for protease activity

Proteolytic activity was determined using 0.5 % azocasein as a substrate as previously described by Deane *et al.* (1986) with minor modifications. Briefly, 0.1 ml aliquots of supernatant were added to 0.1 ml of 0.5% azocasein in 0.1 M Tris-HCl buffer, pH 7.2 (Appendix A.2.8) and incubated at 37 °C. The reaction was stopped 30 min later by the addition of 0.2 ml of ice cold 10% trichloroacetic acid (TCA, Appendix A.2.8) and incubated for a further 30 min at 4 °C. The supernatants were separated from the undigested azocasein by centrifugation at 12 096 (x g) for 5 min. After transferring 0.3 ml of each supernatant to 0.3 ml of 0.5 M NaOH (Appendix A.2.8), the absorbance of the released dye was determined at 440 nm using a Beckman spectrophotometer. For the control, TCA was added before the substrate and immediately incubated at 4 °C. One unit of protease activity is defined as the amount of enzyme that gives an

increase in optical density at 440 nm of 0.1 in 30 min at 37 °C. Each assay was performed in triplicate.

2.3.3.2 Dinitrosalycilic assay for reducing sugars

Amylase activity was measured by the release of reducing sugar (glucose) from starch using dinitrosalycilic acid (DNS) reagent according to the method described by Miller (1959) with minor modifications. Briefly, 35 µl of supernatant was added to 15 µl of 0.5 % soluble starch (Appendix A.2.9) and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 150 µl of DNS (Appendix A.2.9) and boiled for 5 min before cooling rapidly on ice. Following the addition of 800 µl distilled water, the amount of reducing sugar released was determined by measuring the absorbance at 510 nm using a Beckman spectrophotometer. For the assay blank, 35 µl of PBS was added instead of supernatant and the assay proceeded as above. An additional control was prepared by adding DNS simultaneously with the enzyme extract and immediately boiling for 5 min. This control took into account any reducing sugar present in the samples prior to the assay. A standard curve was constructed by plotting light absorbance at 510 nm against known concentrations of glucose. One unit of amylase activity is expressed as the amount of enzyme that is able to liberate 1 mM of glucose in 30 min.

2.3.4 Phenotypic characterization of *Vibrio* sp. SY9

2.3.4.1 Cell shape and Gram stain

The bacterial cell shape and Gram reaction was tested by following the general procedures of Smibert and Krieg (1994). Stained bacteria were viewed under a light microscope equipped with a 100 x oil immersion lens.

2.3.4.2 Motility test

Bacterial motility was determined by viewing an exponentially grown culture of bacterium SY9 under a light microscope using the hanging drop test for motility as described by Smibert and Krieg (1994).

2.3.4.3 Oxidase and catalase tests

The ability of the bacterium SY9 to produce cytochrome oxidase and catalase was determined by using Kovac's reagent and 3% hydrogen peroxide as described by Smibert and Krieg (1994).

2.3.4.4 Nitrate reductase and denitrification tests

The bacterium SY9 was tested for its ability to reduce nitrate to nitrite and carry out denitrification using the standard nitrate reduction and denitrification tests described by Smibert and Krieg (1994).

2.3.4.5 Tests for indole and H_2S production

The standard methods described by Smibert and Krieg (1994) were used to test for indole and H_2S production by the bacterium SY9.

2.3.4.6 Aerobic and anaerobic metabolism of carbon sources

The ability of bacterium SY9 to metabolize a variety of carbon sources aerobically and anaerobically was determined according to the method described by Smibert and Krieg (1994). However, because strain SY9 is a marine bacterium that cannot grow in the absence of NaCl, the NaCl content of the basal media for each carbon test was raised to 3% (w/v). The carbon sources tested were as follows: D-glucose, sucrose, D-fructose, D-galactose, xylose, D-mannose, maltose, cellobiose, arabinose and lactose. Each carbohydrate was added to give a final concentration of 1% (w/v).

2.3.4.7 Test for agarolytic activity

The cell free supernatant of an O/N bacterial culture of SY9 grown in 5 ml marine broth (MB) (Appendix A.1.1) supplemented with 0.05% (w/v) bacteriological agar (Biolab) was tested for its ability to hydrolyze agar using the ferricyanide assay for reducing sugars described by Schroeder (2001).

2.3.4.8 Test for amylase activity

The cell free supernatant of an O/N bacterial culture of SY9 was tested for its ability to hydrolyze starch using the assay described in section 2.3.3.2.

2.3.4.9 Test for gelatinase activity

The standard method described by Smibert and Krieg (1994) was used to test for gelatinase activity by bacterium SY9.

2.3.4.10 Test for alginase activity

The cell free supernatant of an O/N bacterial culture of SY9 grown in 5 ml alginate broth (Appendix A.1.5) was tested for its ability to hydrolyze alginate using the Thiobarbituric Acid Assay (TCA) described by Press and Ashwell (1962).

2.3.4.11 Growth at different temperatures

Bacterium SY9 was grown in MB and incubated at either 4, 22, 30, 37 or 41 °C on an orbital shaker at 100 rpm. Bacterial cultures were scored for growth every 6 hrs. Growth was determined as an absorbance reading at or above 0.1 at 600 nm.

2.3.4.12 Tolerance of 6% and 10% NaCl

The tolerance of bacterium SY9 to 6% and 10% NaCl (w/v) was determined in MB. Cultures were incubated at room temperature on an orbital shaker and scored every 6 hrs for growth. Growth was determined as an absorbance reading at or above 0.1 at 600 nm.

2.3.4.13 API system for bacterial identification

The rapid API 20E system (bioMérieux, Inc.) was used for further physiological identification of bacterium SY9. Bacterium SY9 was grown at room temperature on an orbital shaker at 100 rpm for 12 hrs. Cells were pelleted by centrifugation in a bench-top centrifuge for 1 min, washed twice with API 0.85% NaCl Medium and finally resuspended in an equal volume of API 0.85% NaCl Medium. The API 20E strip was inoculated, read and interpreted according to the manufacturer's instructions.

2.3.5 Determination of the 16S rRNA gene sequence of *Vibrio* sp. SY9

The genomic DNA of bacterium SY9 was extracted as described in Appendix B.1. The isolated DNA (50 ng) was used as a template to amplify the small-subunit rRNA genes by PCR using the primers described by Schroeder (2001) (Appendix B.4.1). The primers were used in amplification reactions in the following combinations: F1 and R5, F1 and R3, F1 and R1, F3 and R5, and F5 and R5 (Fig. 2.2). The PCR was conducted as described in Appendix B.4.2.

The amplified PCR products (10 µl) were analyzed by agarose gel electrophoresis (Appendix B.3) to verify reaction specificity and fragment sizes. The amplified PCR products were subsequently purified using a PCR product purification kit (Roche). The purified 16S rRNA PCR products were sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). All reactions were performed according the manufacturer's instructions. Samples were sequenced using the MegaBACE 500 Sequencer (Amersham Biosciences, Automated Capillary DNA Sequencing System). The sequences were edited and assembled using DNAMAN version 4.13 (Lynnon BioSoft). The homology search was carried

out using the BLASTN algorithm (Altschul *et al.*, 1989) provided by the internet service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nlm.nih.gov/BLAST/).

2.3.6 Phylogenetic tree construction for *Vibrio* sp. SY9

The 24 *Vibrio* 16S rRNA gene sequences (Table 2.4) and the three *Photobacterium* 16S rRNA gene sequences, *Photobacterium augustum* (X74685), *Photobacterium leiognathi* (X74686) and *Photobacterium phosphoreum* (Z19107), were obtained from the GENBANK database. The stretches of sequence common to the 1330 bp sequence of strain SY9 (Fig. 2.3) were aligned using the optimal alignment option of DNAMAN version 4.13 (Lynnon BioSoft). Alignments were exported in clustal format for phylogenetic and molecular evolutionary analysis using MEGA version 2.1 (Kumar *et al.*, 2001). A Neighbour Joining (NJ) tree (Saitou and Nei, 1987) was constructed for the data set using MEGA. The Kimura 2-parameter (pairwise distances) model (Kimura, 1980) was used for distance estimation. The reliability of the inferred phylogenetic tree was assessed using the bootstrap test (Felsenstein, 1985). A thousand replicates were tested with a random starting seed.

A strict consensus parsimony (MP) tree was inferred using MEGA on the same sequence data set. Parsimony analysis was performed using the close-neighbour-interchange heuristic search. The initial tree was generated by randomly selecting a sequence and adding it to the growing tree on a randomly selected branch (random addition tree option). The reliability of the inferred phylogenetic tree was assessed using the bootstrap test (Felsenstein, 1985). A thousand replicates were tested with a random starting seed.

2.3.7 Determination of G+C (mol%) content of *Vibrio* sp. SY9

The genomic DNA of bacterium SY9 was extracted according to the method described in Appendix B.1. The G+C (mol%) content was determined according to the spectrophotometric method described by Ulitzur (1972).

2.3.8 Electron microscopy

Bacterium SY9 was grown in MB at room temperature on an orbital shaker at 100 rpm to mid-exponential phase. Cells were washed gently (twice) in sterile PBS and resuspended in an equal volume of sterile PBS. A carbon coated grid (carbon coated side face down) was placed on the surface of a 10 µl aliquot of the washed cells for 10 min to allow the cells to bind to the positively charged grid. The grid was then removed and placed onto a 10 µl suspension of dH_2O for 1 min before being stained with a 2% uranyl acetate (pH 5) solution (Appendix A.2.7) for 10 min (uranyl acetate solution forms crystals and needs to be centrifuged briefly before use). The stain was removed with two 1 min washes with dH_2O before placing the grid onto Whatman filter paper to air-dry (Schroeder, 2001). The sample was viewed on a Zeiss Electron Microscope 109 (Zeiss).

2.3.9 Phenotypic characterization of *Cryptococcus* sp. SS1 and *Debaryomyces* sp. AY1

2.3.9.1 Microscopic examination of non-filamentous vegetative cells

Yeasts SS1 and AY1 were cultured in 5 ml of yeast-peptone-D-glucose (YPD) broth (Appendix A.1.3) at room temperature on an orbital shaker at 100 rpm for 24 hrs. Subsequently, 1 ml of each culture was transferred to 50 ml of fresh YPD media in a 1 litre conical flask and grown to mid-exponential phase. The cells were examined under a light microscope to determine the shape and size of the cells and hence the mode of reproduction (i.e. by budding, splitting or both) (Barnett *et al.*, 1983).

2.3.9.2 Microscopic examination of ballistoconidia

The ability of yeasts SS1 and AY1 to form ballistoconidia, a key feature of yeasts belonging to the family Sporobolomycetaceae (includes the genera *Bullera, Sporobolomyces* or *Sporidiobolus*) was tested using the method described by Barnett *et al.* (1983). Briefly, Petri dishes containing YPD agar (Appendix A.1.4) were inoculated with the yeast to be tested in lines along two diameters at right angles. Each inoculated dish was inverted over another Petri dish

bottom, also containing YPD agar, onto which a sterile microscope slide was placed. Inoculated dishes were taped together with parafilm to maintain sterility and prevent the plates from drying out. Plates were incubated for up to three weeks at room temperature and the lower Petri dish periodically examined for the presence of colonies due to discharged ballistoconidia. Furthermore, the slides on the lower Petri dish were microscopically examined for the presence of ballistoconidia.

2.3.9.3 Microscopic examination of ascospores

Yeast strains SS1 and AY1 cells were examined for the presence of ascospores following the method described by Barnett *et al.* (1983). Briefly, cells were grown on YPD agar at room temperature for three days before being examined under a light microscope for the presence of ascospores. If ascospores were not seen, cells were incubated for longer and examined every week for up to six weeks.

2.3.9.4 Aerobic and anaerobic metabolism of carbon sources

The ability of yeasts SS1 and AY1 to utilize certain organic compounds as the sole major source of carbon, both aerobically and anaerobically, was determined according to the method described by Barnett *et al.* (1983). Briefly, large Durham tubes (150 mm x 12 mm) were filled with 10 ml of yeast extract medium (0.5% (w/v) Yeast extract, Biolab) and 50 mM test sugar. Additionally, an insert tube (50 mm x 6 mm) was placed in each Durham tube to monitor gas production. The carbon sources tested were as follows: D-glucose, sucrose, D-fructose, D-galactose, D-mannitol, D-mannose, D-ribose, maltose, cellobiose, threonine, arabinose and lactose.

2.3.9.5 Production of extracellular starch-like compounds

The ability of yeasts SS1 and AY1 to produce extracellular starch-like polysaccharides, a key characteristic of yeasts belonging to the genus *Cryptococcus*, was determined according to the method described by Barnett *et al.* (1983).

2.3.9.6 Urease test

The ability of yeasts SS1 and AY1 to hydrolyze urea was determined according the method described by Barnett *et al.* (1983).

2.3.9.7 Diazonium blue B (DBB) test

Yeast strains SS1 and AY1 were grown in YPD broth and incubated at room temperature for at least 10 days before performing the DBB test according to the method described by Barnett *et al.* (1983).

2.3.9.8 Growth at different temperatures

Yeast strains SS1 and AY1 were grown in YPD broth and incubated at either room temperature, 30, 37 or 41 °C on an orbital shaker at 100 rpm. Yeast cultures were scored for growth every 6 hrs. Growth was determined as an absorbance reading at or above 0.1 at 600 nm.

2.3.10 Determination of the 18S rRNA gene sequence of *Cryptococcus* sp. SS1 and *Debaryomyces* sp. AY1

The genomic DNA of *Cryptococcus* sp. SS1 and *Debaryomyces* sp. AY1 was extracted as described in Appendix B.1. The isolated DNA (50 ng) was used as a template to amplify the 18S rRNA genes by PCR using the primers described by White *et al.* (1990) (reviewed in Li *et al.*, 1996) (Appendix B.5.1). The primers were used in amplification reactions in the following combinations: NS1 and NS8, and NS3 and NS8 (Fig. 2.6). The PCR was conducted as described in Appendix B.5.2.

The amplified PCR products (10 µl) were analyzed by agarose gel electrophoresis (Appendix B.3) to verify reaction specificity and fragment sizes. The amplified PCR products were subsequently purified using a PCR product purification kit (Roche). The purified 18S rRNA PCR products were sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit

(Amersham Pharmacia Biotech). All reactions were performed according the manufacturer's instructions. Samples were sequenced using the MegaBACE 500 Sequencer (Amersham Biosciences, Automated Capillary DNA Sequencing System). The sequences were edited and assembled using DNAMAN version 4.13 (Lynnon BioSoft). Homology searches were carried out using the BLASTN algorithm (Altschul *et al.*, 1989) provided by the internet service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nlm.nih.gov/BLAST/).

2.3.11 Phylogenetic tree construction for *Cryptococcus* sp. SS1 and *Debaryomyces* sp. AY1

The 18S rRNA gene sequences of *Cryptococcus* sp. SS1 and *Debaryomyces* sp. AY1 determined in this study were aligned with the sequences of 41 other Basidiomycete (23 sequences) and Ascomycete (18 sequences) sequences obtained from the GENBANK database (Fig. 2.9). The stretches of sequence common to the 1592 bp sequence of strain SS1 (Fig. 2.7) and the 1593 bp sequence of strain AY1 (Fig. 2.8), were aligned using the optimal alignment option of DNAMAN version 4.13 (Lynnon BioSoft). Alignments were exported in clustal format for phylogenetic and molecular evolutionary analysis using MEGA version 2.1 (Kumar *et al.*, 2001). A Neighbour Joining (NJ) tree (Saitou and Nei, 1987) and a strict consensus parsimony (MP) tree was constructed for the data set using MEGA as described in section 2.3.6. The reliability of the inferred phylogenetic trees were assessed using the bootstrap test (Felsenstein, 1985). A thousand replicates were tested with a random starting seed.

2.3.12 Determination of bacterial and yeast growth curves

The growth profiles of strains SY9, SS1 and AY1 were determined to investigate the relationship between viable cell numbers (cfu ml^{-1}) and absorbance at 600 nm. Furthermore, the deduced growth profiles were used to determine the length of the lag phase (λ), the maximum specific growth rate (μ) and the maximum biomass yield (A) of each strain. Due to the exponential growth of most bacteria and yeast, the logarithm of the relative population size [$y = \ln (N/N_o)$] is usually plotted against time (Zwietering *et al.*, 1990). The maximum specific growth rate can then be estimated from the slope of the tangent drawn to the inflection of the sigmoid curve

(Baranyi and Pin, 1999). Thus, the specific growth rate at any time is the slope of the curve at that point and is defined by the following equation:

$$\ln (N/N_o) = \mu \times t \qquad \text{(Zizhou, 2001),}$$

where N_o is the initial cell concentration, N is the final cell concentration, μ is the maximum specific growth rate and t is the time. The lag phase (λ) is defined as the x-axis intercept of this tangent and the maximum biomass yield (A) is the maximum cell density reached (Zwietering *et al.*, 1990).

2.3.12.1 *Vibrio* sp. SY9 growth curve determination

Bacterium SY9 was grown in 5 ml of MB at room temperature on an orbital shaker at 100 rpm for 12 hrs. Subsequently, 500 µl of culture was transferred into 50 ml of fresh media and incubated for a further 12 hrs before inoculating two 5 litre conical flasks, each containing 500 ml of fresh media, so as to achieve a final absorbency at 600 nm of 0.01. The cultures were incubated at room temperature on an orbital shaker at 100 rpm, with triplicate samples taken from each culture on an hourly basis for determination of the number of colony-forming units (cfu) and the optical density at 600 nm. For cfu determination, serial dilutions were made of each sample from which aliquots of 100 µl were spread-plated onto Petri dishes containing MA. The Petri dishes were incubated for 18 hrs at room temperature after which the colony-forming units (cfu) were counted.

2.3.12.2 *Cryptococcus* sp. SS1 and *Debaryomyces* sp. AY1 growth curve determination

Yeast strains SS1 and AY1 were each grown in 5 ml of YPD broth at room temperature on an orbital shaker at 100 rpm for 24 hrs. Subsequently, 500 µl of each culture was transferred into 50 ml of fresh media and incubated for a further 24 hrs before inoculating four 5 litre conical flasks, two flasks per culture, each containing 500 ml of fresh media, so as to achieve a final absorbency at 600 nm of 0.1. The cultures were incubated at room temperature on an orbital shaker at 100

rpm, with triplicate samples taken from each culture on an hourly basis for determination of the number of the colony-forming units (cfu) and optical density at 600 nm. For cfu determination, serial dilutions were made of each sample from which aliquots of 100 µl were spread-plated onto Petri dishes containing YPD agar. The Petri dishes were incubated for 72 hrs at room temperature after which the colony-forming units (cfu) were counted.

2.4 Results

2.4.1 Isolation of putative probiotic strains

The selective media used for the isolation of enteric microorganisms from *H. midae* yielded a total of 21 culturable microbial isolates which differed in colony morphology and pigmentation. The quantitative enzyme analysis conducted on these isolates showed that the majority of these isolates were capable of hydrolyzing all of the protein and starch substrates tested (Table 2.1). However, the protease and amylase enzymes produced by different isolates clearly had different substrate specificities, as indicated by the differences in enzyme activity of each isolate on the different substrates. Furthermore, one of the isolates, strain SY9, exhibited high protease activity on all of the protein substrates and a second isolate, strain SS1, exhibited exceptionally high amylase activity on both starch substrates. An additional comparative enzyme analysis conducted on strain SY9, SS1 and the yeast strain AY1 supported the above findings (Fig. 2.1). These results clearly demonstrated the high protease activity of strain SY9 on all of the protein substrates, particularly Fishmeal (Fig. 2.1a), and the extremely high amylase activity of strain SS1 (Fig. 2.1b), in relation to the other strains tested, on both starch substrates. Furthermore, data from this experiment revealed that yeast AY1 is capable of hydrolyzing all of the protein and starch substrates tested. As a result of these findings, strains SY9, SS1 and AY1 were selected for further analysis.

2.4.2 Classification of *Vibrio midae* SY9

The nucleotide sequences of the products obtained from PCR amplification (Fig. 2.2) of the bacterium SY9 16S rRNA gene were edited and assembled in DNAMAN version 4.13 (Lynnon BioSoft) resulting in a full-length sequence of 1444 bp in length (Fig. 2.3). A BLAST search of the GENBANK database revealed that the bacterium SY9 16S rRNA gene sequence showed high identity to a number of *Vibrio* species (Table 2.2).

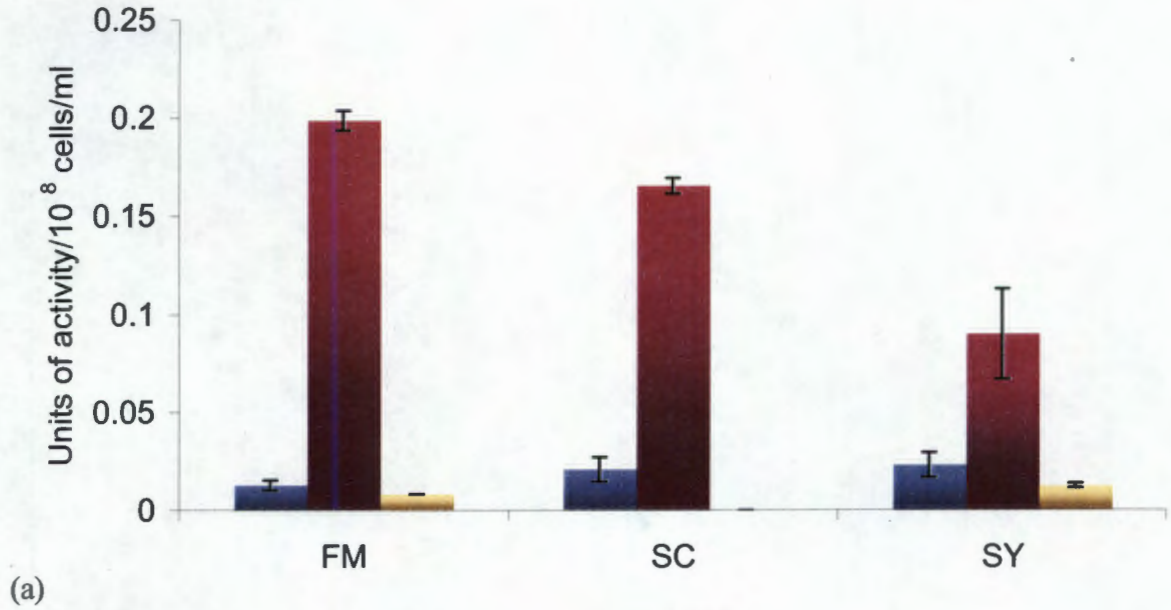
Table 2.1. Extracellular enzyme activity and substrate specificity of the proteases and amylases of the enteric microorganisms isolated from the digestive tract of *H. midae*

Isolate	Fishmeal[a]	Sunflower seed[a]	Soya[a]	PS-starch[b]	SS-starch[b]
SC1	0.057 ± 0.001	0.055 ± 0.004	0.013 ± 0.001	0.074 ± 0.010	0.048 ± 0.012
SC2	0.078 ± 0.002	0.123 ± 0.000	0.003 ± 0.001	0.089 ± 0.002	0.062 ± 0.009
SC3	0.282 ± 0.025	0.069 ± 0.001	0.016 ± 0.001	0.069 ± 0.003	0.026 ± 0.001
SC4	0.114 ± 0.056	0.068 ± 0.002	0.002 ± 0.001	0.066 ± 0.002	0.021 ± 0.002
SC5	0.159 ± 0.001	0.028 ± 0.001	0.021 ± 0.006	0.073 ± 0.002	0.028 ± 0.004
SC7	0.057 ± 0.001	0.119 ± 0.000	0.007 ± 0.001	0.129 ± 0.003	0.069 ± 0.004
SC8	0.061 ± 0.004	0.098 ± 0.002	0.001 ± 0.000	0	0.044 ± 0.014
SC9	0.119 ± 0.000	0.052 ± 0.005	0.002 ± 0.000	0.084 ± 0.002	0.056 ± 0.008
SY1	0.085 ± 0.010	0.341 ± 0.003	0.071 ± 0.018	0	0.026 ± 0.003
SY2	0.056 ± 0.013	0.102 ± 0.003	0.038 ± 0.007	0.062 ± 0.002	0.012 ± 0.003
SY3	0	0.082 ± 0.001	0.040 ± 0.009	0.038 ± 0.000	0.012 ± 0.005
SY4	0.041 ± 0.002	0.189 ± 0.005	0.028 ± 0.004	0.072 ± 0.001	0.025 ± 0.002
SY5	0.036 ± 0.007	0.204 ± 0.003	0.104 ± 0.006	0.078 ± 0.001	0.018 ± 0.002
SY6	0.012 ± 0.001	0.211 ± 0.004	0.039 ± 0.012	0.051 ± 0.000	0.016 ± 0.002
SY7	0.007 ± 0.001	0.176 ± 0.005	0	0.074 ± 0.001	0.018 ± .0007
SY8	0.012 ± 0.006	0.211 ± 0.002	0.032 ± 0.007	0.046 ± 0.002	0.017 ± 0.007
SY9	0.281 ± 0.056	0.318 ± 0.012	0.155 ± 0.008	0.011 ± 0.003	0.016 ± 0.006
PS2	0.218 ± 0.026	0	0.020 ± 0.002	0.010 ± 0.001	0
PS3	0.335 ± 0.005	0	0.050 ± 0.040	0.024 ± 0.008	0
PS4	0.317 ± 0.060	0	0	0.010 ± 0.002	0
SS1	0.006 ± 0.002	0	0.002 ± 0.001	3.92 ± 0.083	4.68 ± 0.072

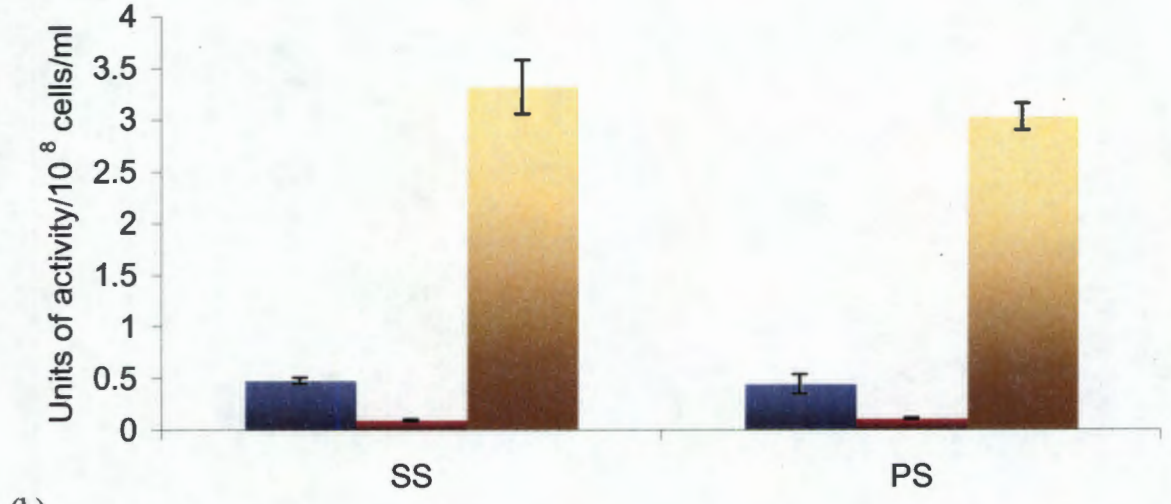
Data represents the mean ± standard error of triplicate samples

[a] Units of protease activity/10^8 cfu/ml

[b] Units of amylase activity/10^8 cfu/ml



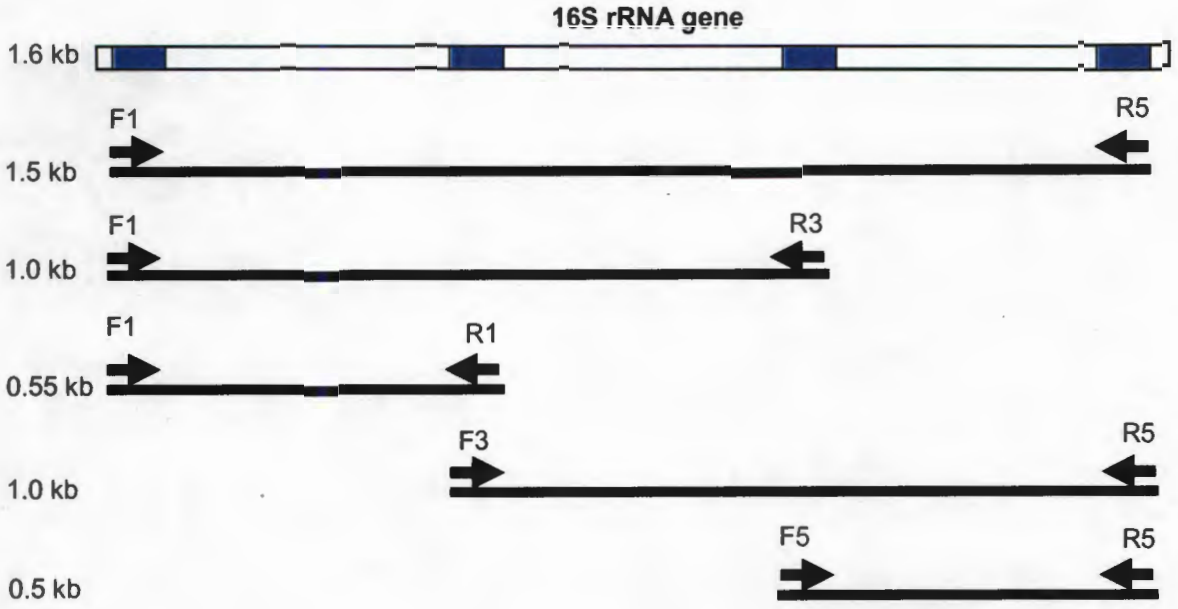
(a)



(b)

Figure 2.1. Comparative protease (a) and amylase (b) activities of isolates AY1 (■), SY9 (■) and SS1 (■) on the different protein and starch substrates included in Abfeed. Data represents the mean ± standard error.

(a)



(b)

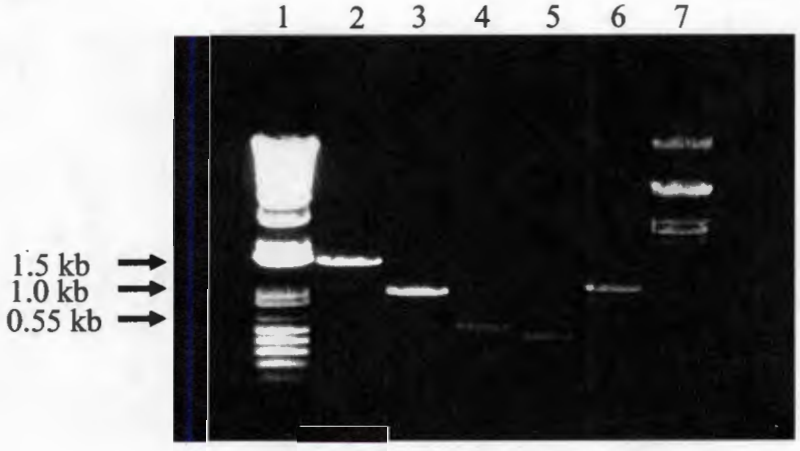


Figure 2.2. (a) Diagram showing the PCR strategy employed to amplify the 16S rRNA gene of bacterium SY9 using the five primer pairs described in section 2.3.5. F1, F3 and F5 represent the forward primers and R1, R3 and R5 the reverse primers. The blue shaded areas represent highly conserved regions of the 16S rRNA gene that occur in most Eubacteria. The sizes depict the length of the PCR products. (b) The amplified bacterium SY9 16S rRNA gene PCR products separated on a 1% TAE agarose gel. Lane 1: 1 kb ladder (Roche), Lane 2: F1/R5 primer combination, Lane 3: F3/R5 primer combination, Lane 4: F1/ R1 primer combination, Lane 5: F5/R5 primer combination, Lane 6: F1/R3 primer combination, Lane 7: lambda DNA digested with *Pst*I.

```
1     AACGAGTTAT CTGAACCTTC GGGGAACGAT AACGGCGTCG AGCGGCGGAC GGGTGAGTAA
61    TGCCTAGGAA ATTGCCCTGA TGTGGGGGAT AACCATTGGA AACGATGGCT AATACCGCAT
121   GATGCCTACG GGCCAAAGAG GGGGACCTTC GGGCCTCTCG CGTCAGGATA TGCCTAGGTG
181   GGATTAGCTA GTTGGTGAGG TAAGGGCTCA CCAAGGCGAC GATCCCTAGC TGGTCTGAGA
241   GGATGATCAG CCACACTGGA ACTGAGACAC GGTCCAGACT CCTACGGGAG GCAGCAGTGG
301   GGAATATTGC ACAATGGGCG CAAGCCTGAT GCAGCCATGC CGCGTGTGTG AAGAAGGCCT
361   TCGGGTTGTA AAGCACTTTC AGTCGTGAGG AAGGTGGTGT AGTTAATAGC TGCATTATTT
421   GACGTTAGCG ACAGAAGAAG CACCGGCTAA CTCCGTGCCA GCAGCCGCGG TAATACGGAG
481   GGTGCGAGCG TTAATCGGAA TTACTGGGCG TAAAGCGCAT GCAGGTGGTT TGTTAAGTCA
541   GATGTGAAAG CCCGGGGCTC AACCTCGGAA TAGCATTTGA AACTGGCAGA CTAGAGTACT
601   GTAGAGGGGG GTAGAATTTC AGGTGTAGCG GTGAAATGCG TAGAGATCTG AAGGAATACC
661   GGTGGCGAAG GCGGCCCCCT GGACAGATAC TGACACTCAG ATGCGAAAGC GTGGGGAGCA
721   AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT GTCTACTTGG AGGTTGTGGC
781   CTTGAGCCGT GGCTTTCGGA GCTAACGCGT TAAGTAGACC GCCTGGGGAG TACGGTCGCA
841   AGATTAAAAC TCAAATGAAT TGACGGGGGC CCGCACAAGC GGTGGAGCAT GTGGTTTAAT
901   TCGATGCAAC GCGAAGAACC TTACCTACTC TTGACATCCA GAGAACTTTC CAGAGATGGA
961   TTGGTGCCTT CGGGAACTCT GAGACAGGTG CTGCATGGCT GTCGTCAGCT CGTGTTGTGA
1021  AATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTATCC TTGTTTGCCA GCGAGTAATG
1081  TCGGGAACTC CAGGGAGACT GCCGGTGATA AACCGGAGGA AGGTGGGGAC GACGTCAAGT
1141  CATCATGGCC CTTACGAGTA GGGCTTACAC ACGTGCTTAC AATGGCGCAT ACAGAGGGCG
1201  GCCAACTTGC GAAAGTGAGC GAATCCCAAA AAGTGCGTCG TAGTCCGGAT TGGAGTCTGC
1261  AACTCGACTC CATGAAGTCG GAATCGCTAG TAATCGTGGA TCAGAATGCC ACGGTGAATA
1321  CGTTCCCGGG CCTTGTACAC ACCGCCCGTC ACACCATGGG AGTGGGCTGC AAAAGAAGTA
1381  GGTAGTTTAA CCTTCGGGGG GACGCTTACC ACTTTGTGGT TCATGACTGG GGTGAAGTCG
1441  TAAC
```

Figure 2.3. Nucleotide sequence (1444 bp) of the 16S rRNA gene of *Vibrio midae* SY9. The underlined sequence was not used in the phylogenetic analysis described in section 2.3.6. The above sequence was submitted to GENBANK and assigned the accession number AY940169.

The bacterium SY9 formed a distinct cluster with *V. natriegens*, *V. pelagius*, *V. alginolyticus* and the rest of the '*Vibrio* core organisms' (bordered by *V. natriegens* and *V. harveyi*) (Fig. 2.4). This cluster exhibited a high bootstrap value with the neighbor-joining analysis (bootstrap 68%), indicating the existence of a strong association between the bacterium SY9 and the '*Vibrio* core organisms'. Furthermore, within this cluster, bacterium SY9 formed a close association with *V. natriegens, V. pelagius, V. alginolyticus* and *V. parahaemolyticus*.

Table 2.2. The top 11 sequence similarities as obtained from the BLAST search of the GENBANK database with the 1444 bp 16S rRNA gene sequence of the bacterium SY9.

Species	**% sequence similarity**	**GENBANK accession number**
Vibrio sp. CJ11052	99	AF500207.1
Vibrio parahaemolyticus	99	BA000031.2
Bacterium K2-74 16S	99	AY345403.1
Vibrio sp. Ex25	99	AF319769.1
Vibrio sp. NLEP97-1598	99	AF410778
Unidentified bacterium clone K2-S-4	99	AY344374.1
Vibrio campbellii strain 90-69B3	99	AY738129.1
Vibrio sp. 98CJ11027	99	AF246980
Vibrio sp. KYJ 962	99	AY542526.1
Vibrio sp. NAP-4	99	AF064637
Vibrio alginolyticus	99	AF513447.1

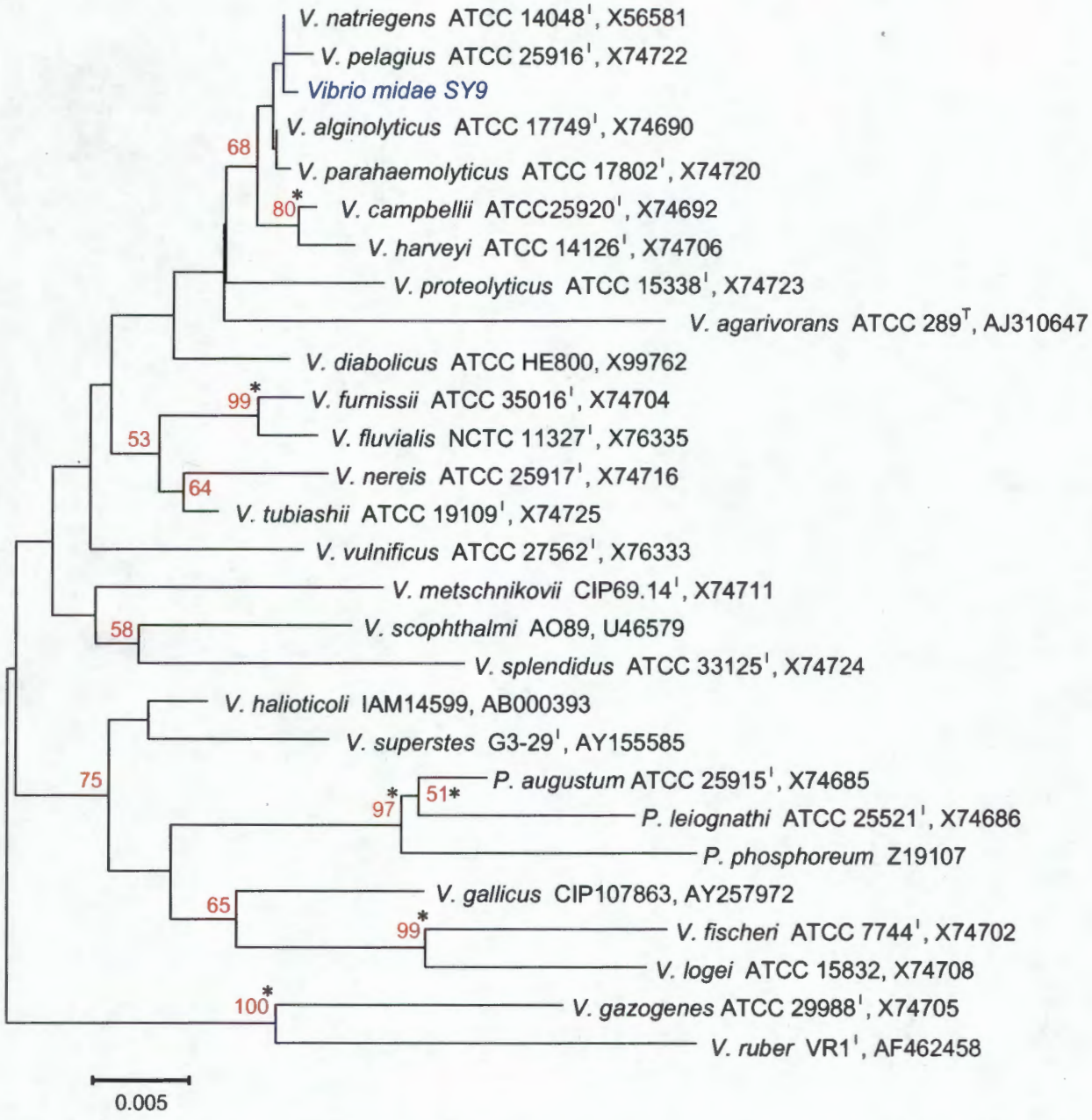


Figure 2.4. A phylogenetic tree derived from a distance-matrix analysis of the 16S rRNA gene sequences of *Vibrio midae* SY9, strains of the genus *Vibrio*, and strains of the closely related genus *Photobacterium* using MEGA version 2.1 (Kumar *et al.*, 2001). An alignment of 1330 characters was used. Numbers at the nodes indicate the bootstrap values retrieved from 1000 replicates (frequencies less than 50% not shown). *, >60 bootstrap value for parsimony analysis. Numbers next to specific names correspond to the strain and accession numbers for the 16S rRNA gene sequences. The bar depicts 1 base substitution per 200 nucleotides.

Table 2.3. Summary of physical and phenotypic characteristics of the bacterium SY9.

Characteristic	*Vibrio midae* SY9
Gram stain	Negative
Cell shape	Straight rod
Growth on TCBS	Yellow
Luminescence	-
Colony pigmentation	-
Motility	+
Polar flagellum	+
Growth in air	+
Na^+ required for growth	+
Nitrate reduction	+
Production of:	
Catalase	+
Oxidase	+
Urease	-
β-galactosidase	-
Indole	+
Acetoin	+
H_2S	-
Thornley's arginine dihydrolase	-
Lysine decarboxylase	+
Ornithine decarboxylase	+
Tryptophane deaminase	-
Hydrolysis of:	
Agar	-
Alginate	+
Starch	+
Gelatin	+
Utilization of:	
D-glucose, sucrose, D-fructose, D-galactose, xylose, D-mannose, maltose, cellobiose	+
arabinose, inositol, D-sorbitol, L-rhamnose, amygdalin and lactose	+
Growth at:	
4, 22, 30, 37 and 41 °C	+
Tolerance to NaCl at:	
6 and 10%	+
Acid production from:	
D-glucose, sucrose and maltose	+
G+C (mol%)	45

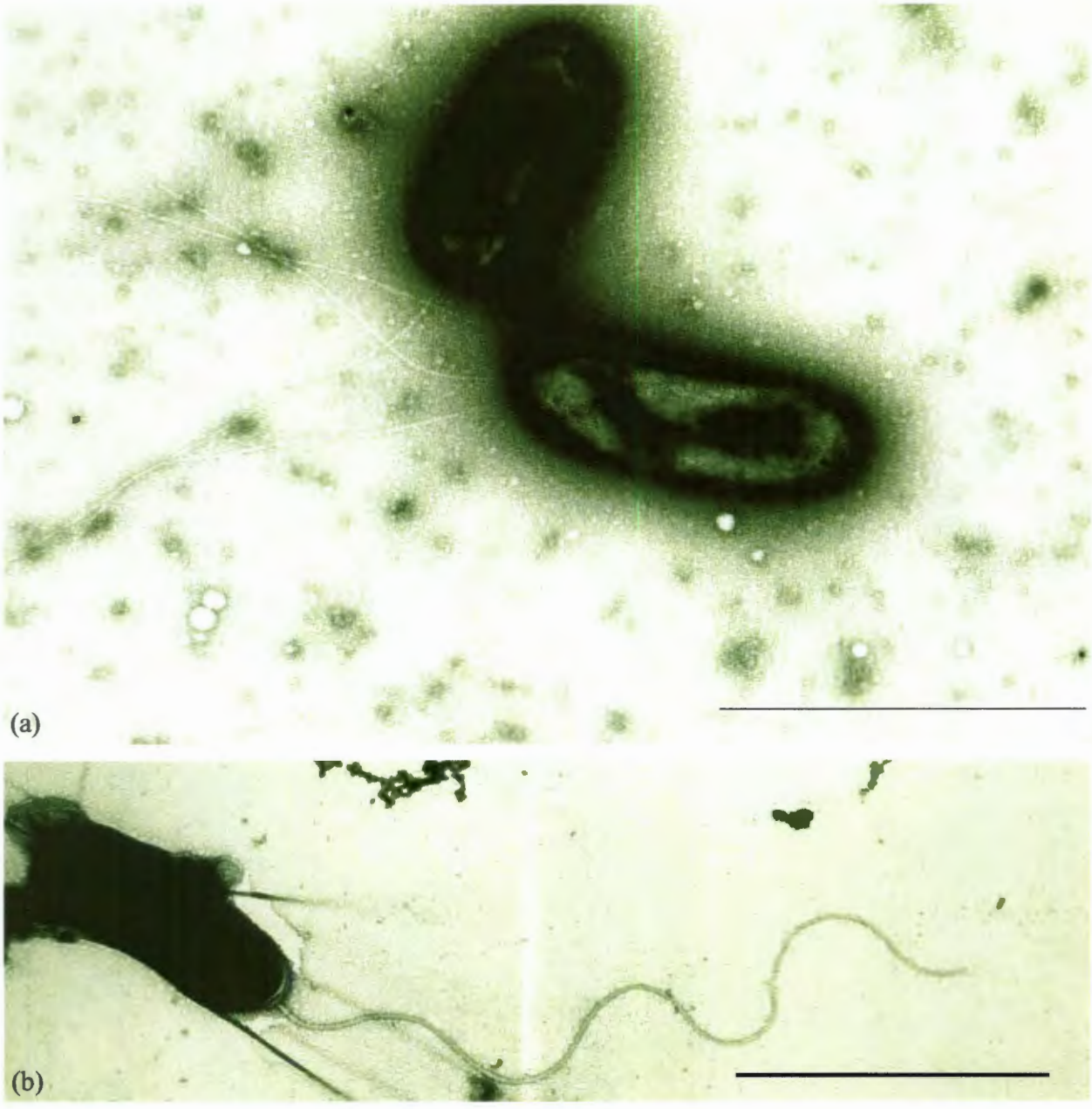


Figure 2.5. Electron micrographs showing the rod shape of *Vibrio midae* SY9 cells. (a) Cells with one and three polar flagella can be seen and (b) a cell with a clearly visible single polar flagellum. Bar: 2.0 µm.

Table 2.4. Differential characteristics of species belonging to the genus *Vibrio*

Characteristic	*V. natriegens* ATCC 14048^T	*V. pelagius* ATCC 25916^T	*Vibrio midae* SY9	*V. alginolyticus* ATCC 17749^T	*V. parahaemolyticus* ATCC 17802^T	*V. campbellii* ATCC25920^T	*V. harveyi* ATCC 14126^T	*V. proteolyticus* ATCC 15338^T	*V. diabolicus* ATCC HE800	*V. nereis* ATCC 25917^T	*V. tubiashii* ATCC 19109^T
Polar flagella	-	-	+	+	+	+	+	+	+		
Swarming	-	-	+	+	+	-	-	+	+	-	
Growth on TCBS		+	Y	Y	G	G	Y/G				
Straight rods	+	+	+	+	+	+	+	+	+	+	+
Pigmentation	-	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	+	-	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	
Acetoin production	-	-	+	+	-	-	-	+		-	
Lysine decarboxylase	-	-	+	+					+		-
Indole	-	-	+	+					+		+
Acid from sucrose	+	+	+	+	-	-	d	-			
Growth at: 4 °C	+	v	+	-	-	-	-	-	-	d	
40 °C	+	-	+	+	+	-	d	+	+	d	
G+C (mol%)	46-47	45-47	44.84	45-47	46-47	46-48	46-48	50.5	49.6	46-47	
Production of:											
Amylase	d	-	+	+	+	+	+	+	+	-	+
Gelatinase	d	-	+	+	+	+	+	+		d	
Alginase	-	+	+	-	-	-	-	-		-	
Agarase	-	-	-	-							
Chitinase	-	d		+	+	+	+	+	-	d	
Utilization of:											
Sucrose	+	+	+	+	-	-	d	-	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+
D-galactose	+	+	+	d	+	-	d	-	+	-	+
Xylose	-	-	+	-	-	-	-	-	-	-	-
D-mannose	d	v	+	d	+	d	+	+	+	-	+
D-ribose	+	+		+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	d	-	+	-	-	d	+	-	-	-	+
Arabinose	+	-	-	-	d	-	d	-	-	-	-
Inositol	d	-	-	-	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-	+	-	-	-
L-rhamnose	+	-	-	-	-	-	-	-		-	
Amygdalin			-						+		+
Lactose	-	v		-	-	-	-	-	-	-	-
Melibiose	v	-		-	-	-	-	-	-	-	-
L-arginine	+	+		+	+	-	d	+	-	+	-
Accession	X56581	X74722	AY940169	X74690	X74720	X74692	X74706	X74723	X99762	X74716	X74725
Reference	Ruimy *et al.*, 1994; Baumann *et al.* 1984	Ruimy *et al.*, 1994; Baumann *et al.* 1984	This study	Ruimy *et al.*, 1994; Baumann *et al.* 1984	Ruimy *et al.*, 1994; Baumann *et al.* 1984	Ruimy *et al.*, 1994; Baumann *et al.* 1984	Ruimy *et al.*, 1994; Baumann *et al.* 1984	Ruimy *et al.*, 1994; Baumann *et al.* 1984	Raguénés *et al.*, 1997	Ruimy *et al.*, 1994; Baumann *et al.* 1984	Raguénés *et al.*, 1997

Table 2.4. Continued

V. agarivorans ATCC 289^T	*V. fluvialis* NTCC 11327^T	*V. furnissii* ATCC 35016^T	*V. vulnificus* ATCC 27562^T	*V. splendidus* ATCC 33125^T	*V. scophthalmi* AO89	*V. metschnikovii* CIP69.14^T	*V. gazogenes* ATCC 29988^T	*V. ruber* VR1^T	*V. halioticoli* IAM14599	*V. superstes* G3-29^T	*V. gallicus* CIP107863	*V. fischeri* ATCC 7744^T
+		+		+	+				-	-	-	
	-		-	-		-	-	-	-	-	-	-
G	Y		G	+		G			+	+		
+	d	d	-	-	+	d	-	+	+	+	+	+
-	-	-	-	-		-	R	R	-	-	B	Y
-	+	+	-	+	+	-	-	-	-	-	-	-
+	+	+	+	+	+	-	-	+	+	+	+	d
+	+	+	+	+	+	-	-	-	+	+	+	+
	-	-	-	-		-	-		-	-	-	-
-	+	-	+	-	-	d		-	-	-	-	
-		-		+	-			-	+	+		
-	+		-	+		+	+	+	-	-	-	-
-	-		-	-	-	-	-	-	-	-	-	-
-	+		+	-	-	+	+	-	-	-	-	-
44.8	49-51	50	46-48	45-46	44-46	46-48	47.1	45.8	41.6	48-49	43-45	39-41
	+		+	+	-	+	+	+	-	-	-	-
	+		+	+	-	+	+	+	-	-	-	-
	-		-	d	-	-	-		+	+	+	-
+				-				-	-	-	-	
	+		+	+		+	-		-	-	-	+
	+		+	+	-	+	+	+	-	+	-	-
	+		+	+	+	+	+	+	+	+	+	+
	-		+	-	-	+	+		+	-	-	-
	+		+	+	-	+	+	+	d	+	-	+
	+		+	-	-	+	+		-	+	+	+
	+		+	-	-	+	+	+	d	+	-	+
	-		-	-	-	+	+	+	-	+	+	+
	+		-	+	-	+	+		d	+	-	+
	+		+	-	-	+	+	+	-	-	-	-
	-		-	-	-	d	+	-	-	-	-	-
	-		-	-	-	d	+	-	-	-	-	-
	-		-	-	-	-	-					-
+	-		d	+	-	d	-	+	-	+	-	-
+	-		-	+	-	-			-	+	-	-
	+		+	+		+	-					-
AJ310647	X76335	X74704	X76333	X74724	U46579	X74711	X74705	AF462458	AB000393	AY155585	AY257972	X7470
Macán *et al.*, 2001	Aznar *et al.*, 1994	Brenner *et al.*, 1983	Aznar *et al.*, 1994	Sawabe *et al.*, 1998	Cerdà-Cuéllar *et al.*, 1997	Ruimy *et al.*, 1994; Baumann *et al.*, 1984	Ruimy *et al.*, 1994; Baumann *et al.* 1984	Shieh *et al.*, 2003	Sawabe *et al.*, 1998	Hayashi *et al.*, 2003	Sawabe *et al.*, 2004	Ruimy *et al.*, 1994;

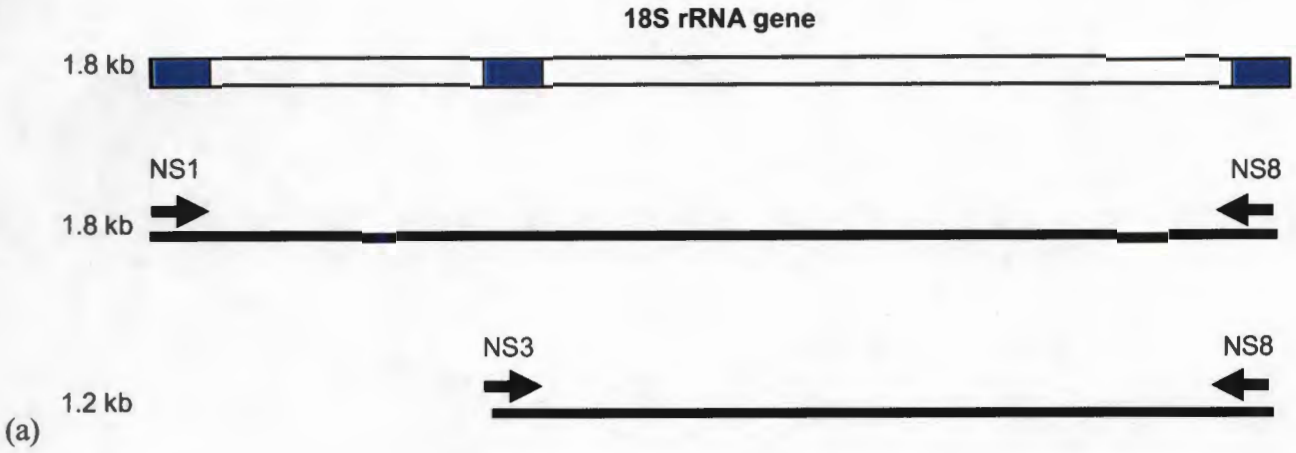
The physical and phenotypic characteristics of the bacterium SY9, which confidently place it within the genus *Vibrio*, are summarized in Table 2.3. The bacterium is a non-pigmenting, motile, oxidase-positive, catalase-positive, Gram-negative rod with between one and three polar flagella of approximately 6.6 μm in length. Cells are ~0.7 μm in diameter and ~2 μm in length (Fig. 2.5). Cells are non-luminous and swarm on marine agar plates. They develop yellow colonies on thiosulphate-citrate-bile-sucrose (TCBS, Difco) agar (i.e. ferments sucrose). The bacterium is capable of growing at 4 °C and 42 °C, with an optimum growth temperature of between 22 – 37 °C. It is strictly halophilic, unable to grow in media lacking NaCl, and is able to grow at NaCl concentrations of up to 10%. Cells were fermentative in the oxidative/fermentation test, fermenting glucose with gas production, and gave a positive Voges-Proskauer test result. Cells reduced nitrate to nitrite and tested positive for lysine decarboxylase, ornithine decarboxylase and indole production. Test results for arginine dihydrolase, tryptophane deaminase, urease and H_2S production were negative. The bacterium tested positive for the utilization of D-glucose, sucrose, D-fructose, D-galactose, xylose, D-mannose, maltose and cellobiose. Of the carbohydrates tested, it was unable to utilize arabinose, inositol, D-sorbitol, L-rhamnose, amygdalin and lactose. The bacterium was capable of hydrolyzing gelatin, starch and alginase, but not agar. The G + C content of the DNA is 44.84% (Table 2.3).

The differential characteristics of the species belonging to the family Vibrionaceae, whose 16S rRNA gene sequences have been deposited in the GENBANK database, are shown in Table 2.4. Of the isolates that clustered with strain SY9 in the 16S rRNA phylogenetic tree (Fig. 2.4), the physiological characteristics of *V. alginolyticus* are the most similar to those exhibited by SY9. However, *V. alginolyticus* differs from strain SY9 in its ability to grow at 4 °C, degrade alginate and utilize cellobiose and xylose. Of the other members of this cluster, *V. pelagius* is also similar to strain SY9. However, this strain differs from SY9 in a number of physiological features, including the ability to produce arginine dihydrolase, lysine decarboxylase, acetoin and indole and the ability to produce amylase and gelatinase. The other members of this cluster, including *V. natriegens*, *V. parahaemolyticus* and *V. harveyi*, vary in biochemical profile to a greater degree when compared to strain SY9. Of the species that are able to degrade alginate, one of the key physiological features of strain SY9, *V. pelagius* is most similar to SY9. Other species which

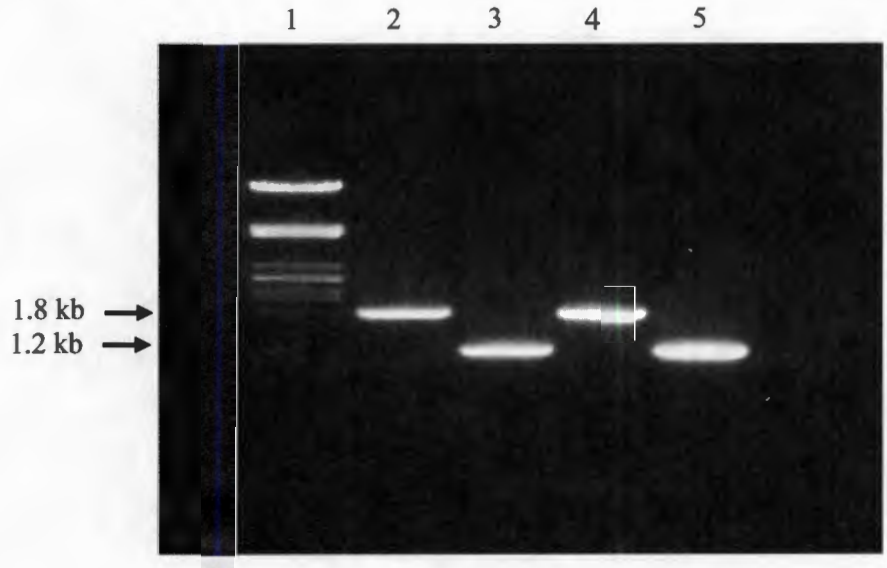
are able to degrade alginate include *V. halioticoli* and *V. gallicus*, however these two strains differ in a large number of physiological features from strain SY9.

2.4.3 Classification of *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1

The nucleotide sequences of the products obtained from PCR amplification (Fig. 2.6) of the 18S rRNA gene of yeasts SS1 and AY1 were edited and assembled in DNAMAN version 4.13 (Lynnon Biosoft) resulting in full-length sequences of 1709 and 1630 bp in length for yeast SS1 and AY1 respectively (Fig. 2.7 and 2.8). A BLAST search of the GENBANK database revealed that the yeast SS1 18S rRNA gene sequence showed high identity to a number of organisms belonging to the genera *Cryptococcus* and *Bullera*, whereas the yeast AY1 18S rRNA gene sequence showed high identity to a number of organisms belonging to the genera *Debaryomyces* and *Candida* (Table 2.5). The 18S rRNA phylogenetic analysis confirmed the above findings, with yeast SS1 forming a distinct cluster with the members of the Basidiomycetous yeast genera *Cryptococcus* and *Bullera* and yeast AY1 forming a distinct cluster with members of the Ascomycetous yeast genera *Debaryomyces* and *Candida* (Fig. 2.9). Both these clusters exhibited very high bootstrap values (100%) with the neighbour joining (NJ) analysis, indicative of a very strong association. Furthermore, within the Basidiomycetous yeast genera, yeast SS1 formed a distinct cluster with *Cryptococcus cellulolyticus, C. laurentii* and *Bullera pseudoalba*, again supported by a high bootstrap value (98%). Conversely, yeast AY1 branched deeply within the clade belonging to members of the Ascomycetous yeast genera and formed a distinct cluster with all of the members of the *Debaryomyces* genus tested in this study (bootstrap 54%). However, the high bootstrap value (89%) supporting the association between yeast AY1 and *D. hansenii* within this cluster, suggests that these two species are closely related. This association is supported by the BLAST search of the GENBANK database with the yeast AY1 18S rRNA gene sequence, which showed that yeast AY1 had high identity (99%) to a number of *Debaryomyces hansenii* strains (Table 2.5).



(a)



(b)

Figure 2.6. (a) Diagram showing the PCR strategy employed to amplify the 18S rRNA gene of yeast strains SS1 and AY1 using the two primer pairs described in section 2.3.10. NS1 and NS3 represent the forward primers and NS8 the reverse primer. The blue shaded areas represent highly conserved regions of the 18S rRNA gene that occur in most fungi. The sizes depict the approximate lengths of the PCR products. (b) The amplified 18S rRNA PCR products of yeast strains SS1 and AY1 separated on a 1% TAE agarose gel. Lane 1: lambda DNA digested with *Pst*I, Lane 2: NS1/NS8 primer combination of SS1, Lane 3: NS3/NS8 primer combination of strain SS1, Lane 4: NS1/NS8 primer combination of strain AY1, Lane 5: NS3/NS8 primer combination of strain AY1.

```
1     CCATGCATGT CTAAGTATAA ACAAATTCAT ACTGTGAAAC TGCGAATGGC TCATTAAATC
61    AGTTATAGTT TATTTGATGG TATCTTGCTA CATGGATAAC TGTGGTAATT CTAGAGCTAA
121   TACATGCTGA AAAGCCCCGA CTTCTGGGAG GGGTGTATTT ATTAGATAAA AAACCAATGG
181   GTGAAAGCCC TCTATGGTGA TTCATAATAA CTTCTCGAAT CGCATGGCCT TGCGCCGGCG
241   ATGCTTCATT CAAATATCTG CCCTATCAAC TTTCGATGGT AGAGTAGTGG TCTACCATGG
301   TATCAACGGG TAACGGGGAA TTAGGGTTCG ATTCCGGAGA GGGAGCCTGA GAAACGGCTA
361   CCACATCCAA GGAAGGCAGC AGGCGCGCAA ATTACCCAAT CCCGACACGG GGAGGTAGTG
421   ACAATAAATA ACAATACAGG GCCCTATTGG GTCTTATAAT TGGAATGAGT ACAATTTAAA
481   TCCCTTAACG AGGAACAACT GGAGGGCAAG TCTGGTGCCA GCAGCCGCGG TAATTCCAGC
541   TCCAGTAGCG TATATTAAAG TTGTTGCAGT TAAAAAGCTC GTAGTCGAAC TTCGGGCCTG
601   GCGGGACGGT CCGCCTTACG GTGTGTACTG TCCGGCCGGG TCTTACCTCT TGGTGAGGCC
661   GTATGCTCTT TACTGGGTGT GCGGTGGAAC CAGGAATTTT ACCTTGAGAA AATTAGAGTG
721   TTCAAAGCAG GCATTTGCCC GAATACATTA GCATGGAATA ATAGAATAGG ACGTGCGGTT
781   CTATTTTGTT GGTTTCTAGG ATCGCCGTAA TGATTAATAG GGACGGTCGG GGGCATTAGT
841   ATTCCGTTGC TAGAGGTGAA ATTCTTAGAT TTACGGAAGA CTAACTTCTG CGAAAGCATT
901   TGCCAAGGAC GTTTTCATTG ATCAAGAACG AAGGTTAGGG GATCAAAAAC GATTAGATAC
961   CGTTGTAGTC TTAACAGTAA ACTATGCCGA CTAGGGATCG GGCCACGTTA ATTTCTGACT
1021  GGCTCGGCAC CTTACGAGAA ATCAAAGTCT TTGGGTTCTG GGGGGAGTAT GGTCGCAAGG
1081  CTGAAACTTA AAGGAATTGA CGGAAGGGCA CCACCAGGTG TGGAGCCTGC GGCTTAATTT
1141  GACTCAACAC GGGGAAACTC ACCAGGTCCA GACATAGTAA GGATTGACAG ATTGATAGCT
1201  CTTTCTTGAT TCTATGGGTG GTGGTGCATG GCCGTTCTTA GTTGGTGGAG TGATTTGTCT
1261  GGTTAATTCC GATAACGAAC GAGACCTTAA CCTGCTAAAT AGCCAGGCCG GCTTTGGCTG
1321  GTCGTCGGCT TCTTAGAGGG ACTGTCGGCG TTTAGCCGAC GGAAGTTTGA GGCAATAACA
1381  GGTCTGTGAT GCCCTTAGAT GTTCTGGGCC GCACGCGCGC TACACTGACT GAGCCAGCGA
1441  GTTTATCACC TTGGCCGAGA GGCCTGGGTA ATCTTGTGAA ACTCAGTCGT GCTGGGGATA
1501  GAGCATTGCA ATTATTGCTC TTCAACGAGG AATACCTAGT AAGCGTAAGT CACCAACTTG
1561  CGTTGATTAC GTCCCTGCCC TTTGTACACA CCGCCCGTCG CTACTACCGA TTGAATGGCT
1621  TGGTGAGATC TCCGGATTGG CGTTGGGGAG CCGGCAACGG CACCCCTTGG CCGAGAAGCT
1681  GATCAAACGT GGTCATTTAG AGGAAGAAA
```

Figure 2.7. Nucleotide sequence (1709 bp) of the 18S rRNA gene of *Cryptococcus* sp. SS1. The underlined sequence was not used in the phylogenetic analysis described in section 2.3.11. The above sequence was submitted to GENBANK and assigned the accession number AY940170.

```
1       AATGGCTCAT TAAATCAGTT ATCGTTTATT TGATAGTACC TTTACTACTT GGATAACCGT
61      GGTAATTCTA GAGCTAATAC ATGCTAAAAA TCCCGACTGT TTGGAAGGGA TGTATTTATT
121     AGATAAAAAA TCAATGCTTT TCGGAGCTCT TTGATGATTC ATAATAACTT TTCGAATCGC
181     ATGGCCTTGT GCTGGCGATG GTTCATTCAA ATTTCTGCCC TATCAACTTT CGATGGTAGG
241     ATAGTGGCCT ACCATGGTTT CAACGGGTAA CGGGGAATAA GGGTTCGATT CCGGAGAGGG
301     AGCCTGAGAA ACGGCTACCA CATCCAAGGA AGGCAGCAGG CGCGCAAATT ACCCAATCCC
361     GACACGGGGA GGTAGTGACA ATAAATAACG ATACAGGGCC CTTTCGGGTC TTGTAATTGG
421     AATGAGTACA ATGTAAATAC CTTAACGAGG AACAATTGGA GGGCAAGTCT GGTGCCAGCA
481     GCCGCGGTAA TTCCAGCTCC AATAGCGTAT ATTAAAGTTG TTGCAGTTAA AAAGCTCGTA
541     GTTGAACCTT GGGCTTGGTT GGCCGGTCCG CCTTTTTGGC GAGTACTGGA CCCAACCGAG
601     CCTTTCCTTC TGGCTAACCT TTCGCCCTTG TGGTGTTTGG CGAACCAGGA CTTTTACTTT
661     GAAAAAATTA GAGTGTTCAA AGCAGGCCTT TGCTCGAATA TATTAGCATG GAATAATAGA
721     ATAGGACGTT ATGGTTCTAT TTTGTTGGTT TCTAGGACCA TCGTAATGAT TAATAGGGAC
781     GGTCGGGGGC ATCAGTATTC AGTTGTCAGA GGTGAAATTC TTGGATTACC TGAAGACTAA
841     CTACTGCGAA AGCATTTGCC AAGGACGTTT TCATTAATCA AGAACGAAAG TTAGGGGATC
901     GAAGATGATC AGATACCGTC GTAGTCTTAA CCATAAACTA TGCCGACTAG GGATCGGGTG
961     TTGTTCTTTT TTTGACGCAC TCGGCACCTT ACGAGAAATC AAAGTCTTTG GGTTCTGGGG
1021    GGAGTATGGT CGCAAGGCTG AAACTTAAAG GAATTGACGG AAGGGCACCA CCAGGAGTGG
1081    AGCCTGCGGC TTAATTTGAC TCAACACGGG GAAACTCACC AGGTCCAGAC ACAATAAGGA
1141    TTGACAGATT GAGAGCTCTT TCTTGATTTT GTGGGTGGTG GTGCATGGCC GTTCTTAGTT
1201    GGTGGAGTGA TTTGTCTGCT TAATTGCGAT AACGAACGAG ACCTTAACCT ACTAAATAGT
1261    GCTGCTAGCT TTTGCTGGTA TAGTCACTTC TTAGAGGGAC TATCGATTTC AAGTCGATGG
1321    AAGTTTGAGG CAATAACAGG TCTGTGATGC CCTTAGACGT TCTGGGCCGC ACGCGCGCTA
1381    CACTGACGGA GCCAACGAGT ATTAACCTTG GCCGAGAGGT CTGGGAAATC TTGTGAAACT
1441    CCGTCGTGCT GGGGATAGAG CATTGTAATT ATTGCTCTTC AACGAGGAAT TCCTAGTAAG
1501    CGCAAGTCAT CAGCTTGCGT TGATTACGTC CCTGCCCTTT GTACACACCG CCCGTCGCTA
1561    CTACCGATTG AATGGCTTAG TGAGGCCTCC GGATTGGTTT AAAGAAGGGG GCAACTCCAT
1621    CTTGGAACCG
```

Figure 2.8. Nucleotide sequence (1630 bp) of the 18S rRNA gene of *Debaryomyces hansenii* AY1. The underlined sequence was not used in the phylogenetic analysis described in section 2.3.11. The above sequence was submitted to GENBANK and assigned the accession number AY940171.

The physical and phenotypic characteristics of yeasts SS1 and AY1 are summarized in Table 2.6. The vegetative cells of the yeast SS1 produce slimy colonies when grown on YPD agar that are either cream, tan or pink in color. The cells are spherical or oval, approximately 8 μm in diameter and reproduce by budding (Fig. 2.10a). The yeast tested negative for the formation of ballistoconidia and the presence of ascospores. It grows optimally at a temperature of ~22 °C, but does not grow at temperatures at or above 30 °C. It grows in the presence of 3% (w/v) sodium chloride, but NaCl is not essential for growth. It is positive for the utilization of D-glucose, sucrose, D-fructose, D-galactose, D-mannitol, D-ribose, maltose, cellobiose, arabinose, threonine and lactose. Of the carbohydrates tested, none could be fermented by yeast SS1. The yeast tested positive for the Diazonium blue B reaction, urea hydrolysis and the production of extracellular starch-like compounds.

The vegetative cells of yeast AY1 produce fluffy white or cream colonies when grown on YPD agar. The cells are spherical in shape, between 8 and 10 μm in diameter and reproduce by budding (Fig. 2.10b). The yeast tested negative for the formation of ballistoconidia, positive for the presence of ascospores and can grow at temperatures of up to 30 °C. It can grow in the presence of 3% NaCl, but NaCl is not essential for growth. It is positive for the utilization of D-glucose, fructose, sucrose, D-galactose, D-mannitol, D-ribose, maltose, cellobiose and arabinose. Of the carbohydrates tested, all except threonine and lactose could be utilized by the yeast and it was able to ferment fructose, D-glucose and mannose. The yeast tested negative for the Diazonium blue B reaction, urea hydrolysis and the formation of extracellular starch-like compounds.

Table 2.5. The top 10 sequence similarities to yeasts SS1 and AY1 as obtained from the BLAST search of the GENBANK database with the 1709 bp 18S rRNA gene sequence of yeast SS1 and the 1630 bp 18S rRNA gene sequence of strain AY1.

18S rRNA gene sequence of yeast SS1		
Species	**% sequence similarity**	**GENEBANK accession number**
Cryptococcus cellulolyticus	99	AB032624.1
Unidentified fungus sp. QPX	99	AF261665
Cryptococcus laurentii	99	AB032640.1
Bullera pseudoalba	99	D31660.1
Cryptococcus aureus	99	AB085795.1
Bullera unica	98	D78330.1
Cryptococcus flavescens	98	AB085797.1
Cryptococcus flavescens	98	AB085796.1
Bullera penniseticola	98	AB005452.1
Bullera hannae	98	D78327.1
18S rRNA gene sequence of yeast AY1		
Debaryomyces hansenii var. hansenii	99	AB106349.1
Debaryomyces sp. strain:MBIC4210	99	AB022440.1
Debaryomyces hansenii	99	AB070854.1
Candida sp. BG99-8-11-1-4-1	99	AY242150.1
Debaryomyces hansenii var. hansenii	99	AB013590.1
Debaryomyces hansenii var. hansenii	99	AB013568.1
Debaryomyces hansenii var. fabryi	99	AB013567.1
Debaryomyces hansenii	99	DHA508273
Debaryomyces hansenii	99	X58053.1
Candida psychrophila	99	AB013528.1

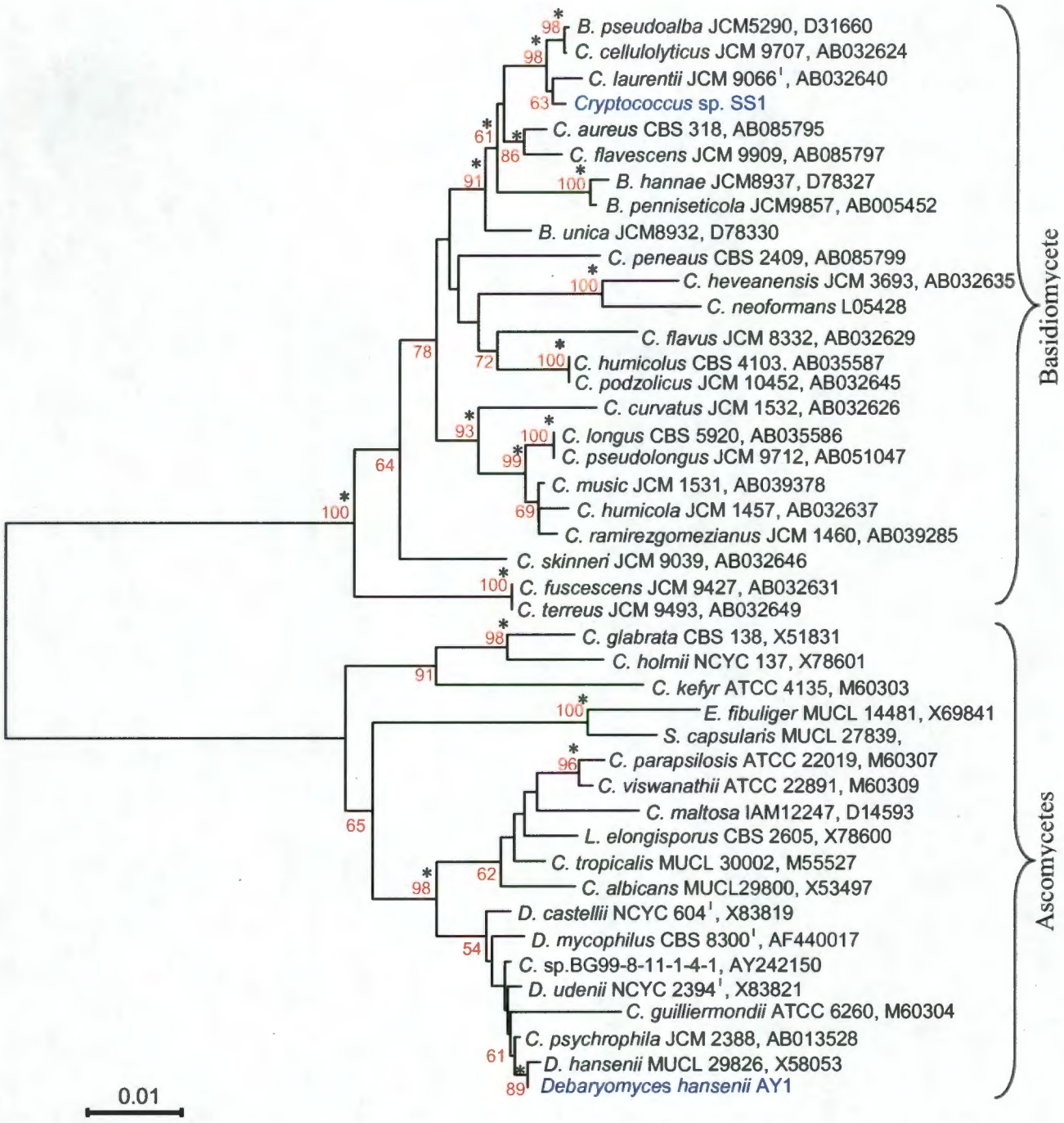


Figure 2.9. A phylogenetic tree derived from a distance-matrix analysis of the 18S rRNA gene sequences of *Cryptococcus* sp SS1 and *Debaryomyces hansenii* AY1 with 23 Basidiomycete and 18 Ascomycete sequences using MEGA version 2.1 (Kumar *et al.* 2001). Numbers at the nodes indicate the bootstrap values retrieved from 1000 replicates (frequencies less than 50% not shown). *, >60 bootstrap value for parsimony analysis. Numbers next to specific names correspond to the strain and accession numbers for the 18S rRNA gene sequences. The bar depicts 1 base substitution per 100 nucleotides.

Table 2.6. Summary of the physical and phenotypic characteristics of yeast strains SS1 and AY1

Characteristic	*Cryptococcus* sp. SS1	*Debaryomyces hansenii* AY1
Cell shape	Spherical/oval	Spherical
Colony pigmentation	Slimy/Pink	White/Cream
Budding	Polar	Multilateral
Formation of ballistoconidia	-	-
Presence of ascospores	-	+
Starch formation	+	-
Urea hydrolysis	+	-
DBB test	+	-
Growth in 3% NaCl broth	+	+
NaCl required for growth	-	-
Utilization of: **D-glucose, sucrose, D-fructose, D-galactose, D-mannitol, D-ribose, maltose, cellobiose, arabinose** **Threonine, lactose** **Fermentation of:** **Fructose, D-glucose, mannose**	 + + -	 + - +
Growth at: **22 °C** **30 °C** **37 °C** **41 °C**	 + - - -	 + + - -

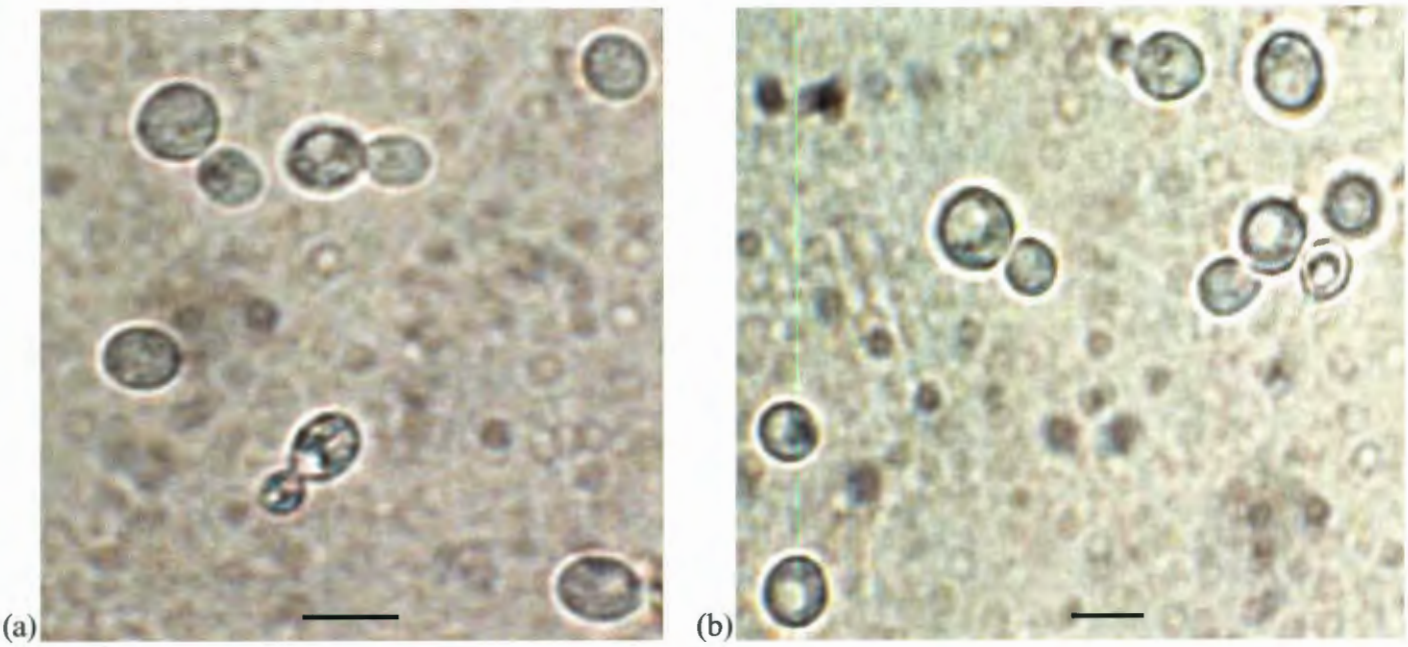
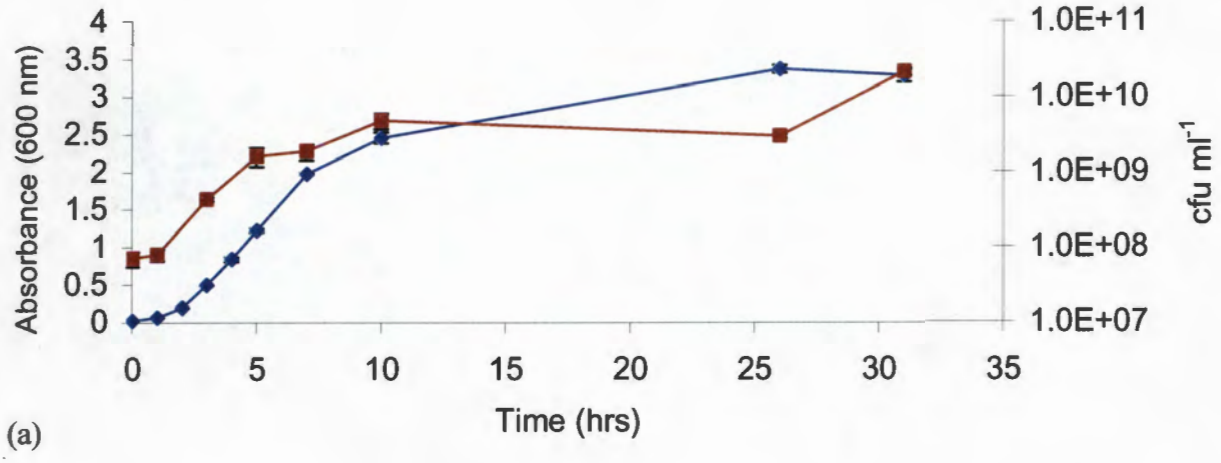


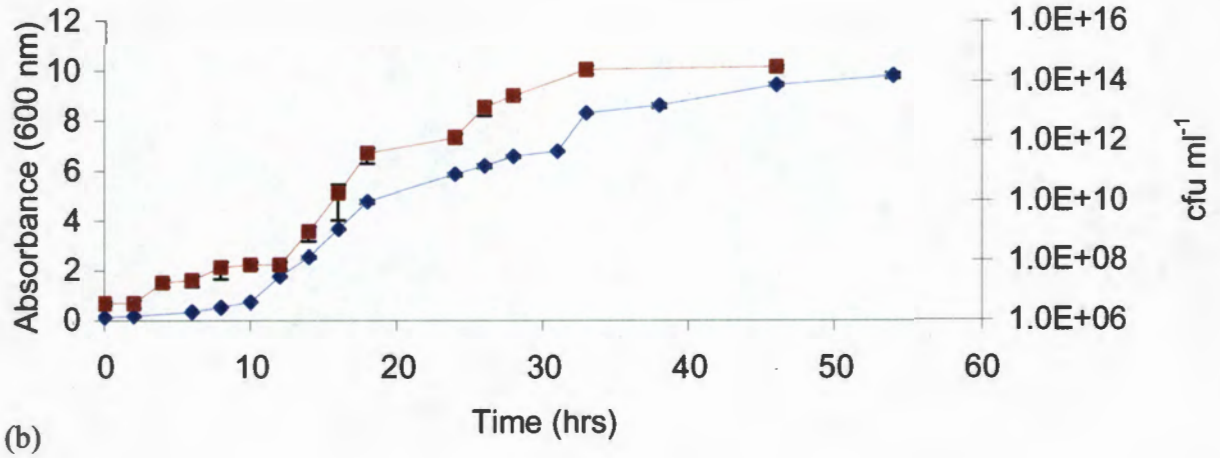
Figure 2.10. Vegetative cells of *Cryptococcus* sp. SS1 (a) and *Debaryomyces hansenii* AY1 (b) grown in YPD broth for approximately 48 hrs at room temperature. Bars, 10 µm.

2.4.4 Bacterial and yeast growth curve analysis

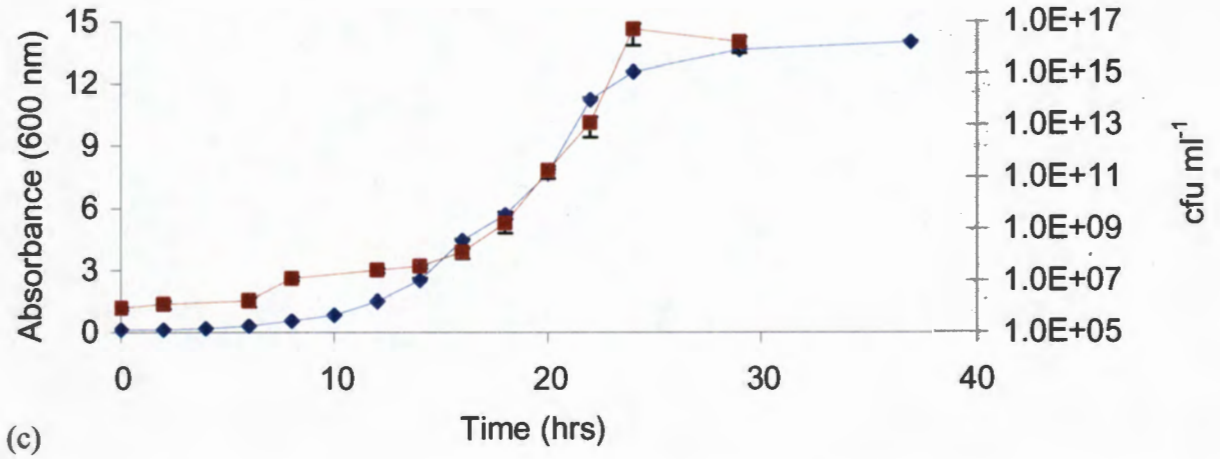
Shake-flask cultivation of *Vibrio midae* SY9 in marine broth resulted in a lag phase (λ) of approximately 1.56 hrs and a maximum specific growth rate (µ) of 0.37 h^{-1} at room temperature (Fig. 2.11a). The maximum biomass yield of strain SY9 was approximately 2 x 10^{10} cfu ml^{-1}, at 3.3 OD units, after 31 hrs of growth. Shake-flask cultivation of *Cryptococcus* sp. SS1 in YPD broth resulted in a lag phase (λ) of approximately 6.4 hrs and a maximum specific growth rate (µ) of 0.18 h^{-1} at room temperature (Fig. 2.11b), as apposed to *Debaryomyces hansenii* AY1, which had a longer lag phase, approximately 11 hrs, and a higher maximum specific growth rate, approximately 0.28 h^{-1} at room temperature (Fig. 2.11c). Furthermore, the maximum biomass yield (A) of yeast AY1 was higher, approximately 5 x 10^{16} cfu ml^{-1} (13 OD units) after only 24 hrs of growth, compared to strain SS1, approximately 3 x 10^{15} cfu ml^{-1} (10 OD units) after 46 hrs of growth (Fig. 2.11).



(a)



(b)



(c)

Figure 2.11. The growth profiles of strains SY9 (a), SS1 (b) and AY1 (c) showing the relationship between absorbance at 600 nm (♦) and the total number of colony forming units (cfu) per ml (■).

2.5 Discussion

Erasmus (1996) performed a detailed analysis of the types of enteric bacteria that colonize the different regions of the digestive tract of *Haliotis midae*. Furthermore, she used a number of different algal polysaccharides as substrates to examine the role of microbes in the digestive physiology of *H. midae*. She clearly showed that bacteria occur throughout the digestive tract of *H. midae* that are capable of hydrolyzing the complex algal polysaccharides agarose, carrageenan, carboxymethylcellulose (CMC), laminarin and alginic acid found in the seaweeds *Ecklonia maxima* and *Gracilaria gracilis*. In a similar study, Harris *et al.* (1998a) isolated bacteria from the digestive tract of the greenlip abalone, *Haliotis laevigata* Donovan, that were able to hydrolyze CMC, agar and starch. Hamid *et al.* (1979) (reviewed in Ringø and Birkbeck, 1999) isolated bacteria from the digestive tract of the grey mullet, *Mugil cephalus* L., which exhibited protease, amylase, chitinase and lecithinase activity and Gatesoupe *et al.* (1997) isolated a bacterium from the seabass larva, *Dicentrarchus labrax*, that exhibited protease, phospholipase, amylase and lipase activity. These results are similar to findings in the present study where microflora isolated from the digestive tract of *H. midae* exhibited protease and amylase activity.

Data presented in this study demonstrates that the gut microflora of *H. midae* are able to hydrolyze the substrates included in artificial abalone feed formulations. In fact, most of the isolated bacteria could utilize all of the substrates tested. To my knowledge, this is the first study to report the isolation of enteric microflora from the gut of *H. midae* capable of degrading protein and starch substrates included in artificial abalone feeds. Furthermore, the enzyme analysis conducted in this study showed that different bacterial strains had vastly different enzyme activities, similar to the results obtained by Erasmus *et al.* (1997). One bacterium, designated *Vibrio midae* SY9, exhibited high protease activity on all of the protein substrates tested and a yeast strain, designated *Cryptococcus* sp. SS1, exhibited amylase activity at least 7 times greater than any of the other amylase producing strains. These properties were deemed potentially advantageous to the nutrition of *H. midae* fed artificial feeds rich in protein and starch. As a result, these two strains were selected for further analysis. Since it has been shown that yeasts and yeast extracts have immunostimulatory properties, which can confer resistance

against a wide range of pathogens (Miles *et al.*, 2001; Suphantharika *et al.*, 2003), yeast strain AY1 was also selected for further analysis. Furthermore, our data showed that this strain was able to hydrolyze all of the protein and starch substrates tested in this study, further validating its use as a potential probiotic strain.

Hamid *et al.* (1979) (reviewed in Ringø and Birkbeck, 1999) found that many of the bacteria isolated from the digestive tract of *M. cephalus* L., which exhibited protease and amylase activity, were members of the *Vibrio* genus. Similarly, Gatesoupe *et al.* (1997) identified a bacterium from *D. labrax*, exhibiting protease and amylase activity, as a *Vibrio*. More specifically, Erasmus *et al.* (1997) showed that a large percentage of the bacteria isolated from the digestive tract of *H. midae* belonged to the *Vibrio* genus, while Sawabe *et al.* (2003) showed that *Vibrio* species, in particular *V. halioticoli*, constitute between 40 – 65% of the indigenous microflora of several Haliotid species, including *H. midae*. Therefore, it is not surprising that the bacterium identified in this study, exhibiting protease and amylase activity, belongs to the genus *Vibrio*. Indeed, the production of these enzymes by *Vibrio* species is fairly common (Table 2.4). Furthermore, it is thought that these enzymes play an important role in the ability of *Vibrio* species to colonize the gut of marine animals (Ringø and Birkbeck, 1999).

The members of the genus *Vibrio* are straight or curved Gram-negative rods, are marine, halophilic, facultatively anaerobic bacteria that are common inhabitants of estuarine and marine waters (Aznar *et al.*, 1994; Shieh *et al.*, 2003). The 16S rRNA gene sequence, phylogenetic analysis and the physical and phenotypic characteristics of bacterium SY9 suggest that it is a member of the *Vibrio* genus. Since this strain is significantly different to the phenotypic characteristics of the other members of the *Vibrio* genus, we propose that the bacterium SY9 be considered a new species and thus be designated *Vibrio midae* SY9. This approach was considered sufficient for allocating novel species to an isolate from the Mediterranean sea, designated *Vibrio agarivorans* sp. nov. (Macián *et al.*, 2001) and to an isolate from the red algae, *Gracilaria gracilis*, designated *Pseudoalteromonas gracilis* B9 (Schroeder *et al.*, 2003).

Sawabe *et al.* (2004) isolated a *Vibrio*, designated *V. gallicus* sp. nov., from the gut of the French abalone, which could be differentiated from *V. halioticoli*, its closest phenotypic relative, on the

basis of four phenotypic traits, and described it as a new species. Similarly, *Vibrio* species, isolated from several Haliotid species, have been described as new species based on 8 – 14 phenotypic differences to their closest relatives (Hayashi *et al.*, 2003; Sawabe *et al.*, 2004; Shieh *et al.*, 2003). In all of these studies, the description of a new species included DNA-DNA hybridization experiments, which is consistent with the suggested criterion for the designation of a species (Murray *et al.*, 1990; Wayne *et al.*, 1987). However, Sawabe *et al.* (2000) (reviewed in Schroeder, 2001) showed that DNA-DNA hybridization data can be limiting, sometimes conflicting with rRNA gene sequence data and physical and phenotypic characteristics, leading to the incorrect differentiation of species. Consequently, Wayne *et al.* (1987) stated that in the event of a disagreement between phenotypic characteristics and DNA-DNA hybridization data, phenotypic characteristics would take precedence, thus stressing the importance of phenotypic characteristics in the description of a new species. Indeed, the levels of relatedness between members of the genus *Vibrio* have been found to be inconsistent when different phylogenetic approaches have been used (Dorsch *et al.*, 1992). However, although DNA-DNA hybridization data can be limiting, it is still considered important when describing a new species. Thus, due to the absence of DNA-DNA hybridization data in this study, as in Schroeder *et al.* (2004) and Macián *et al.* (2001), strain SY9 is tentatively considered as a new species.

The Vibrios are commonly found in estuarine, coastal and oceanic waters and are found associated with a number of marine organisms, including fish, crustaceans and several marine molluscs (Shieh *et al.*, 2003). Members of this genus have had a profound effect on human civilization, particularly the pathogenic strains, such as *V. cholerae* and *V. parahaemolyticus*. The *Vibrio* genus forms the dominant intestinal microflora of many marine invertebrates and has been positively implicated in the health of some of these species (Erasmus, 1996; Sawabe *et al.*, 2003; Vine *et al.*, 2004). *Vibrio halioticoli*, isolated from the digestive tract of several Haliotid species, is thought to contribute significantly towards the digestion of alginate, the major polysaccharide found in Japanese and South African kelps ingested by these animals (Sawabe *et al.*, 2003). Furthermore, a number of *Vibrio* species have also been identified as candidate probiotics (Austen *et al.*, 1995; Gatesoupe, 1997; Gomez-Gil *et al.*, 2002; Gullian *et al.*, 2004; Riquelme *et al.*, 1997). In particular, *V. alginolyticus*, originally isolated from a shrimp hatchery, has been used effectively as a probiotic for shrimp, improving the survival of shrimp larva

exposed to known pathogens (Gatesoupe, 1999). Thus, this genus is fast gaining importance as a dominant member of the indigenous microflora of marine animals that could potentially improve the health of cultured marine organisms.

The members of the *Debaryomyces* genus are characterized by budding cells that produce white or cream colonies, sometimes pseudohyphae, do not form ballistoconidia and reproduce sexually by ascospores (Barnett *et al.*, 1983). The 18S rRNA gene sequence, phylogenetic analysis and the physical and phenotypic characteristics of yeast AY1 suggest that it is a member of the *Debaryomyces* genus. Furthermore, the ability of yeast AY1 to form ascospores excludes it from the genus *Candida*, which was closely associated with yeast AY1 in the phylogenetic analysis. The close association between the *Candida* and *Debaryomyces* genera in the phylogenetic analysis is in agreement with the results obtained by Cai *et al.* (1996). A comparison of the physiological characteristics of several *Debaryomyces hansenii* strains, summarized in Barnett *et al.* (1983), to those exhibited by yeast AY1 indicates a high degree of similarity between yeast AY1 and other *Debaryomyces hansenii* strains. Of the physiological characteristics tested, yeast AY1 differed only in its ability to utilize cellobiose. This data is supported by the close association between yeast AY1 and *D. hansenii* MUCL 29826 (bootstrap 89%) in the phylogenetic analysis and the result of the BLAST search of the GENBANK database with the yeast AY1 18S rRNA gene sequence. Consequently, we propose that the yeast AY1 be assigned to the genus *Debaryomyces*, and thus be designated *Debaryomyces hansenii* AY1. However, this is a tentative assignment since the DNA-DNA hybridization data is still outstanding. Furthermore, due to a lack of adequate phenotypic data published in the literature, a more detailed comparison of yeast AY1 and other members of the *Debaryomyces* genus was not possible. Therefore, we recommend that DNA-DNA hybridization experiments and a more detailed physiological analysis be conducted in order to determine whether the yeast AY1 is a new strain of *D. hansenii*.

The 18S rRNA gene sequence, phylogenetic analysis and the physical and phenotypic characteristics of yeast SS1 suggest that it is a member of the *Cryptococcus* genus. Some of the key diagnostic features of members of this genus, which are exhibited by yeast SS1, include the production of extracellular starch-like polysaccharides and the formation of slimy, pink colonies

when cultured on solid yeast media (Barnett *et al.*, 1983). Although the 18S rRNA gene sequence analysis suggested that yeast SS1 could be a member of the *Bullera* genus, its inability to form ballistoconidia, a key diagnostic feature of members of the *Bullera* genus (Suh *et al.*, 1996; Takashima *et al.*, 2001), exclude it from this genus. Unfortunately, no published phenotypic data was available for *C. cellulolyticus* JCM 9707. However, the physiological characteristics of yeast SS1 closely matched those of *C. laurentii* JCM 9066^T as described by Takashima *et al.* (2003). In fact, of the physiological characteristics tested, the only difference between the two strains was the inability of yeast SS1 to grow at temperatures above 22 °C, compared to *C. laurentii* JCM 9066^T, which has a maximum growth temperature of 35 °C (Takashima *et al.*, 2003). However, we recommend that DNA-DNA reassociation experiments and further physiological characterization be performed in order to determine whether yeast SS1 is a distinct species, as further analysis was not possible due to time constraints. Consequently, we propose that the yeast SS1 be assigned to the genus *Cryptococcus*, and be designated *Cryptococcus* sp. SS1.

Erasmus (1996) identified coccoid shaped bodies in the digestive tract of *H. midae* that were similar in shape and size to yeast cells previously observed in the gut of rainbow trout and turbot (Andlid *et al.*, 1995). Andlid *et al.* (1995) identified the yeast cells as *Debaryomyces hansenii* (strain HF1) and Tovar *et al.* (2002) subsequently tested strain HF1 as a probiotic for the sea bass, *Dicentrarchus labrax*. The present study confirms the presence of yeast cells in the digestive tract of *H. midae*, supporting the observations made by Erasmus (1996).

Vine *et al.* (2004) suggested that putative probionts with the shortest lag period (λ) and fastest growth rate (μ) would potentially out-compete other microorganisms in the digestive tract of the host, thus enabling them to exert their probiotic effect. Based on these assumptions and the data obtained in this study, *V. midae* SY9 is considered as the best candidate probiotic out of the three selected strains. *V. midae* SY9 had the fastest growth rate and the shortest lag period, which could potentially enable it to out-compete other microbes *in vivo*. Furthermore, the types of enzymes secreted by *V. midae* SY9 could potentially enable it to effectively colonize the digestive tract of *H. midae*. However, growth is not the sole criterion useful for screening potential probiotics (Vine *et al.*, 2004). Other factors, such as production of antagonistic

metabolites, the secretion of extracellular enzymes and the ability to attach to the intestinal tract of the host organism are also important (Ringo and Birkbeck, 1999; Vine *et al.*, 2004). Furthermore, if the growth profiles of the putative probionts are known, then microbes with a slow growth rate (μ) and long lag phase (λ) could be cultured in large numbers before being added to experimental feeds or the aquaculture system (Vine *et al.*, 2004). Indeed, this approach has been used in several studies (Douillet and Langdon, 1994; Rengpipat *et al.*, 1998; Robertson *et al.*, 2000). Although the two yeast strains had a slower growth rate (μ) and longer phase (λ) compared to *V. midae* SY9, the maximum biomass yield of the two yeast strains was far greater than *V. midae* SY9. Collectively these results mean that the three putative probionts, SY9, SS1 and AY1, can be successfully cultivated to high cell densities for incorporation into commercial abalone feed formulations. Furthermore, it must be noted that all of these strains have been cultured in shake-flasks and that fermentation parameters still need to be optimized in order to generate high cell-density cultures, which could substantially improve cell biomass, enzyme formation and enzyme secretion (Lee *al.*, 1999; Riesenberg and Guthke, 1999). In fact, a project is underway in our laboratory to optimize the fermentation parameters of the three candidate strains identified in this study, which could make these strains even more desirable as putative probiotics for the enhancement of growth and disease resistance in *H. midae*.

CHAPTER 3

THE EFFECT OF DIETARY SUPPLEMENTATION WITH *VIBRIO MIDAE* SY9, *CRYPTOCOCCUS* SP. SS1 AND *DEBARYOMYCES HANSENII* AY1 ON THE GROWTH AND NUTRITION OF FARMED *HALIOTIS MIDAE*

CONTENTS

3.1 Summary

Vibrio midae SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1 were evaluated for their potential use as probiotics for farmed *H. midae*. A mixture of the three probiotic strains were added to a local commercial abalone feed so as to achieve a final culturable cell concentration of each probiont of approximately 1.0×10^7 cfu g^{-1} of dried feed. The optimal storage temperature for maximum stability of each probiont in the probiotic-supplemented feed was determined to be 4 °C, with no significant decrease ($P<0.05$) in culturable cell counts over a 6 week storage period for strains SY9 and AY1. Furthermore, we have shown that *H. midae* fed the probiotic-supplemented diet have an improved growth rate compared to animals not fed probiotics. The growth rate of small (20 mm) and large (67 mm) abalone was improved by 8% and 34%, respectively, in two separate eight-month farm trials. *In situ* protease and amylase assays showed that probiotic treatment significantly increased ($P<0.05$) both protease and amylase activity in the intestinal region of the digestive tract of animals fed the probiotic-supplemented feed. This correlated with a significant increase ($P<0.05$) in the amount of protein digestion and absorption measured in this region of the abalone gut.

3.2 Introduction

Improving the slow growth rate of farmed *Haliotis midae* remains one of the primary challenges of aspirant abalone farmers in South Africa. While research has shown that the growth rate of *H. midae* is faster in captivity than in wild populations (Cook, 1990), it still takes approximately 5 years for cultured abalone to reach a market size of 100 g, representing a substantial investment to local abalone farmers. Initial research on improving the growth rate of cultured abalone focused primarily on ways of obtaining higher feed conversion efficiencies through the use of various artificial diets and optimizing growth conditions, such as water temperature, pH, ammonia and oxygen availability (Britz and Hecht, 1997; Cook, 1990; Harris *et al.*, 1998b). However, as postulated by Erasmus *et al.* (1997), another potentially important aspect is the role of microorganisms present in the digestive tract.

The suggestion that exogenous enzymes produced by gut microflora may be of benefit to the host animal (Ringø and Birkbeck, 1999; Vitalis *et al.*, 1988) prompted researchers to investigate the effect of selected microorganisms on the growth of various marine species. Douillet and Langdon (1994) showed that the addition of a specific bacterium as a food supplement to xenic cultures of *Crassostrea gigas* larvae enhanced the growth of the larvae. Nogami and Maeda (1992) isolated a bacterial strain from a crustacean culture pond and showed that it was able to enhance the growth of the crab, *Portunus trituberculatus*, while Rengpipat *et al.* (2000) showed that a bacterium, *Bacillus* S11, enhanced the growth of the black tiger shrimp *Penaeus monodon*. More recently, Sawabe *et al.* (2003) suggested a possible positive association between the bacterium *Vibrio halioticoli* and several Haliotid species from which the bacterium was isolated. However, it was Erasmus *et al.* (1997) who first suggested that enteric bacteria of the South African abalone *H. midae* could enhance the growth rate of the host.

Erasmus *et al.* (1997) showed that bacteria exist throughout the digestive tract of *H. midae* that are capable of breaking down complex polysaccharides such as laminarin, carboxymethylcellulose (CMC), alginate, agarose and carrageenan, which are found in *Ecklonia maxima* and *Gracilaria gracilis*. Furthermore, a comparison of polysaccharolytic activity between bacteria-free and normal abalone showed that enteric bacteria improved hydrolysis of

the above mentioned algal polysaccharides (Erasmus *et al.*, 1997). Erasmus *et al.* (1997) therefore suggested that bacterial polysacharases could have a beneficial effect on the growth of *H. midae* and that dietary supplementation with specifically selected bacteria, with enhanced enzyme activities, could lead to an improvement in the growth rate of this species. In fact, a preliminary study carried out in our laboratory showed that a bacterium with enhanced alginate lyase activity, isolated from *H. midae*, was able to enhance the growth of farmed *H. midae* fed a diet of *Ecklonia maxima*, a brown seaweed with a cell wall composed primarily of alginate (ten Doeschate, *unpublished data*). Similarly, this concept can be extended to other diets, such as artificial diets with a high protein content. If the constituents of the diet are known, then selected bacteria, capable of hydrolyzing protein as well as other components, could be included in the diet to assist the host organism with digestion. Indeed, De Schrijver and Ollevier (2000) demonstrated that dietary supplementation with the potential probiotic bacterium, *Vibrio proteolyticus*, tended to enhance apparent protein digestibility in the juvenile turbot, *Scophthalmus maximus*. However, the effect that this had on growth of *S. maximus* was not investigated.

A problem still facing South African abalone farmers is the identification of an optimal diet for cultured *H. midae*. Feed makes up a large percentage of production costs on abalone farms, with the majority of South African abalone farmers feeding their animals a diet of either *E. maxima* or *G. gracilis*, and in some cases, protein-based artificial diets (Sales and Britz, 2001). However, rapid expansion of the local industry has placed mounting pressure on natural seaweed stocks, particularly *E. maxima*. As a result, a regular supply of large quantities of this seaweed is becoming increasingly problematic, forcing abalone farmers to progressively use more artificial diets (Britz, 1996), as has already occurred internationally (Serviere-Zaragoza *et al.*, 1997). The identification of a cost-effective, nutritionally complete artificial diet is therefore of paramount importance to the local industry, promoting a great deal of research in this field. Furthermore, in order to achieve maximal growth rates on artificial diets, the rate of nutrient digestion and absorption by the abalone needs to be maximised (Britz and Hecht, 1997).

The aim of this study will therefore be to determine the effect of inclusion of the three putative probionts, *Vibrio midae* SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1, on the

growth of *H. midae*. Another practical aspect of the use of probiotics in abalone diets that needs to be determined is the possible positive effect that they could have on the digestion and absorption of feed. Therefore, an additional aim of this study will be to obtain information in this regard. After feeding the animals a diet with or without probiotic supplementation, the amount of protein digested and absorbed in different segments of the digestive tract of *H. midae* will be investigated.

3.3 Materials and methods

All media and solutions used in this study are listed in Appendix A.

3.3.1 Media and culture conditions

Media and culture conditions for the three putative probionts are as described in section 2.3.12.

3.3.2 Preparation of experimental diets

The basal 'Abfeed' diet, formulated and supplied by Sea Plant Products Limited, consisted of fishmeal, starch, vitamins and minerals. Two diets were tested: (a) the basal diet (Basal) and (b) the basal diet supplemented with a mixture of the three putative probionts (SY9, SS1 & AY1) (Probiotic). Each probiont was added to the feed to achieve a final concentration of approximately 10^7 viable cells g^{-1} dried feed. Feed was prepared every three weeks for the duration of the growth trials and stored in clean, plastic bags at room temperature until used. Batches were routinely analyzed to ensure bacterial viability and cell number. For the *in situ* enzyme analysis and protein digestibility study, 0.5% chromic oxide (w/v) (Sigma) was included in the two dietary treatments to mark the particulate material for protein digestibility measurements (Shipton and Britz, 2001a). This approach was employed as chromic oxide was shown to be an effective marker for protein digestibility as opposed to the total faecal collection method (Shipton and Britz, 2001a). As in Maguire *et al.* (1993) and unlike Shipton and Britz (2001a), who only examined faecal pellets for determining nutrient digestibility, we did not separate the three faecal types as we were comparing protein digestibility in different regions of the digestive tract in two groups of animals (one group fed the probiotic-supplemented feed and the other fed a basal feed). However, it must be noted that when nutrient digestibilities are determined for the formulation of biologically and economically optimized diets, of the three faecal types produced by *H. midae*, Type 1 faeces provides the most reliable estimate for apparent protein digestibility in this species (Shipton and Britz, 2001a).

In order to determine the optimal storage temperature for the probiotic feed, batches of feed were stored at 4 °C, room temperature, 30 °C and 37 °C for up to 6 weeks. Cell numbers were determined by homogenizing 1 g quantities of feed (in triplicate) in 3 ml of sterile PBS (Appendix A.2.4) and spread-plating serial dilutions onto MA (Appendix A.1.2) and YPD agar (Appendix A.1.4) to culture the bacterial and yeast strains respectively. Cell numbers were recorded after incubating SY9 for 18 hrs and SS1 and AY1 for 3 – 4 days and viability recorded as the number of cfu g^{-1} of dried feed.

3.3.3 Growth trials

The effect of the dietary inclusion of the three putative probionts on the growth of *H. midae* was assessed in two separate growth experiments conducted on Sea Plant Products' commercial abalone farm in the Western Cape Province, South Africa. Abalone were kept in baskets (0.8 x 0.5 x 0.5 m) suspended in large concrete raceways under standard farming conditions (Fig. 3.1). Seawater, pumped directly from the sea, flowed through the raceways at a rate of 360 L/h and was constantly aerated. The first growth trial was conducted on abalone with an average initial size of 20 mm and maintained at an initial stocking density of 2000 animals/basket. The second growth trial was conducted on abalone with an average initial size of 67 mm and maintained at an initial stocking density of 200 animals/basket. For each growth trial there were four baskets, with abalone in two of the baskets fed the basal diet and animals in the remaining two baskets fed the probiotic-supplemented diet (Fig. 3.1).

Abalone were fed on a daily basis, with each basket receiving an equal amount of feed. All uneaten feed was removed from the baskets each evening before the addition of fresh feed. The duration of each experiment was between eight and ten months. Thereafter, the probiotic treatment was discontinued and all animals were fed the basal feed for a further two to three months. Weight and shell length measurements were taken every three months throughout the experimental period. Weight was recorded to the nearest 0.01 g using an electronic balance and shell length to the nearest 0.01 mm using electronic vernier callipers.

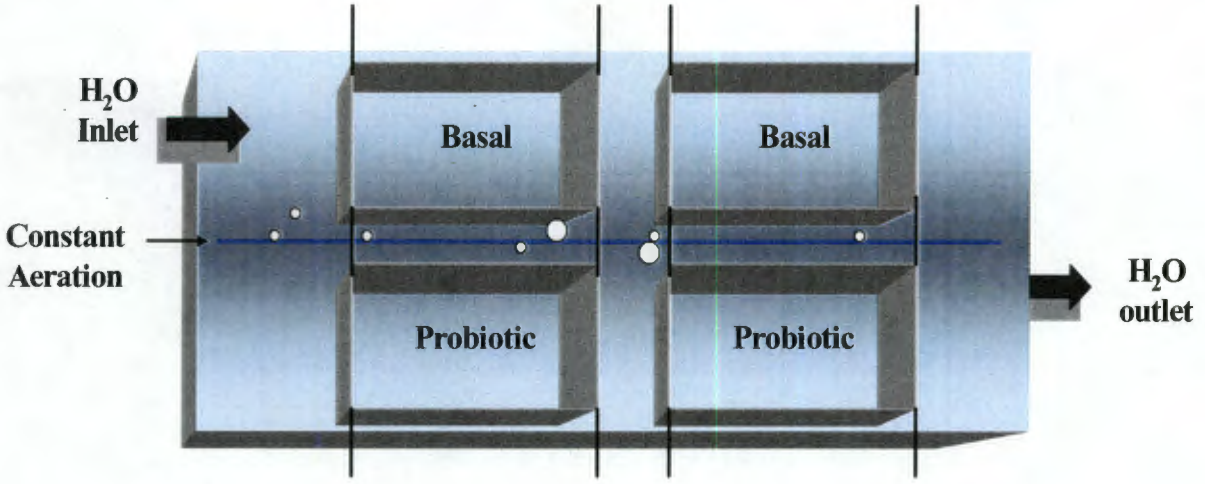


Figure 3.1. Schematic representation of the experimental design of the growth trials conducted on the 20 mm and 67 mm abalone.

The total increase in length and mass at each sampling time was calculated by subtracting the mean final weight or length from the mean initial weight or length. Monthly growth rate in shell length (mm/month) was calculated using the formula:

$$\text{Monthly growth rate} = ((L_t - L_o)/t)*30$$

where L_o is the mean initial abalone shell length and L_t is the mean abalone shell length at time t (days). Monthly growth rate in weight was calculated according to the formula:

$$\text{Monthly growth rate} = ((W_t - W_o)/t)*30$$

where W_0 is the mean initial abalone weight and W_t is the mean abalone weight at time t (days).

Each experiment was performed in duplicate and a total of approximately 200 animals, consisting of four randomly selected groups from each basket, were measured on each occasion. The monthly growth rate, in weight and shell length, was calculated for each group in order to

obtain an average growth rate for each of the baskets. Furthermore, data obtained from duplicate baskets in each growth trail was grouped for statistical analysis. The stocking density of the baskets was adjusted after each measurement according to farm requirements and availability of space.

3.3.4 *In situ* enzyme assays and protein digestibility

Haliotis midae (52.92 ± 1.36 mm shell length; 18.58 ± 1.82 g wet weight) were maintained in two large polyethylene tanks as described in section 2.3.1. Each tank contained 20 abalone, which were acclimated for a period of 4 weeks on the basal diet containing 0.5% chromic oxide. Two weeks prior to the start of the experiment abalone in each tank were further subdivided into four separate 2.5 L plastic containers. The top of each container was covered with a plastic mesh and in order to prevent abalone from eating their faeces, a plastic mesh was placed 2 cm from the bottom of each container. Animals in one tank were fed the basal diet (0.5 % chromic oxide) whereas animals in the second tank were fed the probiotic-supplemented diet (0.5 % chromic oxide).

Because abalone are erratic feeders and do not feed every evening (Shipton and Britz, 2001a), animals were starved for 3 days prior to the start of the experiment in order to ensure that upon presentation of the experimental feeds, there would be a rapid feed response in which all abalone would feed to satiation. Preliminary data showed that this 3 day starving period resulted in minimal feed remaining in the digestive tract prior to feeding and resulted in a rapid feeding response upon presentation of the experimental diets. Before the start of the experiment, all tanks were thoroughly cleaned and the water flow rate was reduced to approximately 6 L hr^{-1}. Animals were fed at 06h00 and the tanks covered and left in the dark so that the animals would feed. Uneaten feed was removed eighteen hours later at 24h00. Nine hours after the uneaten feed was removed, faeces were removed from each basket by washing the contents of the basket through a 100 μm mesh, which retained the faecal material. All of the animals were removed and immediately sacrificed. In a preliminary experiment, it was found that this time period resulted in the presence of digesta throughout the digestive tract, thus allowing a protein digestibility analysis to be conducted on the contents of all regions of the abalone digestive tract.

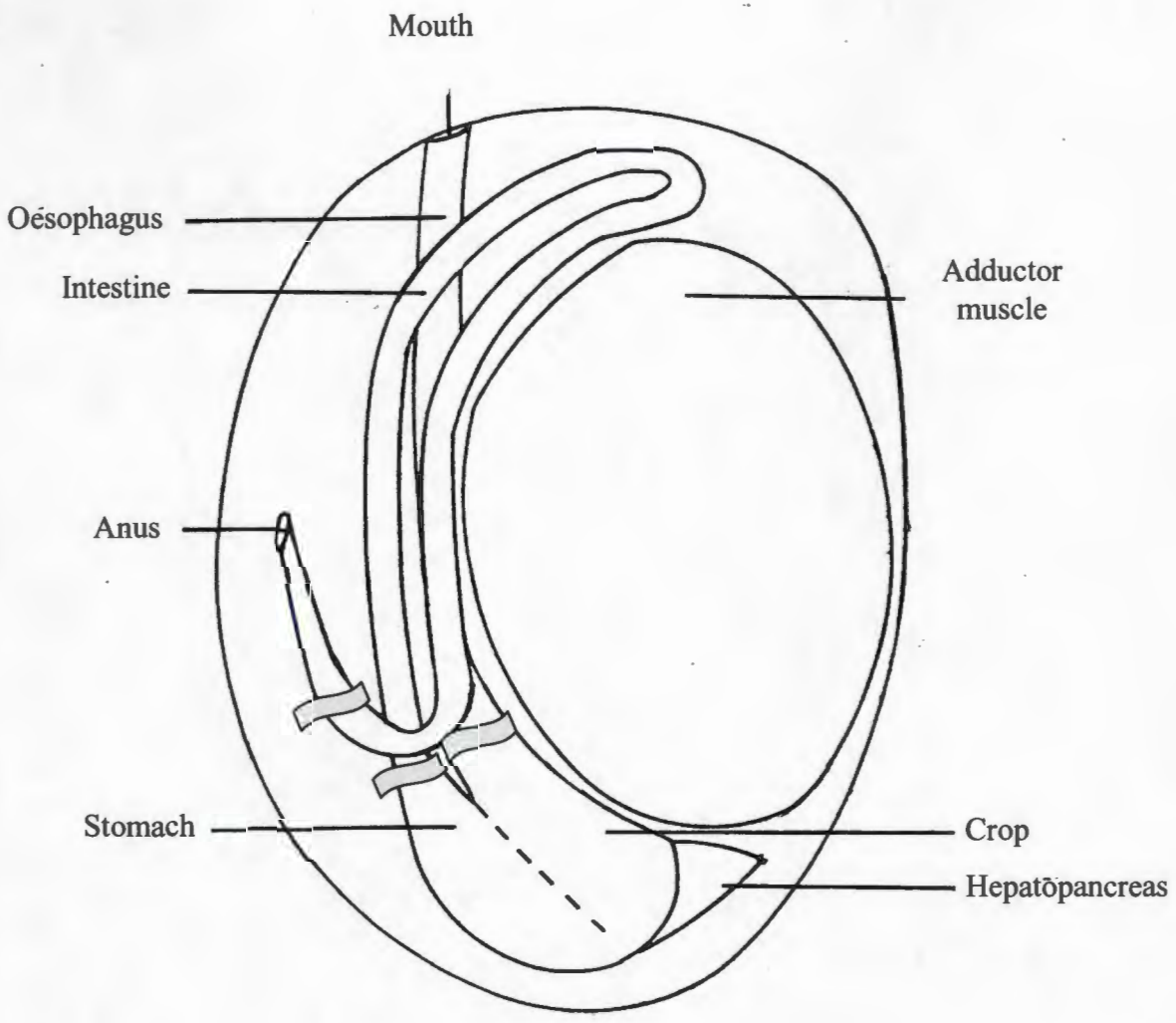


Fig 3.2. Schematic representation (anterior view) of the gut of *Haliotis midae* showing the portions of the digestive tract excised for *in* situ enzyme and protein digestibility analysis. The green bows indicate where sterile cotton was used to tie-off the digestive tract to prevent spillage during dissection. The organs removed for this analysis were the crop/stomach and intestine. Figure adapted from Erasmus *et al.* (1997).

Initially, the entire digestive tract was aseptically removed and placed on ice. Sterile cotton was used to tie-off the digestive tract before the crop, after the stomach and immediately before the anus in order to prevent spillage from the digestive tract during dissection. The crop/stomach and the intestine were then carefully dissected (Fig. 3.2) and the contents from the digestive segments gently extruded and collected separately. As the amount of digesta from individual abalone was insufficient for the analysis, samples obtained from the five abalone in each of the small containers were pooled. Equal quantities, by weight, of the pooled samples were resuspended in 10 ml PBS and centrifuged at 12 000 x g for 10 min at 4 °C. Supernatants were stored at – 20 °C, while the pellets and faeces were dried in an oven at 60 °C for 24 hours and subsequently ground with a pestle and mortar. Supernatants were retained for the determination of extracellular protease and amylase activity and the pellets for the determination of total nitrogen and chromic oxide in the digesta.

3.3.4.1 Enzyme assays

Protease and amylase activity of supernatants from the crop/stomach regions and intestine of *H. midae* were determined as described in sections 2.3.3.1 and 2.3.3.2.

3.3.4.2 Total protein determination

The oven-dried samples were aliquotted into triplicate 1 mg (±0.001 mg) samples using an electronic Satorius Micro Scale. Total nitrogen was measured using a CHNS–932 analyzer (LECO Co-orp., St Joseph, M.I., USA) and values multiplied by 6.25 to get an estimate of total protein.

3.3.4.3 Chromic oxide determination

The amount of chromic oxide in the samples was determined spectrophotometrically according to Divakkaran *et al.* (2002) with some modifications. Briefly, 40 mg of each oven-dried sample, in duplicate, was ashed at 600 °C for 4 hrs and subsequently added to 4 ml perchloride reagent (Appendix A.2.16) in 50 ml thick-walled Duran flasks. Four glass beads (2 mm diameter) were

added to each flask to prevent bumping during boiling. Flasks were heated on a temperature-controlled hot plate in a fume hood. A thermometer in a glass beaker filled with glass beads was placed at the centre of the hot plate to record temperature. The flasks were placed on the hot plate and heated to a temperature range of 210 – 220 °C for 10 – 12 minutes before being removed and allowed to cool. Once cooled, the liquid was quantitatively transferred to a volumetric flask and made up to 25 ml by repeatedly rinsing the flask with distilled water. Known quantities of chromic oxide (2 – 6 mg) were similarly treated in order to generate a standard curve. An ashed feed sample, lacking chromic oxide, served as a blank. The amount of chromic oxide in the ashed samples was determined by measuring the absorbance of the oxidized solutions at 370 nm using a Beckmann DU70 spectrophotometer. The samples that were made up to 25 ml were directly measured at 370 nm without any further dilution, whereas the standard oxidized chromic oxide solutions were serially diluted with distilled water to generate a concentration range of 20, 16, 12, 8, 4, and 2 μg ml^{-1}. The absorbance values of the dilution series were used to generate a regression equation to calculate the concentration of chromic oxide in the samples. Each sample was read at least three times and values converted to percentage chromic oxide.

Apparent protein digestibility (APD), which is indirectly based on the ratios of marker to nutrient in the feed and marker to nutrient in the digesta or faeces, was calculated from the protein and chromic oxide content of the feeds, digesta and faeces using the formula for digestibility described by Sales and Britz (2002):

$$\text{APD (\%)} = 100 \times \left[1 - \left[\frac{\text{\%marker in feed}}{\text{\%marker in the digesta or faeces}}\right] \times \left[\frac{\text{protein concentration in faeces}}{\text{protein concentration in feed}}\right]\right]$$

3.3.5 Statistical analysis

All data is presented as means and standard error. For comparison of two means, paired or unpaired student t-test were used where appropriate. For multiple comparisons, data was analyzed by one-way or two-way ANOVA where appropriate. When the effects of ANOVA were significant, the Tukey test was used to test for significant differences between sample means. Prior to statistical analysis, the cell count data underwent natural log transformations. Significant differences were established at $P<0.05$ and $P<0.1$.

3.4 Results

3.4.1 Number of culturable probiotics in the feed

In order to determine the optimal storage temperature of the probiotic-supplemented feed, the number of culturable probiotic cells in feed stored at various temperatures was determined. When feed was stored at a constant temperature of either 30 °C or 37 °C, the number of culturable probiotic microorganisms was considerably reduced, with minimal survival of cells after three weeks of storage (data not shown), compared to feed stored at either 4 °C or room temperature (R/T) (Fig. 3.3).

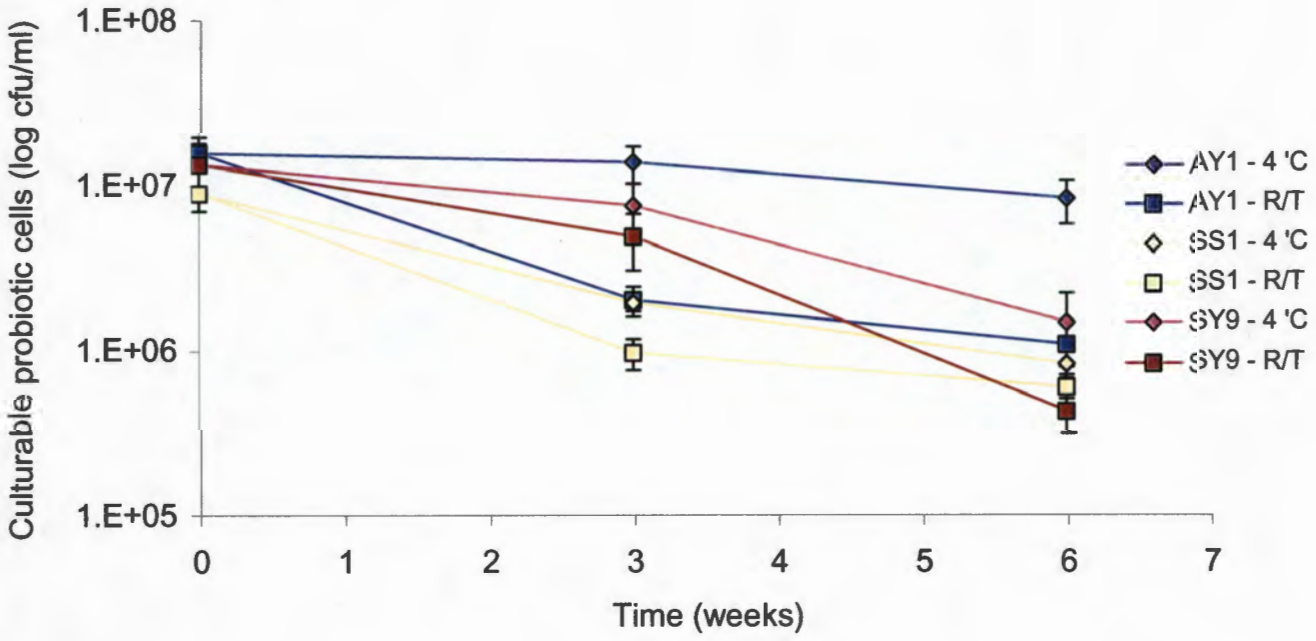


Figure 3.3. Viability of probiotic microorganisms in feed (cfu/gram feed) stored at a constant temperature of 4 °C and room temperature (~22 °C) over the course of 6 weeks. Data represents the mean ± standard error of three batches of probiotic Ab feed at each temperature.

When feed was stored at the latter temperatures, there was no significant difference ($P<0.05$, one-way ANOVA) in the number of culturable probiotic cells after 3 weeks, with the exception of strain SS1, at 4 °C and R/T, and AY1 at R/T. After 6 weeks of storage at R/T, there was a significant decrease ($P<0.05$) in the viability of all three strains. Conversely, when feed was stored at 4 °C, there was no significant difference ($P<0.05$) in the number of culturable probiotics after 6 weeks, with the exception of strain SS1. Although SS1 cell numbers decreased significantly ($P<0.05$) after 3 weeks of storage at 4 °C, from 9.0×10^6 to 1.9×10^6, their was no further decrease ($P<0.05$) from week 3 to 6. In general, the viability of all three strains was better when stored at a constant temperature of 4 °C.

3.4.2 Growth trials

In order to determine the effect of dietary inclusion of the three putative probionts on the growth of *H. midae*, growth experiments were conducted on both small (20 mm) and large (67 mm) abalone fed a diet with or without probiotic-supplementation. The mean length, mass and growth rate of 20 mm abalone fed the probiotic diet was not significantly ($P<0.05$) improved after 102 days of treatment (Fig. 3.4). However, mean length (mm) and growth rate (mm/month) was significantly ($P<0.05$) improved after 180 days of probiotic treatment and remained significant ($P<0.05$) up until the end of the 252 day growth trial (Fig. 3.4a). Similarly, the mean weight (grams) and growth rate (grams/month) of 20 mm abalone fed the probiotic diet was significantly ($P<0.1$) improved after 180 days and remained higher than the control animals up until the end of the 252 day growth trial, although this difference was not significant ($P<0.1$) (Fig. 3.4b). However, once the probiotic treatment was stopped and both groups of abalone were fed the basal feed for a further 84 days, the difference in growth rate between the animals formally fed the probiotic-supplemented diet and the control animals was no longer significant ($P<0.05$) (Fig. 3.4).

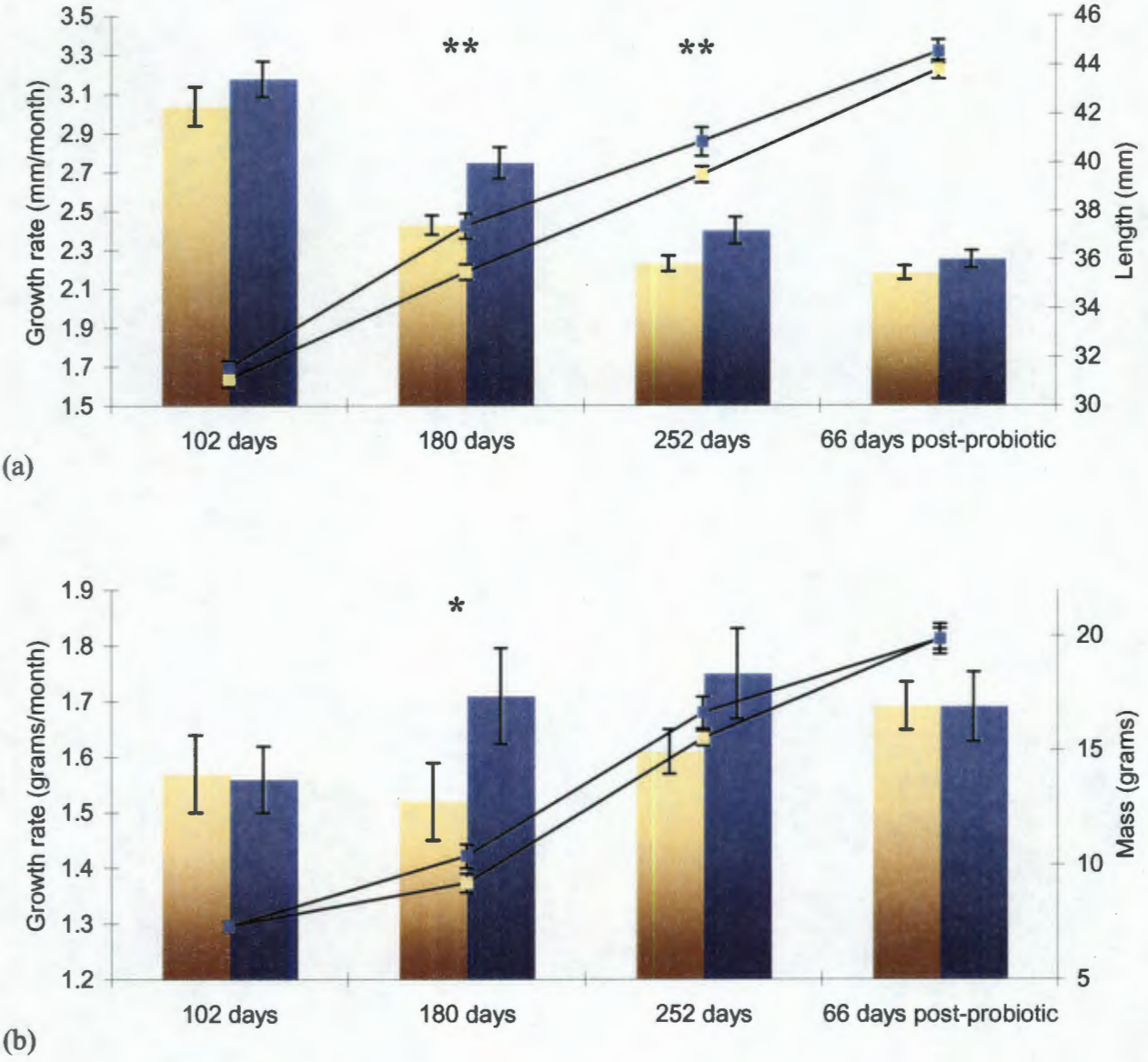
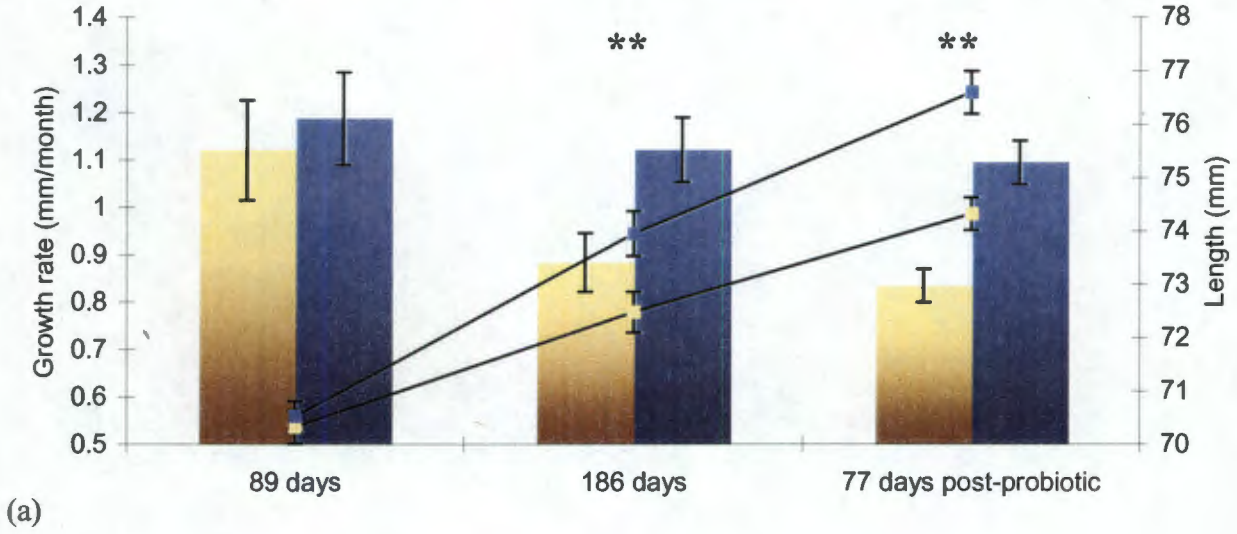
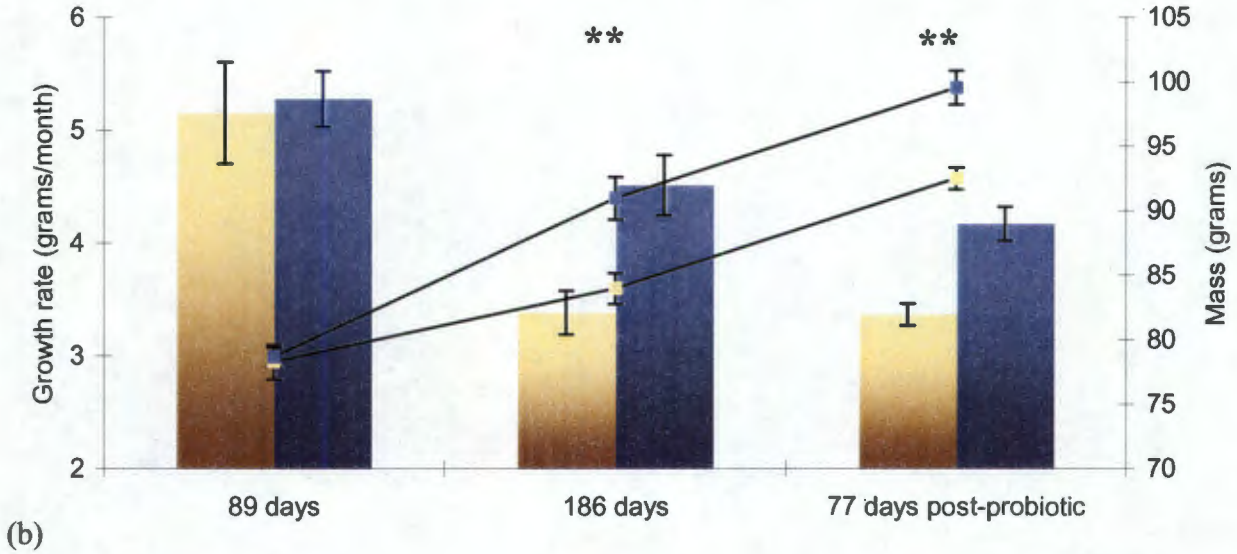


Figure 3.4. Mean length and growth rate in length (mm/month) (a) and mean mass and growth rate in weight (grams/month) (b) of *Haliotis midae* fed the basal feed (▪) or the probiotic feed (■) during the growth trial conducted on 20 mm abalone. Lines represent growth in length and mass and bars represent growth rate in length and mass. Data represents mean ± S.E. **($P<0.05$) and *($P<0.1$) (student t-test) represent a significant difference between means of abalone fed the basal or probiotic diet.



(a)



(b)

Figure 3.5. Growth rate in length (mm/month) (a) and growth rate in weight (grams/month) (b) of *Haliotis midae* fed the basal feed (▪) or the basal feed supplemented with probiotics (■) during the growth trial conducted on 67 mm abalone. Lines represent growth in length and mass and bars represent growth rate in length and mass. Each data point represents the mean ± S.E. **Represents a significant difference ($P<0.05$, student t-test) between means of the growth rate of abalone fed the basal or probiotic diet.

The mean length, mass and growth rate of 67 mm abalone fed the probiotic diet was not significantly improved ($P<0.05$) after 89 days of treatment (Fig. 3.5). However, there was a significant ($P<0.05$) improvement in the length, mass and growth rate of abalone after 186 days of probiotic treatment, with this difference remaining significant ($P<0.05$) 77 days after the probiotic treatment had ended, during which time both groups of abalone were fed the basal feed.

Overall, there was a greater percentage improvement in growth rate in the larger abalone (67 mm), with a 33% and 35% improvement in length and mass respectively, compared to the smaller abalone (20 mm), which exhibited a 7% and 8% improvement in length and mass, respectively. Furthermore, the mean growth rate in length was much greater in the smaller animals, whereas the mean growth rate in mass was much higher in the larger animals. The mean growth in weight increased by 7% over a 256 day experimental period for the small (20 mm) abalone and by 8% over the 186 day experimental period for the large (67 mm) abalone.

3.4.3 *In situ* enzyme assays

To determine whether the probiotic strains are contributing towards the pool of digestive enzymes in the digestive tract of *H. midae*, the protease and amylase activity of the supernatants from the intestine and crop/stomach region of abalone fed the basal and the probiotic diet were compared. Protease and amylase activity was higher in the crop/stomach region than in the intestine, regardless of whether the abalone had been fed the basal or the probiotic diet (Fig. 3.6). However, intestinal protease and amylase activity was significantly higher ($P<0.05$) in animals fed the probiotic diet as opposed to the basal diet. There was no significant difference ($P<0.05$) between the protease and amylase activity in the crop/stomach region of abalone fed the basal diet and those fed the probiotic diet.

3.4.4 *In situ* protein digestion and absorption

In order to determine whether the three probiotic strains have an affect on protein digestion in *H. midae*, the extent of protein digestion and absorption was examined in the faeces and different regions of the digestive tract of *H. midae* fed a diet with or without probiotic-supplementation.

The extent of protein digestion and absorption increased in both dietary groups as the gut contents moved along the digestive tract (Fig. 3.7). The amount of protein digestion and absorption measured in the crop/stomach region of the digestive tract was low, with no significant difference ($P<0.05$) in the percentage of undigested protein between animals fed the basal diet or the probiotic diet. Overall, the percentage digestion and absorption of protein was higher in animals fed the probiotic feed, with a statistically significant difference ($P<0.05$) measured at the site of the intestine. The percentage protein remaining in the faeces was higher (28.7%) in animals fed the basal diet compared to animals fed the probiotic diet (20.5%), however this difference was not statistically significant ($P<0.05$).

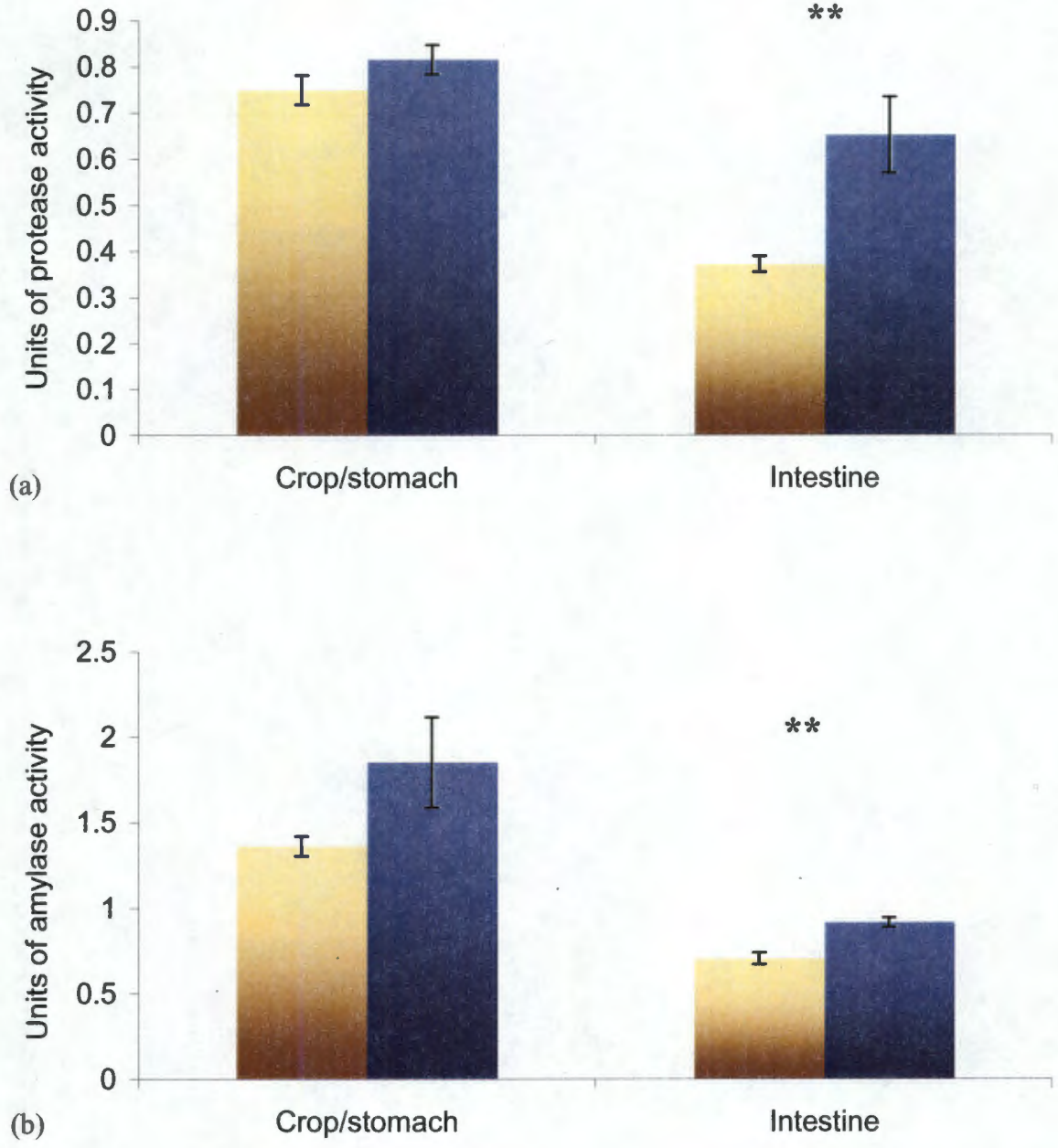


Figure 3.6. *In situ* protease (a) and amylase (b) activity in different regions of the digestive tract of *H. midae* fed the basal diet (□) versus the probiotic diet (■). Each data point represents the mean ± SE of at least three data points. ** Represents a significant difference ($P<0.05$, student t-test) between the means of two treatments.

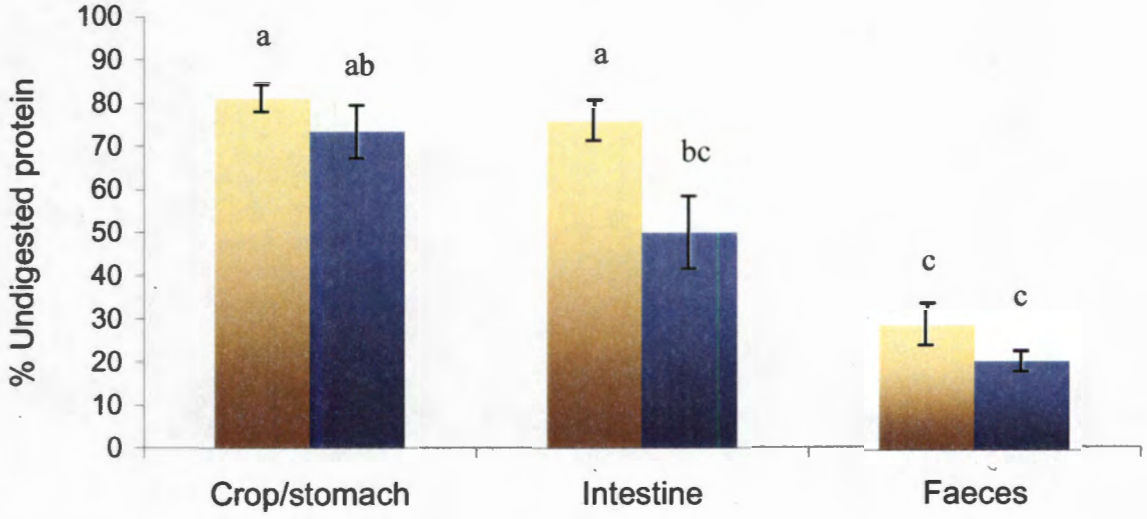


Figure 3.7. Protein digestion and absorption along the digestive tract of *H. midae* fed either the basal diet (■) or the probiotic diet (■). Values for each region represents the percentage protein left in the feed at the time of sampling. Data is presented as the mean (±S.E). Different letters indicate a significant difference ($P<0.05$, two-way ANOVA) between values.

3.5 Discussion

The present study reports for the first time an enhancement in the growth rate of *H. midae* as a result of supplementing their feed with a mixture of the three putative probionts, *Vibrio midae* SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1. Growth rates were improved by up to 8% in the smaller sized abalone and up to 33% in the larger sized abalone. Furthermore, mean growth in weight increased by 7% over a 252 day experimental period for the smaller sized abalone and 8% over a 186 day experimental period for the larger sized abalone. Similar improvements in growth due to dietary-supplementation with probiotics have been reported in fish. Lara-Flores *et al.* (2003) demonstrated an improvement in the growth of the Nile Tilapia, *Oreochromis niloticus*, due to dietary supplementation with either *Saccharomyces cerevisae* or a mixture of *Streptococcus faecium* and *Lactobacillus acidophilus*. Vázquez-Juárez *et al.* (1993) (reviewed in Lara-Flores *et al.*, 2003) reported an improvement in the growth rate of cultured rainbow trout receiving a yeast-supplemented diet and Noh *et al.* (1994) and Bogut *et al.* (1998) (reviewed in Gatesoupe, 1999; Lara-Flores *et al.*, 2003) demonstrated an improvement in growth and feed efficiency of Israeli carp as a result of dietary-supplementation with the bacterium, *S. faecium*. Furthermore, Kennedy *et al.* (1998) (reviewed in Gomez-Gil *et al.*, 2000) reported an improvement in the growth of various marine fish larvae, including snook, red drum and stripped mullet, as a result of a Gram-positive probiotic bacterium and Gatesoupe (1989) (reviewed in Gatesoupe, 1999; Gomez-Gil *et al.*, 2000) demonstrated that disinfected rotifers, pre-inoculated with *Bacillus toyoi*, enhanced the growth rate of turbot larvae. The effective use of probiotics for growth enhancement in shrimp (Garriques and Arevalo, 1995; Gullian *et al.*, 2004; Rengpipat *et al.*, 1998; Rengpipat *et al.*, 2000) and bivalves (Douillet and Langdon, 1994) has also been documented, although not as extensively. These studies support the findings of the present study, where dietary-supplementation with the probionts SY9, SS1 and AY1 enhanced the growth of cultured *H. midae*.

In the present study, each probiotic was added to the feed so as to achieve a final culturable cell concentration of approximately 10^7 cfu g^{-1} of dried feed. This approach is similar to Rengpipat *et al.* (1998), where the probiotic bacterium, *Bacillus* S11, was added to shrimp feed at a

concentration of 10^{10} cfu g^{-1} of dried feed and Douillet and Langdon (1994), where the probiont, designated CA2, was added to an algal suspension, used as feed for oyster larvae, at a concentration of 10^5 cfu ml^{-1}. In both these studies, the growth of shrimp and oyster larvae were enhanced by 76% and 22% respectively. As one of the proposed functions of the probiotics tested in this study is to add to the pool of digestive enzymes within the gut of *H. midae*, the presence of culturable probiotic cells in the feed is essential. Hence, optimal storage conditions for maximum stability of the probiotics in the feed are important. Consequently, we found the optimal storage temperature for maximum stability or viability of each probiotic in the feed to be 4 °C. Similar results were obtained by Robertson *et al.* (2000), where *Carnobacterium* sp., a probiont for Atlantic salmon and rainbow trout, was shown to be most stable in feed stored at a temperature of 4 °C for a period of up to 6 months.

The growth rate of the small (20 mm) abalone recorded in this study is similar to the results reported by Sales and Britz (2001). However, the slow growth rate recorded for the large (67 mm) abalone fed the basal diet was unexpected. A factor that could have attributed to the slow growth rate was the high stocking density of the abalone on the farm during the growth trial with large (67 mm) abalone. It must be noted that the abalone fed the basal and the probiotic-supplemented feed were stocked at the same density as abalone on the rest of the farm for the duration of the experiment and that similar growth rates were recorded for abalone fed the basal feed on the rest of the farm. Indeed, it has been reported that one of the main growth-inhibiting factors in intensive aquaculture systems is overstocking or overpopulation (Lara-Flores *et al.*, 2003). However, the greater percentage improvement in the growth rate of large (67 mm) abalone receiving the probiotic-supplemented feed in this study, suggests that the probiotic effect is more pronounced under adverse conditions, thus exemplifying the benefits of dietary-supplementation with probiotics. Coincidently, Lara-Flores *et al.* (2003) showed that overstocked Tilapia receiving a probiotic-supplemented diet grew faster than animals fed a non-supplemented feed. These authors postulated that high stocking densities adversely effect feed digestibility and that administration of probiotics can mitigate these adverse effects (Lara-Flores *et al.*, 2003). However, factors such as the initial size of an animal and different dietary protein requirements between large and small abalone cannot be discounted as a reason for the difference in growth rate between the small and large abalone recorded in this study.

Previous work by Britz and Hecht (1997) demonstrated that in order to promote maximal growth in *H. midae*, smaller abalone (10 mm shell length) require a higher dietary protein content compared to larger abalone (36 mm shell length). Similarly, Uki and Watanabe (1992) (reviewed in Shipton and Britz, 2001b), showed that protein digestibility (the digestion and absorption of protein) in *Haliotis discus hannai* decreased with increasing animal size, suggesting that larger abalone require a higher dietary protein content. Furthermore, Knauer *et al.* (1996) showed that juvenile *H. midae* (3 – 11 mm shell length) can increase their digestive protease levels in response to a high protein diet, suggesting that the digestive physiology of a young abalone is highly adaptable. Therefore, the substantial improvement in the growth rate of large (67 mm) abalone recorded in this study may be attributed to the increased intestinal proteolytic activity generated by the exogenous protease enzymes secreted by *V. midae* SY9, which would enhance digestion of the complex proteins included in the basal diet, thus increasing the rate at which they can be assimilated by the host animal. This finding supports the theories of Uki and Watanabe (1992) (reviewed in Shipton and Britz, 2001b) and Lara-Flores *et al.* (2003), suggesting that the additional proteolytic enzymes secreted by *V. midae* SY9 help improve protein digestibility in adult abalone.

Supplementation of the basal feed with the three putative probionts resulted in a significant increase in protein digestion and absorption within the intestine. This finding is similar to that obtained by De Schrijver and Ollevier (2000), who investigated protein digestion in juvenile *S. maximus* and showed that dietary-supplementation with a potential probiont, *V. proteolyticus*, resulted in increased digestion and absorption of protein, particularly in the distal portion of the gastrointestinal tract. In their study, it was suggested that *V. proteolyticus* was able to stimulate protein degradation in the digestive tract of juvenile turbot as soon as pH requirements for bacterial growth were fulfilled. They recorded pH values below 5 in the stomach, whereas pH values in the intestine and rectum were 6 and 7, respectively. Harris *et al.* (1998a) showed that the crop of *Haliotis laevigata* Donovan has a pH of 5.28, whereas a pH of 6.64 occurs from the intestine to the rectum. The stomach of *H. midae* has been reported to have a pH of approximately 5.2 (Knauer *et al.*, 1996). In this study, increased digestion and absorption of protein in the intestine was accompanied by an increase in intestinal protease activity in abalone

fed the probiotic diet. However, there was no significant difference in enzyme activity in the crop/stomach of animals fed the probiotic diet and those fed the basal feed. These results may therefore indicate that the probiotic microorganisms are more active in the distal portion of the digestive tract of *H. midae* where the pH could be more suited to the requirements of the probionts. In fact, similar results were recorded for intestinal amylase activity in *H. midae*, suggesting that the increased amylase activity could result in increased digestion and absorption of starch in the intestine of abalone fed the probiotic diet. However, a similar assessment of starch digestion and absorption in the digestive tract of *H. midae* would have to be conducted in order to substantiate this theory.

Even though the amount of protease activity in the crop/stomach was much greater than in the intestine regardless of the treatment, the amount of protein absorption was minimal. This finding is similar to that obtained by De Schrijver and Ollevier (2000). In their study the authors stated that although proteolytic enzymes are produced and secreted in the stomach, this region is not considered a site for absorption of amino acids and peptides. Indeed, Harris *et al.* (1998a) characterized the digestive tract of the greenlip abalone *Haliotis laevigata* Donovan and showed that the crop/stomach possessed mainly columnar secretory cells. These authors conclude that the crop/stomach is important in digestion, but not absorption. This data supports the findings of the present study where the amount of protease activity was high in the crop/stomach region of the digestive tract but the amount of protein absorbed was minimal.

Data obtained from the *in situ* enzyme and protein digestibility assays may also indicate that the probiotic microorganisms colonize the intestinal region of the digestive tract of *H. midae*. Abalone have a relatively long intestine, with numerous folds and grooves, providing ample surfaces for microbial colonization (Harris *et al.*, 1998a). The hindgut of bivalves has been shown to be the most heavily colonized region of the digestive tract, and the accumulation of bacteria in the hindgut of both bivalves and abalone has been suggested to be due to the extended passage time of food through this region (Harris *et al.*, 1998a). Studies conducted on *Haliotis rubra* indicate that faeces are produced up to seven days after cessation of feeding, indicating that there is ample opportunity for microbial colonization. Erasmus *et al.* (1997) showed that both the number and diversity of bacteria in the digestive tract of *H. midae* is greatest in the

intestine compared to other regions of the digestive tract. This, together with the results of this study, suggests that the probiotic microorganisms may be colonizing the intestinal region of the digestive tract of *H. midae*.

Feed represents a large percentage of the production costs of an abalone farm and as such, the economic success of a farm depends in part on good growth rates and efficient feed utilization by abalone. Furthermore, increased growth rates of farmed abalone will lead to a faster turnover rate of abalone on a farm, directly translating to increased profits for the abalone farmer. Thus, the ability of the probiotic microorganisms tested in this study to enhance protein digestibility and improve the growth rate of farmed *H. midae* is of great value to the local abalone mariculture industry. Furthermore, the probiotic microorganisms were shown to be relatively stable in feed over an extended period of time when stored at the correct temperatures. Collectively, this data indicates that probiotic-supplemented feed has great potential as a means for improving the profitability of the abalone mariculture industry.

CHAPTER 4

THE EFFECT OF *VIBRIO MIDAE* SY9, *CRYPTOCOCCUS* SP. SS1 AND *DEBARYOMYCES HANSENII* AY1 ON THE HEALTH AND DISEASE RESISTANCE OF FARMED *HALIOTIS MIDAE*

CONTENTS

4.1 Summary

The present study clearly demonstrates the immunostimulatory affect of the three putative probionts, *Vibrio midae* SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1, included in the diet fed to farmed *Haliotis midae*. To our knowledge, this is the first study to report probiotic immunostimulation in abalone. Furthermore, enhancement of the immune response in *H. midae* resulted in increased survival following challenge with the pathogenic bacterium *Vibrio anguillarum*. Seven days after challenging with *V. anguillarum*, the probiotic-fed animals had a 62% survival rate compared to a 25% survival rate for non-treated animals. Furthermore, the number, phagocytic activity and respiratory burst of circulating haemocytes was significantly higher ($P<0.05$) in probiotic treated animals compared to non-treated animals following challenge with *V. anguillarum*. Histological analysis showed that the digestive glands of animals receiving probiotics were bacteria-free, whereas the digestive glands of 70% of the animals receiving the non-supplemented feed had a high bacterial load.

4.2 Introduction

Infectious diseases are considered one of the main barriers to the successful development and continuation of molluscan and shrimp aquaculture as they limit production in terms of quality, quantity and regularity (Bachère *et al.*, 1995). Internationally, the aquaculture industry has experienced relatively severe disease problems due to the lack of control of microorganisms in rearing systems (Roche, 1999; Olafsen, 2001). Viral, bacterial, rickettsial, chlamydial and protozoan pathogens have been shown to be involved in mass mortalities of molluscs and crustaceans (Mailhe *et al.*, 1995). More recently, abalone farmers have also experienced mass mortalities of both adult abalone and spat larvae as a result of bacterial infections (Cheng *et al.*, 2004b).

Although disease control is an inherent component of any intensive animal production system, controlling disease in the aquatic environment is further complicated by the intimate relationship that exists between pathogens and their host and the frequent use of open production systems (Olafsen, 2001). Broad-spectrum antimicrobials have been extensively used as a means of disease control on many aquaculture facilities and unfortunately remain the method of choice for many farmers (Gram *et al.*, 2001). However, as excessive antimicrobial use can lead to the emergence and spread of bacterial resistance (Verschuere *et al.*, 2000), its use in animal husbandry has been banned in several countries (Gram *et al.*, 2001). Consequently, greater emphasis has been placed on improved husbandry through better nutrition, improved water quality and lower stocking densities, and the use of vaccines and non-specific immunostimulants such as β-1,3 glucans, peptidoclycans and lipopolysaccharides (Robertson *et al.*, 2000).

The use of probiotics for disease prevention in aquaculture has also become increasingly popular and has received a great deal of attention due to an increasing demand for environment-friendly aquaculture. Microorganisms have a critical role in aquaculture systems, because water quality and disease control are directly related and closely affected by microbial activity (Pillay, 1992). Intensive cultivation often alters the composition of the indigenous protective flora of cultured organisms. This is often due to alterations in diet, where animals are fed artificial diets and feed is often added at high concentrations to the water, which results in an excellent medium for the

growth of heterotrophic or opportunistic bacteria. Indeed, many diseases in aquaculture are caused by the growth of opportunistic pathogens (Olafsen, 2001). Furthermore, stress caused by handling or transport of organisms and adverse water quality conditions can render animals more susceptible to infectious diseases. The adverse effects of stress on the immune status of oysters and abalone (Lactose *et al.*, 2002; Malham *et al.*, 2003) and the adverse effects of low dissolved oxygen and high ammonia on the susceptibility of shrimps to infection (Cheng and Cheng, 2002; Cheng *et al.*, 2002) are well documented. More specifically, the adverse effects of changing water temperature, high ammonia and high salinity on the immune response of abalone and their susceptibility to disease has also been well documented (Cheng *et al.*, 2004a; Cheng *et al.*, 2004b; Cheng *et al.*, 2004c; Martello *et al.*, 2000; Martello and Tjeerdema, 2001). These studies have clearly shown that stress can alter the susceptibility of aquacultured animals to disease. However, there is mounting evidence that suggests that both the health and survival of organisms in intensive rearing systems is improved by manipulating the gut microflora with "probiotic" microorganisms and/or prebiotics, which can be added to the diet to promote the growth of beneficial bacteria (Olafsen, 2001).

Several studies have reported the beneficial effects of administering probiotics for improved disease resistance. A study carried out by Gatesoupe (1994) (reviewed in Skjermo and Vadstein, 1999; Verschuere *et al.*, 2000) clearly showed that the addition of lactic acid bacteria to the enrichment medium of rotifers, used as a live feed for the turbot larvae *Scophthalmus maximus*, increased the larval survival rate when challenged with a pathogenic *Vibrio*. *Pseudomonas fluorescens* (AH2) was shown to be strongly inhibitory against *V. anguillarum* in model systems and it was found that this effect could be transferred to an *in vivo* situation where the mortality rate in rainbow trout infected with *V. anguillarum* was significantly reduced by the addition of the probiotic bacterium to the tank water (Gram *et al.*, 1999). Improved disease resistance has also been observed in cod (*Gadus morhua)* fry fed a dry feed containing *Carnobacterium divergens* for three weeks prior to a challenge with *V. anguillarum* (Gildberg *et al.*, 1997), while recently, the use of a *Carnobacterium* sp. as a probiotic for Atlantic salmon and rainbow trout has been reported (Robertson *et al.*, 2000). Rengpipat *et al.* (2000) and Meunpol *et al.* (2003), showed that the survival of the black tiger shrimp (*Penaeus monodon*), fed the probiont *Bacillus* S11, was increased compared with non-treated shrimp. It was also shown that the administration

of this strain enhanced immunity in shrimp, as measured by the percentage phagocytosis and the phagocytic index (PI) of the haemolymph (Rengpipat *et al.*, 2000). Similarly, Gullian *et al.* (2004) isolated a bacterium, *Bacillus* P64, that stimulated the immune system of *P. vannamei* and Suphantharika *et al.* (2003) reported immunostimulation in *P. monodon* through dietary supplementation with yeast β-glucans. Chang *et al.* (2003) then demonstrated that this immunostimulatory effect could be transferred to an *in vivo* situation where the survival of *P. monodon* was improved following challenge with white spot syndrome virus (WSSV). In bivalve molluscs, the addition of bacterium CA2 as a food supplement to xenic cultures of *Crassostrea gigas* larvae was found to consistently enhance the growth of the oyster larvae during different seasons of the year (Douillet and Langdon, 1994). Riquelme *et al.* (1997) isolated a *Vibrio*, which produced substances inhibitory to a *V. anguillarum*-related strain, that protected scallop larvae against subsequent infection with *V. anguillarum*. Furthermore, *Aeromonas media* A199 was shown to prevent the death of *C. gigas* larvae following *in vivo* challenge with *Vibrio tubiashii*. Thus, probiotics have been shown to be effective in a wide range of species for enhanced immunity and survival.

In abalone, as in other invertebrates possessing an innate immune response, the circulating blood cells represent the first line of defense against potential pathogens. These circulating haemocytes have been divided into two distinct cells types, basophilic and eosinophilic cells, which are thought to play distinctive roles in the innate immune response of invertebrates (Pipe *et al.*, 1997). Upon haemocytes encountering foreign particles or pathogenic organisms, a metabolic reaction known as the respiratory burst reaction, characterized by the production of reactive oxygen intermediates (ROIs), is initiated (Martello and Tjeerdema, 2001; Ordás *et al.*, 2000a; Pipe, 1992). The respiratory burst is an enzyme cascade, initiated through the activation of a membrane-bound NAD(P)H-oxidase in the plasma membrane of haemocytes, that partially reduces oxygen to produce a complex array of reactive oxygen species, including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and hypochlorous acid (HOCl) (Ordás *et al.*, 2000a). All of these molecules are highly reactive, microbiocidal metabolites that can function independently or in concert with lysosomal enzymes, which are also released from haemocytes upon appropriate stimulation (Pipe, 1992). Due to the primary role of haemocytes in invertebrate immunology, assays for measuring phagocytosis and the

production of ROIs have been developed in order to provide an overall measure of the immune status of invertebrates (Bland *et al.*, 2001; Esteban *et al.*, 1998; Ordás *et al.*, 2000a; Pipe *et al.*, 1995; Volety *et al.*, 1999). Furthermore, these techniques have been tried and tested in several molluscan species in order to study the fundamental aspects of invertebrate immunology as well as to determine the effects of various environmental and chemical factors on the immune status of invertebrates (Cheng *et al.*, 2004a; Cheng *et al.*, 2004b; Cheng *et al.*, 2004c; Coles *et al.*, 1995; Lactose *et al.*, 2001; Lactose *et al.*, 2002; Malham *et al.*, 2003; Martello *et al.*, 2000; Martello and Tjeerdema, 2001; Pipe *et al.*, 1995; Volety *et al.*, 1999).

The first aim of this study will be to determine the combined effect of *Vibrio midae* SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1 on the immune status of *H. midae* before and after being challenged with the pathogenic bacterium *V. anguillarum*. The immune parameters to be monitored will include the total number of circulating haemocytes and the percentage phagocytosis and internal respiratory burst of the circulating haemocytes. The process of phagocytosis can be subdivided into several stages, including chemotaxis, recognition, internalization and killing of foreign particles (Bayne, 1990). Several methods have been developed for visualization of foreign particles phagocytosed by haemocytes, including the use of stained zymosan particles (Pipe *et al.*, 1995) and more recently, the use of fluorescein 5-isothiocyanate (FITC) labeled bacteria (Lactose *et al.*, 2002; Malham *et al.*, 2003). In this study, FITC-labeled *V. anguillarum*, a known pathogen of abalone, will be used in the phagocytosis assay as described by Malham *et al.* (2003). Furthermore, the release of superoxide anion (O_2^-), within phagocytic haemocytes, will be detected utilizing the nitroblue tetrazolium (NBT) reduction assay described by Malham *et al.* (2003). In this assay, the NBT taken up by phagocytic haemocytes is reduced to a water insoluble blue formazan dye by released O_2^-, which can then be spectrophotometrically quantified after been dissolved in dimethylsulphoxide (DMSO) and potassium hydroxide (Pipe *et al.*, 1995). In addition to the measurement of immune parameters, the percentage survival of abalone fed a diet either supplemented with or lacking probiotics will be recorded after being challenged with *V. anguillarum*.

The overt signs of certain stresses, such as environmental contaminants, adverse water quality conditions and pathogenesis, are more often than not preceded by biochemical, physiological

and/or morphological changes in an organism (Harris *et al.*, 1998b). Consequently, histopathology can be used to determine the overall heath of an organism. Harris *et al.* (1998ab) used this approach to determine the effects of chronic exposure of *Haliotis laevigata* Donovan to a range of pH levels, low dissolved oxygen and high levels of ammonia and nitrate. Gullian *et al.* (2004) used histopathology to confirm the probiotic strains administered to *P. vannamei* had no pathogenic effect on the host. Furthermore, Miles *et al.* (2001) used histopathology to monitor differences in the muscle tissue of the striped snakehead, *Channa striata*, injected with PBS or various immunostimulants, following infection with the oomycete *Aphanomyces invadans*. Therefore, an additional aim of this study will be to conduct a histological analysis on *H. midae*, fed a diet either supplemented with or lacking probiotics, that were included in the growth trial (20 mm abalone) (Chapter 3) and challenge experiment.

4.3 Materials and methods

All media and solutions used in this study are listed in Appendix A

4.3.1 Bacterial strains

The three probiotic strains (SY9, SS1 and AY1) and the probiotic-supplemented feed used for the immunology experiment were prepared as described in sections 2.3.12 and 3.3.2 respectively. The pathogen used in this study was *Vibrio anguillarum* 5677, which was previously isolated from diseased *H. midae* by Dr. Anna Mouton (Animal health consultant for the Abalone Farmers Association of Southern Africa (AFASA)). The virulence of *V. anguillarum* 5677 was tested by Dr. Kim ten Doeschate in another study with *Haliotis midae* (unpublished data). *V. anguillarum* 5677 was streaked from a glycerol stock onto tryptone soya agar (TSA) (Biolab) supplemented with 2% NaCl (w/v) and incubated for 48 hrs at room temperature. A single bacterial colony was transferred from the culture plate into 5 ml of tryptone soya broth (TSB) (Biolab) supplemented with 2% NaCl (w/v) and incubated at room temperature for 24 hrs at 100 rpm on an orbital shaker. Subsequently, 100 µl of culture was transferred to 100 ml of fresh media and incubated for a further 24 hrs. Following incubation, cells were adjusted to an optical density of 3.0 at an absorbancy of 600 nm, before being washed twice and resuspended in an equal volume of PBS (Appendix A.2.4). This absorbancy value had previously been determined to equal approximately 1.0×10^{10} cfu ml^{-1} by spread-plating cells onto TSA.

4.3.2 Challenge experiments

Abalone were maintained in four large polyethylene tanks each containing 98 l of aerated and continuously flowing (330 L/h) natural seawater at 15 – 18 °C. Each tank was stocked with 60 abalone (30 animals/basket), which were acclimatized for three weeks before the start of the experiment and fed the basal diet. At the start of the experiment (T = 0), animals in two of the tanks were fed the probiotic-supplemented diet, while the animals in the remaining two tanks were continually fed the basal diet (Fig 4.1).

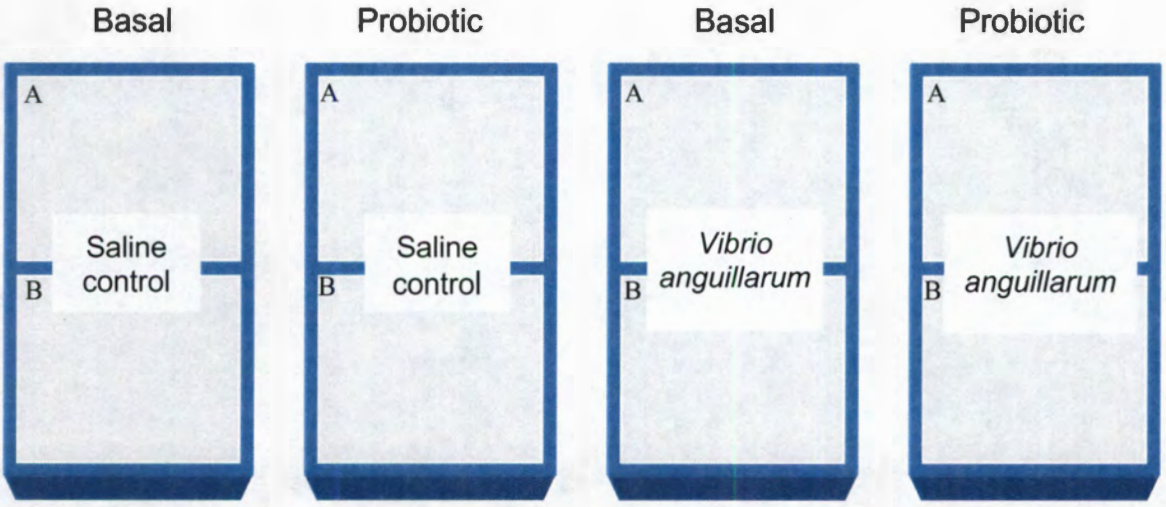


Figure 4.1. Experimental design for the challenge experiment. Each tank contained a total of 60 animals (30 abalone/basket), with duplicate tanks per treatment. When challenged, animals from one basal and one probiotic treatment tank were injected with the pathogen *V. anguillarum*, whereas animals in the remaining two tanks were injected with sterile PBS solution.

The immune parameters (total number of circulating haemocytes, phagocytic rate of the circulating haemocytes and the respiratory burst response of the circulating haemocytes) of the animals were recorded over a two-week period at the following time points: T = 0, 1, 2, 4, 7, and 14 days. Immediately after sampling on day 14, animals in one of the probiotic and control treatment tanks were challenged with an intra-mantle injection of a 0.1 ml bacterial suspension of *V. anguillarum* (10^{10} cfu ml^{-1}) on the right side of the mantle according to Liu *et al.* (2001). Animals in the remaining tanks were injected with sterile PBS and served as controls. Animals were sampled for measurement of their immune parameters at the following time points: T = 15, 16 and 18 days. Mortalities were recorded daily for 1 week post-injection. At the end of the experiment, five dead and five living animals from each tank were sacrificed for histological analysis as described in section 4.3.4.

4.3.3 Measurement of immune status

4.3.3.1 Haemolymph collection

Haemolymph (0.2 – 0.5 ml/abalone) was collected from the pedal sinus using 2ml syringes and 26 G × 1/2 inch needles. At each time point, an equal volume of haemolymph from five animals was pooled and immediately placed on ice to prevent clotting. Different animals were used at each time point so that abalone were not bled more than once. The total number of circulating haemocytes ml^{-1} was determined immediately using a haemocytometer and adjusted to 1.0×10^6 cells ml^{-1} with Modified Hank's Balance Salt Solution (MHBSS) (Appendix A.2.14) to ensure that an equal number of blood cells was used for each assay.

4.3.3.2 Total haemocyte count

Undiluted haemolymph (100µl) was added to 200µl of Alsevers buffer (Appendix A.2.13). The total number of circulating haemocytes of the fixed blood cells was counted with a haemocytometer and a light microscope (100× magnification). Each sample was read in triplicate.

4.3.3.3 Phagocytosis assay

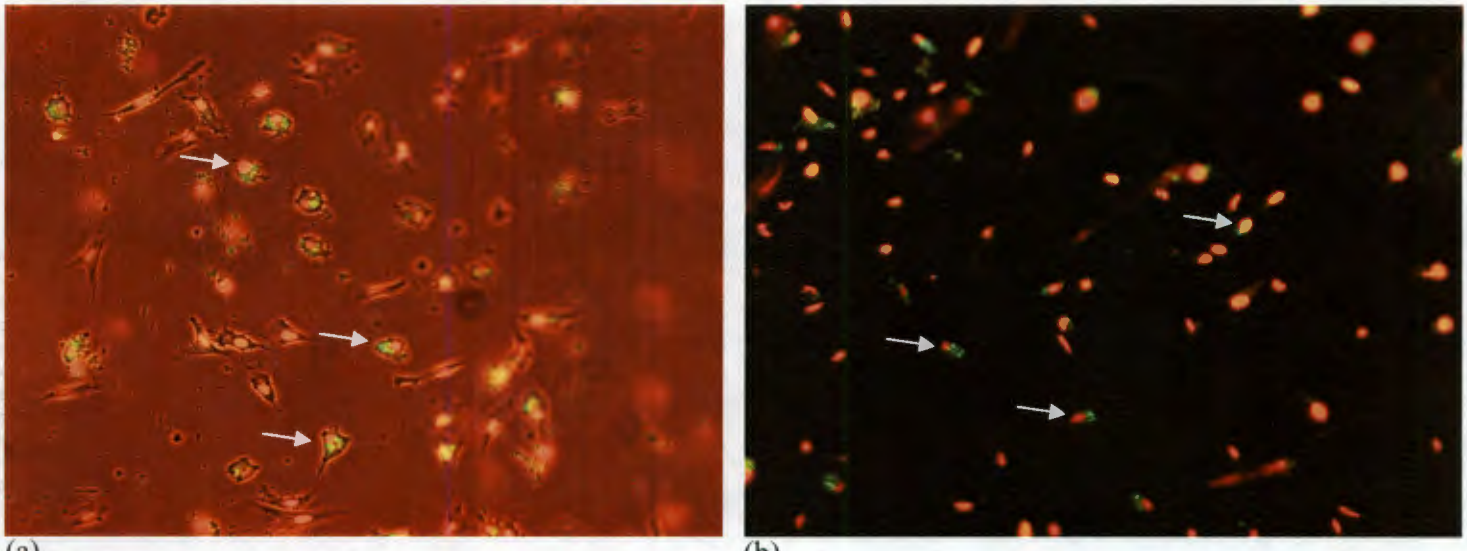
Vibrio anguillarum was grown at 22°C for 24 h in tryptone soya broth (TSB, Biolab) supplemented with 2.5% (w/v) NaCl. The bacteria were killed by the addition of 10% formalin and pelleted by centrifugation at 12 000 x g for 10 min. Cells were then washed twice in sterile PBS before resuspending in 0.1 M $NaHCO_3$ pH 9.0 (Appendix A.2.14) containing 0.1 mg ml^{-1} fluorescein 5-isothiocyanate, isomer 1 (FITC, Sigma) as described by Malham *et al.* (2003). Cells were allowed to label for 1 h at 25 °C in the absence of light. After centrifugation and resuspension in PBS the bacteria were counted with a haemocytometer and a light microscope and diluted to 1.0×10^8 bacteria ml^{-1}. Aliquots of the bacteria were then stored at –20 °C until needed.

One hundred microlitres of haemolymph containing haemocytes at a concentration of 10^6 haemocytes ml^{-1} in MHBSS was placed inside a ring (1.5 cm^2) of silicon prepared on a glass slide that had been soaked in 50% acetic acid (Appendix A.2.14) for 24 h, rinsed thoroughly in distilled water and dried at room temperature between paper towels. The slides were placed in a moist, dark incubation chamber for 20 min to allow the haemocytes to adhere to the glass. One hundred microlitres of FITC-labeled *V. anguillarum* was added to the cells inside the silicon ring. The glass slides were then returned to the moist incubation chamber and incubated in the dark for a further 30 min. Slides were then gently rinsed three times with MHBSS before adding 100 µl of an ethidium bromide (Sigma) solution (50 µg ml^{-1} in PBS, Appendix A.2.14) to the slide to stain the unphagocytosed bacteria. The ethidium bromide solution was removed after 1 min by rinsing the slides with MHBSS and the remaining liquid removed with a pipette. A glass coverslip was placed on top of the silicon ring and gently squeezed down. The slides were stored in the dark prior to counting. Four hundred cells were counted on each slide (prepared in duplicate) using a 488 nm emission filter on an Olympus Fluorescent microscope. Phagocytic haemocytes were easily distinguishable from non-phagocytic cells as they contained green fluorescent bacteria (Fig. 4.2). The percentage phagocytosis was calculated for each slide and the mean and standard error determined for each sample.

4.3.3.4 Nitroblue tetrazolium reduction assay

Debaryomyces sp. AY1 was grown at 22 °C for 24 hrs in YPD broth (Appendix A.1.3). Following incubation, the cells were adjusted to an optical density of 1.0 at an absorbancy of 600 nm and pelleted by centrifugation at 12 000 x g for 10 min. Cells were then washed twice in sterile PBS before being resuspended in an equal volume of this solution. The resuspended cells were disrupted by five cycles of sonication at 95 Watts for 30 sec at 4 °C in a Virsonic Digital 475 Cell Disruptor, with 20 sec cooling intervals between each sonication step. The cell wall debris was then pelleted by centrifugation at 12 000 x g for 10 min before being resuspended in an equal volume of sterile PBS. Aliquots of the yeast cell wall debris were stored at -20 °C until needed.

The intracellular respiratory burst response of circulating haemocytes was measured using the nitroblue tetrazolium (NBT) reduction assay as described by Malham *et al.* (2003) with minor modifications. Briefly, two hundred microlitres of haemolymph, containing haemocytes at a concentration of 10^6 haemocytes ml^{-1} in MHBSS, and two hundred microlitres of the prepared yeast cell wall debris (stimulant) was added to 1.5 ml eppendorf tubes in triplicate for each sampling point. Unlike Malham *et al.* (2003) and like Pipe (1992) and Suphantharika *et al.* (2003), the yeast cell wall debris was added as a stimulant in order to activate the release of reactive oxygen metabolites from haemocytes. Two hundred microlitres of NBT (2 mg ml^{-1} in Tris/HCl buffer containing 2 % NaCl, pH 7.6, Appendix A.2.15) was added to each tube. Tubes containing the stimulant, NBT and an equal volume of MHBSS buffer (no cells) served as blanks. Additional negative controls included tubes containing either diluted cells, the stimulant, NBT solution and superoxide dismutase (SOD, Sigma, 300 U/ml) in MHBSS (serves to verify the specificity of the reaction (Pipe *et al.*, 1995)) or diluted cells and the stimulant with SOD only (serves as a control for the SOD).



(a) (b)

Figure 4.2. Phagocytic and non-phagocytic haemocytes viewed using a 488 nm emission filter on an Olympus Fluorescent microscope (400 X magnification) with (a) and without (b) phase contrast. The white arrows indicate haemocytes that have phagocytosed FITC labeled *V. anguillarum* cells.

The tubes were incubated in the dark for one hour before being centrifuged at 120 x g for 10 min. The supernatant was carefully removed by inverting the tubes and the cells resuspended in MHBSS. The cells were washed a further two times in this manner before the addition of 200 µl methanol (100%) to each tube. The cells were allowed to fixate for 10 min at room temperature before being centrifuged for 10 min at 300 x g. The supernatant was carefully removed and the cells air dried, before rinsing them three times with 200 µl methanol (50%) (Appendix A.2.15). Two hundred and forty microlitres of 2 M KOH (Appendix A.2.15) and 280 µl dimethylsulphoxide (DMSO, Sigma) was added to each tube. The tubes were vortexed and the supernatant transferred to 2 ml cuvettes. The optical density value at 620 nm was measured on a Beckmann DU70 spectrophotometer and the results expressed as OD values/10^6 cells/ml.

4.3.4 Histological analysis

Histological analysis was conducted on abalone employed in the challenge experiments as well as animals included in the growth trials with 20 mm abalone (Chapter 3). The latter animals were sampled (10 animals per sample) over the last four months of the growth trial and included a sample taken two months after the trial had been completed. Once in the laboratory, the shell was carefully removed with a scalpel by severing the adductor muscle as close to the shell as possible. Care was taken not to rupture the digestive tract. Abalone were placed foot down on a dissecting board and five incisions were carefully made at specific points along the digestive tract (Fig. 4.3).

Tissue from regions a, b, c and d (Fig. 4.3) was carefully removed with a scalpel and tweezers by cutting close to the adductor muscle at right angles to the initial incision and gently teasing away the tissue. The excised tissue was placed in an embedding cassette in the same orientation. A portion of the foot muscle from region e (Fig. 4.3) was also sampled and placed in the embedding cassette with the rest of the tissue. The cassettes were incubated in Davidson's solution (Appendix A.2.12) for 48 hours before being transferred to 70% Ethanol (Appendix A.2.4). Following fixation, the tissue was dehydrated through a graded ethanol series to xylene in a Tissue Trek II tissue processor. The dehydrated tissue samples were embedded in paraffin resin and sectioned on a microtome at 3 µm. Sections were carefully mounted onto glass slides,

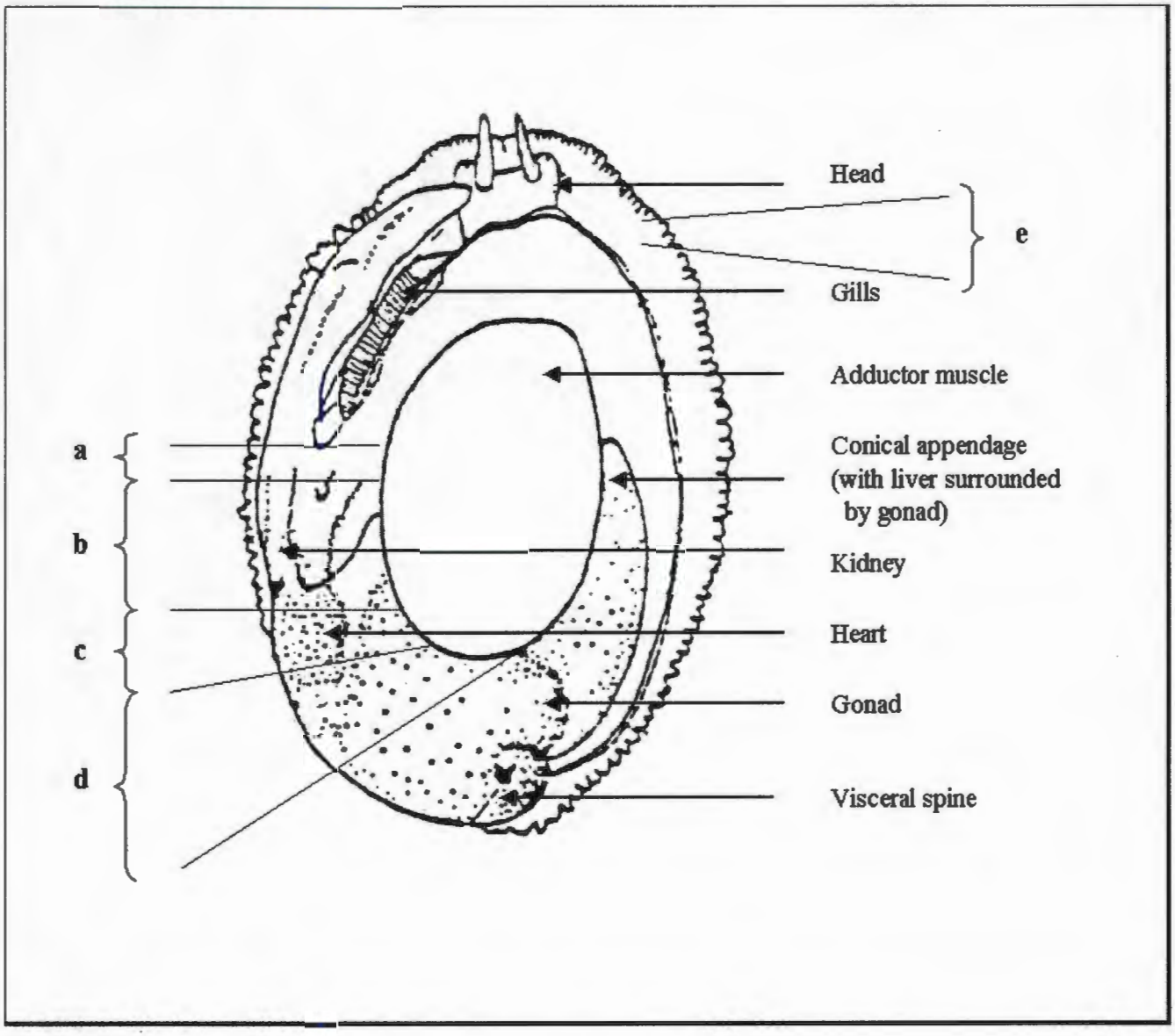


Figure 4.3. Schematic diagram (anterior view) of the abalone *Haliotis midae* showing the major organs and the locations of incisions made for the removal of the various sections for histological analysis. Sections removed included (a) gills and intestine, (b) kidney and heart, (c) heart and stomach, (d) stomach and crop, (e) foot. Figure adapted from Ino (1952).

deparaffinised and stained using standard Harris' Hematoxylin and Eosin (H & E) stain (Hayat, 1993). The sections were examined using an Olympus BX 40 light microscope equipped with a Color View (Soft Imaging System) digital camera.

4.3.5 Statistical analysis

All data is presented as means and standard errors. For comparisons of two means, an unpaired student t-test was used. For multiple comparisons, data was analyzed by two-way ANOVA. When the effects of the ANOVA were significant, the Tukey test was used to test for significant differences between sample means. Prior to statistical analysis, the percentage data underwent natural log transformations. Significant differences were established at $P<0.05$.

4.4 Results

4.4.1 Number of circulating haemocytes

Prior to infection with *Vibrio anguillarum,* there was no significant difference in the total haemocyte count between animals fed either the basal or the probiotic diet (Fig. 4.4). Following infection, the total haemocyte count in abalone fed the basal diet was significantly reduced ($P<0.05$) on days 15 and 16, before recovering on day 18. However, the total haemocyte count in animals fed the probiotic diet was not significantly affected by *V. anguillarum* infection, remaining significantly higher ($P<0.05$) than the haemocyte count in animals fed the basal diet.

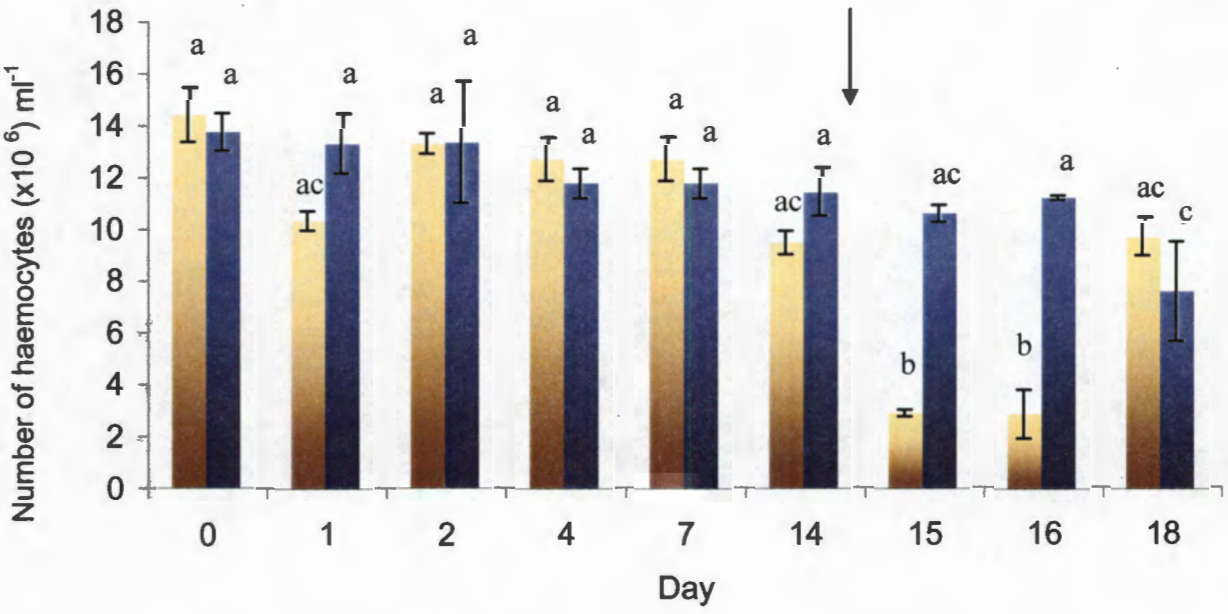


Figure 4.4. The total number of circulating haemocytes ($x10^6$) / ml in animals fed either a basal diet (□) or a probiotic diet (■). The vertical arrow indicates the time of infection with *V. anguillarum*. Data is presented as the mean (±S.E). Different letters indicate a significant difference ($P<0.05$) between values.

4.4.2 Phagocytic activity of circulating haemocytes

The percentage of phagocytic haemocytes was consistently higher in animals fed the probiotic diet compared to animals fed the basal diet, except on days 0, 1 and 18 (Fig. 4.5). Statistically significant differences were recorded on days 2 through to 14, with the exception of day 7, prior to challenge with the pathogen, *V. anguillarum*. Following infection, the phagocytic capability of the circulating haemocytes from animals fed the basal diet was significantly reduced ($P<0.05$) from an average of approximately 26% prior to the challenge, to approximately 8% and 11% on days 15 and 16 respectively, before increasing to 38% on day 18. The phagocytic capability of haemocytes from animals fed the probiotic-supplemented diet did not change significantly ($P<0.05$) immediately following infection, but remained significantly greater ($P<0.05$) than the phagocytic activity of haemocytes from animals fed the basal diet (days 15 and 16).

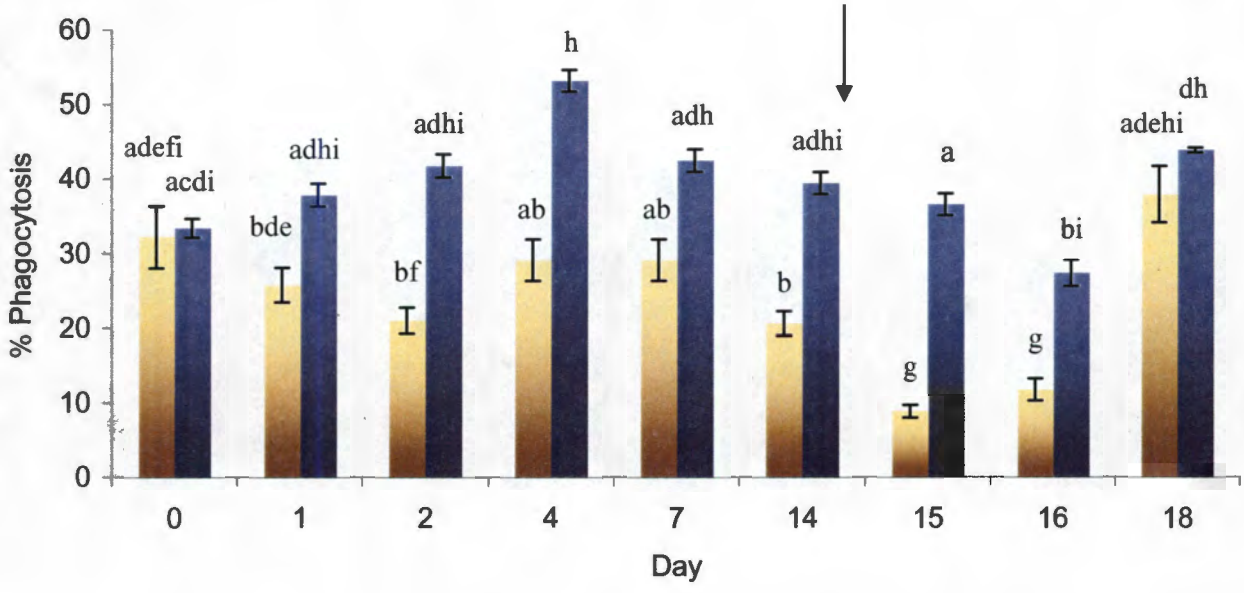


Figure 4.5. The percentage phagocytic circulating haemocytes in animals fed either a basal diet (□) or a probiotic diet (■). The vertical arrow indicates the time of infection with *V. anguillarum*. Data is presented as the mean (±S.E). Different letters indicate a significant difference ($P<0.05$) between values.

4.4.3 Intracellular reactive oxygen production of circulating haemocytes

Prior to infection with *V. anguillarum,* there was no significant difference in intracellular reactive oxygen production by haemocytes from animals fed the basal diet or the probiotic-supplemented diet, except on day 2 (Fig. 4.6). On days 16 and 18, following bacterial infection, reactive oxygen production in haemocytes from abalone fed the basal diet was significantly reduced ($P<0.05$). Conversely, the levels of reactive oxygen production in haemocytes from animals fed the probiotic-supplemented diet did not change significantly ($P<0.05$) following infection, but remained significantly greater ($P<0.05$) than the level of reactive oxygen production in haemocytes from animals fed the basal diet (days 16 and 18).

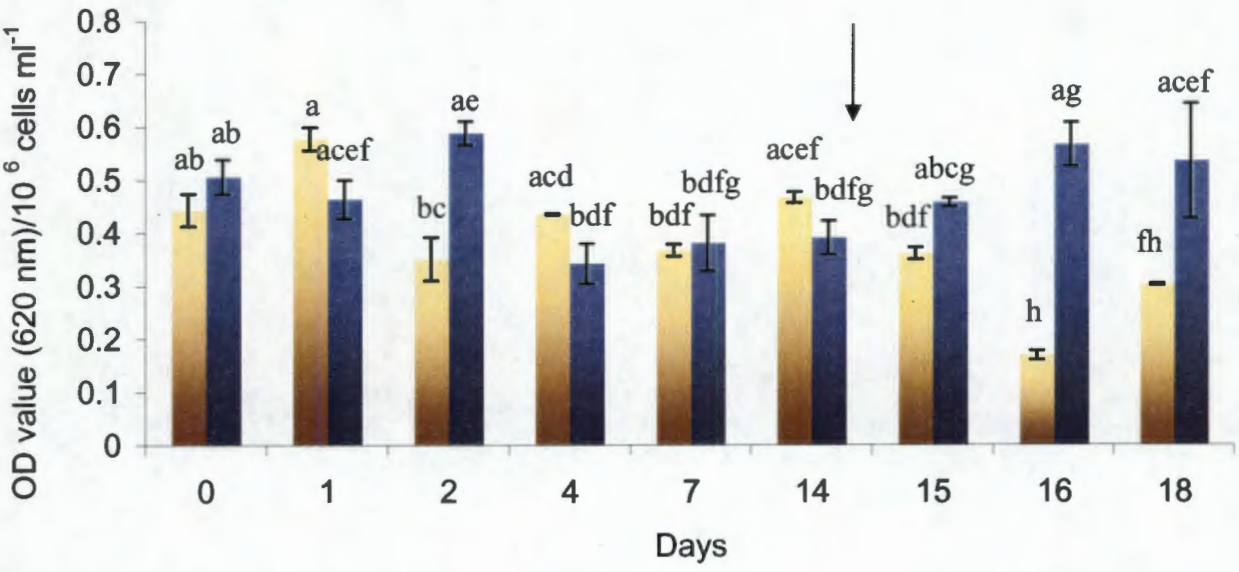


Figure 4.6. The intracellular superoxide anion production of circulating haemocytes in animals fed either a basal diet (□) or a probiotic diet (■). The vertical arrow indicates the time of infection with *V. anguillarum*. Data is presented as the mean (±S.E). Different letters indicate a significant difference ($P<0.05$) between values.

4.4.4 Survival of abalone following challenge

H. midae fed the probiotic-supplemented diet had a higher survival rate compared to animals not fed probiotics, with survival rates of 62% and 29% respectively, seven days after infection with *V. anguillarum* (Fig. 4.7). Abscesses in the mantle of some of the *V. anguillarum*-injected animals (fed either the basal or the probiotic diet) were observed within 2 days post-infection (data not shown). Most mortalities were recorded within four days post-infection.

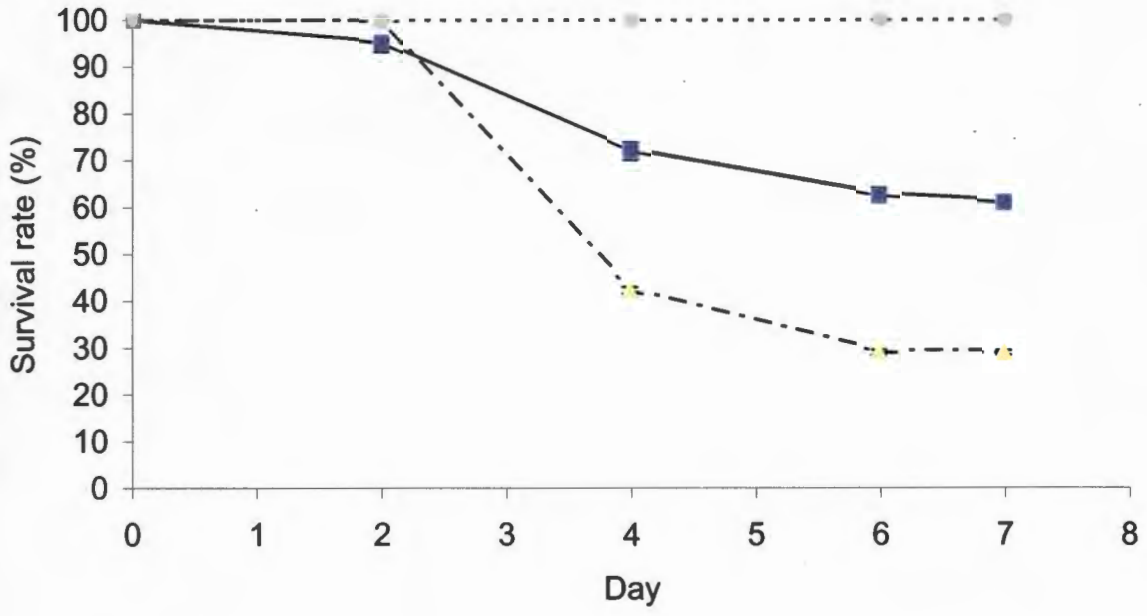


Figure 4.7. Survival (%) of *Haliotis midae* fed either a basal diet (▲) or a probiotic-supplemented diet (■) following challenge with a 100 µl suspension of *V. anguillarum* (1 x 10^{10} cfu ml^{-1}) by intra-mantle injection. Control animals (●) were injected with a 100 µl suspension of PBS. All animals were fed the experimental diets for a period of two weeks prior to infection.

4.4.5 Histology

Histological examination did not identify any differences between the animals fed the basal diet and those fed the probiotic diet following infection with *V. anguillarum*. Most of the animals that died exhibited bacterial invasion of the foot and epipodium, which was sometimes accompanied by bacterial growth in the digestive gland, as well as an enlarged lumen of the right kidney and accumulation of haemocytes in this tissue. Dilation of the tubules of the right kidney was observed in many of the survivors. In one probiotic-fed animal that survived bacterial infection, there was a clear cellular immune response in the foot muscle and right kidney (data not shown).

Histological examination of animals employed in the growth trial conducted on the 20 mm animals (Chapter 3) revealed that the digestive glands of 70% of the abalone that had been fed the basal diet had a high bacterial load. However, the digestive glands of animals fed the probiotic diet were bacteria-free (Fig. 4.8).

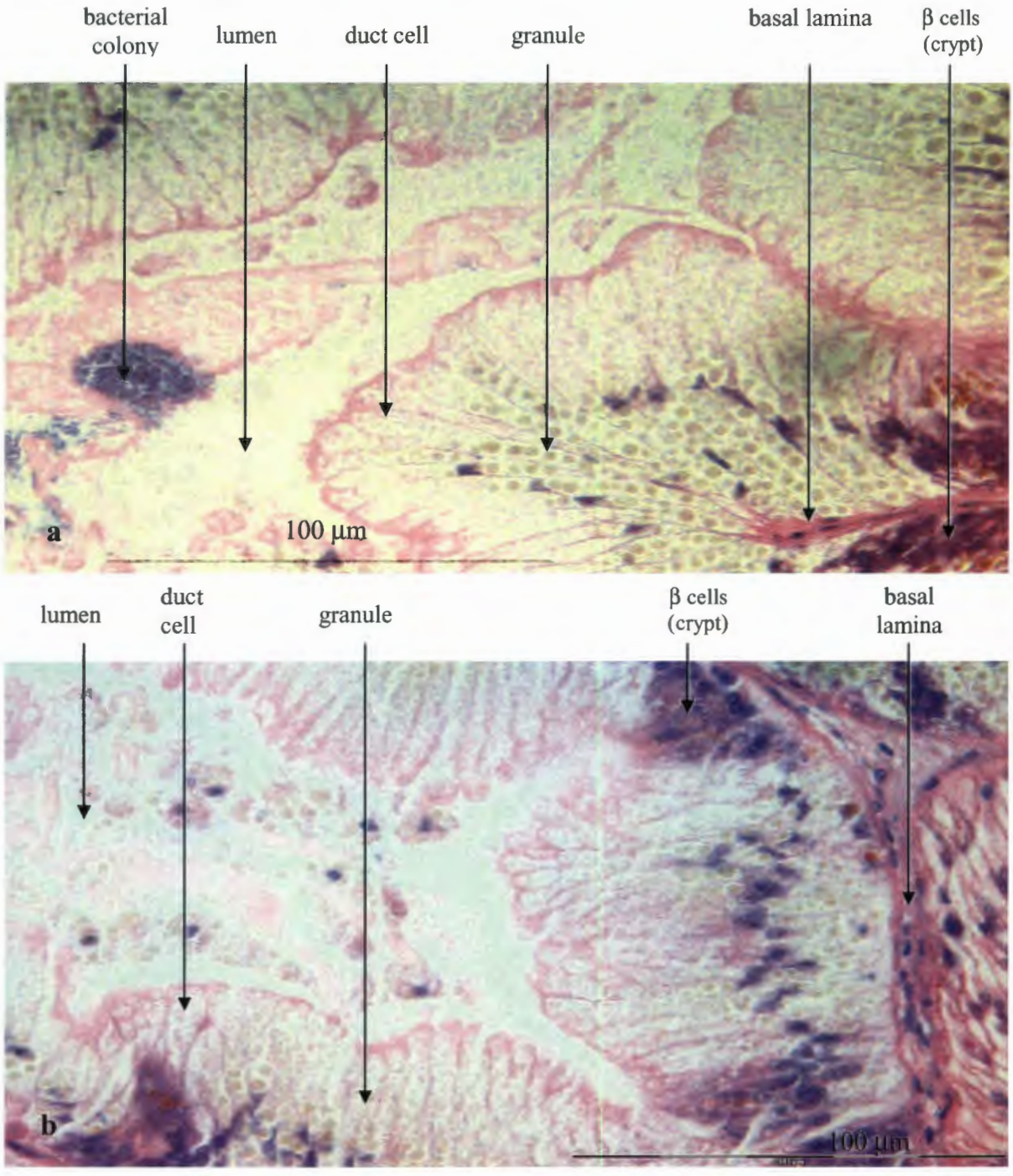


Figure 4.8. Cross-section of digestive gland from *H. midae* fed either the basal diet (a) or the probiotic diet (b). Sections show the extremely tall duct cells with large basal nuclei, supranuclear granules and vacuoles. Dark crypt cells at the base of the duct cells are rich in supranuclear Golgi apparatus and have a high granular iron content. A large bacterial colony is visible in the lumen of the digestive gland from an animal fed the basal diet.

4.5 Discussion

Aquacultured abalone are constantly subjected to a wide range of physical and biological stresses that can have adverse effects on animal growth, health and survival. Lacking an adaptive immune response and due to the nature of abalone farming practices, disease prevention through vaccination and the addition of chemotherapeutics to the water, is not possible or desirable, forcing aquaculture farmers to seek alternative methods for disease control. Contrary to the successful development and use of probiotics in the shrimp farming industry, no studies have been conducted on the development of probiotics for abalone. However, the present study clearly demonstrates the immunostimulatory affect of the three putative probionts, *Vibrio midae* SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1, included in the diet fed to farmed *Haliotis midae*. To our knowledge, this is the first study to report probiotic immunostimulation in abalone.

In the present study, there was no difference in the total haemocyte count (THC) and internal respiratory burst response in abalone fed a diet with or without probiotics prior to infection with *V. anguillarum*. Gullian *et al.* (2004) reported similar results in *P. vannamei* fed a diet supplemented with either *V. alginolyticus* or *Bacillus* P64. In their study, shrimp were constantly fed the probiotic-supplemented diets for a period of 10 days before measuring immune parameters. Although no difference in THC was recorded, these authors did show a significant increase in the hyaline cell population, which is the primary haemocyte involved in phagocytosis (Johansson *et al.*, 2000). It is therefore possible that a differential haemocyte count in this study would show an increase in the proportion of granular haemocytes, the primary phagocytic cell in molluscs (Pipe *et al.*, 1997). Indeed, the increased phagocytic rate in probiotic-fed animals lends support to this theory.

Gullian *et al.* (2004) hypothesized that the lack of superoxide generation in shrimp fed probiotics could be due to the expression of antioxidants such as superoxide dismutase and catalase that neutralize reactive oxygen metabolites in order to prevent self-damage in the host. Similarly, Sung *et al.* (1996) (reviewed in Gullian *et al.*, 2004) showed that the internal respiratory burst response in *P. monodon* immersed in the antigen *V. vulnificus* increased to significant levels six

hours following immersion and then diminished to values lower than the non-immunostimulated control. These results support the findings of the present study, where the only difference in superoxide generation between animals fed a diet either supplemented with or lacking probiotics was recorded after two days. This increase coincided with an increase in the phagocytic rate of circulating haemocytes. However, unlike the respiratory burst rate, the phagocytic rate remained higher in animals fed probiotics up until the end of the experiment. Thus, the lack of increased superoxide generation from days 2 to 14 in this study could be due to the expression of antioxidants, preventing self-damage in *H. midae.*

In contrast to the total haemocyte count and respiratory burst, the phagocytic rate of the circulating haemocytes from *H. midae* was consistently higher in animals fed the probiotic-supplemented diet compared to animals fed the basal diet. Similar results were obtained by Rengpipat *et al.* (2000) after feeding *P. monodon* a diet supplemented with *Bacillus* S11 for a total of 90 days. In an earlier study, Itami *et al.* (1989) demonstrated enhanced phagocytic activity of granular haemocytes isolated from the Kuruma shrimp, *Penaeus japonicus*, following oral administration of the immunostimulant, peptidoglycan. Furthermore, Nikoskelainen *et al.* (2003) demonstrated a significant increase in the phagocytic rate of the rainbow trout, *O. mykiss*, fed a diet supplemented with *L. rhamnosus*. Rengpipat *et al.* (2000) postulated that *Bacillus* S11 cell wall components elicit an immune function in shrimps by acting on granulocytes, thus enhancing the phagocytic activity of haemocytes from probiotic-treated shrimp. It has also been stated that certain virulent pathogens, such as *Aeromonas salmonicida*, can resist both cellular and humoral defense mechanisms in fish, but activated macrophages are able to kill these pathogens. Nikoskelainen *et al.* (2003) therefore postulated that the reason for the increased survival of *O. mykiss* in their study was due to the ability of *L. rhamnosus* to stimulate the immune system. It is therefore possible that the probiotic strains administered to *H. midae* in the present study are acting in a similar manner. In fact, dietary supplementation with the probiotics did enhance the immune response in *H. midae*, resulting in increased survival following challenge with the pathogenic bacterium, *V. anguillarum*. It is therefore possible that the probiotic strains utilized in this study are acting as immunogens for abalone immune defense, priming the immune system of *H. midae* so that it may respond more rapidly upon infection.

It is reasonable to assume that opsonizing factors could be responsible for the enhanced phagocytic rate of haemocytes from abalone fed the probiotic-supplemented diet. Invertebrate lectins take part in humoral defense reactions against bacteria by reacting with non-self ligands and boosting the phagocytic response (Olafsen, 2001). By acting as opsonins, invertebrate lectins which have been released into circulation can improve the survival of an animal because these molecules are then available immediately to help recognize and remove opportunistic or pathogenic organisms (Smith *et al.*, 2003). Under non-immunostimulated conditions, immuno-reactive factors, such as opsonins and anti-bacterial proteins, are stored in granules within haemocytes. However, following appropriate stimulation, these factors are released through a process of regulated exocytosis (Smith *et al.*, 2003; Supungul *et al.*, 2002). In bivalves, natural lectins capable of agglutinating a number of pathogenic *Vibrio* species have been identified (Olafsen, 2001). Furthermore, Hubert *et al.* (1996) isolated several antibacterial proteins from the haemolymph of *Mytilus galloprovincialis* previously immunized by injecting them with bacteria and Charlet *et al.* (1996) isolated both antibacterial and antifungal peptides from immune-challenged *Mytilus edulis*. Similarly, lectins capable of agglutinating vibrios have been identified in crustaceans (reviewed in Marques and Barracco, 2000). Opsonic proteins such as peroxinectin and paenidin have also been identified in crustaceans that are capable of regulating the immune response and stimulating phagocytosis (Johansson *et al.*, 2000; Smith *et al.*, 2003). Marques and Barracco (2000) demonstrated that the phagocytic rate of granular haemocytes from *P. paulensis* was increased approximately three-fold when *V. harveyi* and *B. cereus* were pre-incubated with shrimp serum, suggesting the presence of opsonizing factors in the haemolymph that are capable of enhancing phagocytosis. These findings lend support to our theory that dietary probiotic-supplementation could stimulate the release of opsonizing factors, which could then be responsible for the increased phagocytic rate of haemocytes from probiotic-fed abalone.

The present results clearly demonstrate that following *V. anguillarum* infection, the immune status of *H. midae* not receiving probiotics is severely down-regulated. Chang *et al.* (2003) reported a similar reduction in THC and respiratory burst after injecting *P. monodon* with WSSV. Similarly, Cheng *et al.* (2002) and Holman *et al.* (2004) reported down-regulation of immune parameters in *M. rosenbergii* and *Callinectes sapidus* Rathbun exposed to conditions of hypoxia and hypercapnic hypoxia respectively. The adverse effects of physical stress on the

immune parameters of abalone has also been well documented (Cheng *et al.*, 2004a; Cheng *et al.*, 2004b; Cheng *et al.*, 2004c). These studies have all clearly shown that physical stress causes depression of the immune system, increasing the susceptibility of the animals to disease. Furthermore, Lacoste *et al.* (2002) found that the number and phagocytic activity of circulating haemocytes decreased significantly in *Crassostrea gigas* subjected to mechanical stress. A similar response was reported to occur in the abalone *Haliotis tuburculata* (Malham *et al.*, 2003). Both these studies indicate that there is a strong link between stress and the status of the immune system and suggest that immunostimulation may improve the ability of the animals to respond to infection far more rapidly than non-immunostimulated animals. Indeed, the effect of immunostimulation on the survival of *H. midae* challenged with a pathogenic bacterium is clearly demonstrated in this study.

One of the acute effects of infection in most invertebrates is a rapid reduction in THC per milliliter of haemolymph due to haemocyte degranulation, lysis and the formation of clumps (Smith *et al.*, 2003). Whilst these responses help to sequester the invading organism, it means that for a brief period the size of the host defense armamentarium is reduced and hence its ability to deal with subsequent infections is diminished. The rapid decline in the THC of *H. midae* not fed probiotics seems to be consistent with the role of haemocytes in immune defense. However, abalone fed the probiotic-supplemented diet did not display this trend. In fact, none of the immune parameters changed significantly following infection. These results are similar to Chang *et al.* (2003) who demonstrated a less dramatic decline in the immune parameters of *P. monodon*, fed a diet supplemented with β-glucan and infected with WSSV, and Rengpipat *et al.* (2000) who recorded enhanced phagocytic activity in *P. monodon* following pathogenic infection. These results, together with the data from the present study, suggest that immunostimulated animals have the capacity to respond far more aggressively to infection. The fact that the THC in probiotic-fed abalone remains the same following infection suggests that the haemocytes in circulation have an enhanced capacity to effectively eliminate opportunistic pathogens, resulting in the enhanced survival rate of the abalone fed the probiotic-supplemented diet. Indeed, the enhanced phagocytic rate of haemocytes from probiotic-fed animals, the fact that the phagocytic and respiratory burst rate remained the same following infection, and the enhanced survival of abalone receiving probiotics lends support to this theory.

Abalone fed high protein diets often suffer from a condition called "bloat" during the summer months when raceway water temperatures increase. This condition can also occur when animals are being transported (Abalone farmers; *personal communication*). It is speculated that this condition is due to the proliferation of bacteria in the gut, which leads to contamination of the digestive gland and possible mortality of the animals. The observation that animals fed the probiotic diet during the growth trials conducted in this study lacked bacterial contamination of the hepatopancreas may indicate that the probiotic bacteria prevented proliferation of heterotrophic or opportunistic bacteria within the gut of these animals and consequently, that these animals have an increased stress tolerance. However, further studies would need to be conducted to substantiate this theory.

No histological differences were observed between animals fed either the probiotic or basal diet following exposure to the bacterial pathogen *V. anguillarum*. There was also no evidence of a cellular response, such as bacterial septicemia or the accumulation of haemocytes in infected areas, in animals that survived *V. anguillarum* infection. However, this does not necessarily mean that a cellular response did not occur in these animals. Since cellular responses are normally transient events (Maramorosch and Shope, 1975), they may have occurred prior to the histological examination of the surviving animals, which was performed a week post-infection. What was interesting from this analysis was that many of the surviving animals, fed either the basal or the probiotic diet, exhibited dilation of the right kidney tubules. This is a condition that is usually associated with environmental stress caused by poor water quality (Harris *et al.*, 1998b). However, this histology may also indicate a physiological response to infection as suggested by this study.

The present study clearly demonstrates the immunostimulatory affect of the three putative probionts included in a diet fed to farmed *H. midae*. Furthermore, we have shown that immunostimulation leads to an enhanced capacity of haemocytes from *H. midae* to respond to and effectively eliminate pathogens, leading to increased survival following infection. Abalone receiving the probiotic-supplemented feed also lacked bacterial contamination of the hepatopancreas, suggesting that probiotic-fed animals have an increased stress tolerance compared to abalone not fed probiotics. Collectively, this data suggests that the use of probiotics

in abalone farming represents a viable and highly effective alternative to the use of antibiotics and other chemotherapeutics for improved health and enhanced survival in farmed *H. midae*.

CHAPTER 5

THE COLONIZATION POTENTIAL OF *VIBRIO MIDAE* SY9, *CRYPTOCOCCUS* SP. SS1 AND *DEBARYOMYCES HANSENII* AY1 IN THE DIGESTIVE TRACT OF *HALIOTIS MIDAE*

CONTENTS

5.1 Summary

Viable cell counts and/or *in situ* hybridization were used to evaluate the colonization potential of *Vibrio midae* SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1 in the crop/stomach and intestine of *Haliotis midae*. The mini-Tn10-*gfp-kan* transposon, delivered from pLOFKm*gfp*, transposed at a high frequency (2.4×10^5 cfu ml^{-1}) in *Vibrio midae* SY9.1, resulting in single random chromosomal insertions. Green fluorescent protein (GFP) fluorescence could not be detected in wild-type *V. midae* SY9, whereas low levels of expression could be observed in *V. midae* SY9.1 transconjugants. After feeding *H. midae* with probiotic-supplemented feed for three weeks, the number of culturable probiotic cells reisolated from the *H. midae* digestive tract ranged from 10^6 to 10^7 cfu g^{-1} of crop/stomach or intestinal tissue. 16S and 18S rRNA gene sequencing confirmed the identity of the presumptive probiotic strains. A direct comparison of viable cell-counts and *in situ* hybridization using a *gfp-kan* DNA probe showed that plate counts of *V. midae* SY9.8 were lower in the crop/stomach and intestine of *H. midae* compared to estimates obtained from *in situ* hybridization. Two days after cessation of feeding with the probiotic-supplemented feed there was a significant decrease ($P<0.05$) in the cell numbers of each probiont. This correlated with a significant decrease ($P<0.05$) in intestinal protease and amylase activity in abalone previously fed probiotics. A Pearson product movement correlation analysis revealed a significant positive correlation between *Cryptococcus* sp. SS1 and amylase activity ($r^2 = 0.681$) and *V. midae* SY9.8 and protease activity ($r^2 = 0.711$) in the intestine of *H. midae*. Data from this study suggests that farmed abalone should be fed the probiotic-supplemented feed at least every second day for maximum effect.

5.2 Introduction

The rapidly expanding worldwide aquaculture industry has experienced relatively severe setbacks due to disease outbreaks. Consequently, the use of probiotics has become increasingly popular as a means of improving disease resistance and growth of cultured organisms (Gomez-Gil *et al.*, 2000). Several different mechanisms of action of probiotic microorganisms have been proposed, including competitive exclusion of pathogenic bacteria; production of substances that prevent the growth of pathogenic bacteria; provision of essential nutrients and/or digestive enzymes to enhance the growth of the host organism; and the ability to stimulate the immune system of the host organism (Vershuere *et al.*, 2000). However, all of these proposed modes of action require that the potential probiont is able to reach the location where the probiotic effect is required and is able to successfully colonize this region (Verschuere *et al.*, 2000). Alternatively, the probiont must be introduced at a high dose, continuously or semi-continuously, in order to exert the desired effect (Gatesoupe, 1999). It is therefore essential to evaluate the persistence of a putative probiont in the digestive tract of its proposed host in order to evaluate its probiotic potential and/or devise an effective feeding regime for probiotic administration.

Several direct and indirect methods exist for the detection and quantification of microorganisms within microbial communities (Li *et al.*, 1996; Li *et al.*, 1997). These include the viable-cell count, typically reported as the number of colony forming units (cfu) of the organism of interest per unit area, volume or weight of sample; *in situ* hybridization using DNA-specific probes; competitive enzyme-linked immunosorbent assays (ELISAs) using monoclonal or polyclonal antibodies; and the use of competitive and real-time PCR (Hermansson and Lindgren, 2001; Li *et al.*, 1996). Each of these methods have their advantages and disadvantages. Two of the major drawbacks of the viable-cell count method are that not all viable cells are culturable and the efficiency with which cells are removed from the environment may be low or variable (Li *et al*, 1996). Due to the remarkable improvements that have been made in the field of molecular biology, molecular methods, which are generally more sensitive and precise, are said to have revolutionized the detection, identification and enumeration of microbes in complex ecosystems (Li *et al*, 1996). However, even these techniques have their shortfalls. Quantitative dot blot rRNA hybridization and whole-cell *in situ* fluorescent hybridization techniques are only effective

when the target population accounts for more than 1% of the total bacterial population (Furet *et al.*, 2004). Furthermore, efficient cell lysis, DNA extraction and purification, choice of primers and PCR conditions are all sources of error for indirect methods of bacterial enumeration (Farrelly *et al.*, 1995). Yet, despite the inherent shortfalls of each method, they have all been successfully utilized for the quantification of microbial populations.

Both viable cell counts and ELISAs have been successfully utilized in a number of studies for determining the colonization potential of putative probiotic microorganisms. Robertson *et al.* (2000) fed Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*) a diet supplemented with 4.0×10^6 cfu g^{-1} of *Carnobacterium* sp. before monitoring the fate of the probiotic strain in the digestive tracts of these animals by plating serial dilutions of homogenized intestinal tissue onto selective solid media. Similarly, Gullian *et al.* (2004) assessed the colonization potential of the three putative probionts *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 in shrimp hepatopancreas. In this study, the authors confirmed the identities of the probiotic strains by colony morphology and the arbitrarily primed polymerase chain reaction (AP-PCR) technique. Ringø and Vadstein (1998) used polyclonal antibodies against *Vibrio pelagius* and *Aeromonas caviae* for the detection of these species in the rearing water and gastrointestinal tract of healthy turbot (*Scophthalmus maximus*) larvae exposed to *V. pelagius* and/or *A. caviae*. Later, Ringø (1999) used this technique to determine whether *Carnobacterium divergens*, originally isolated from *S. salar* L., is capable of colonizing the gut of early developing *S. maximus* larvae.

The use of the green fluorescent protein (*gfp*) gene as a reporter and marker in prokaryote and eukaryote cells has also become a popular tool for gaining information on bacterial localization, colonization and gene transfer (Scott *et al.*, 2000). Indeed, Hurst and Jackson (2002) used a series of green fluorescent protein derivatives of the bacterium *Serratia entomophila*, the causative agent of Amber disease, to monitor the fate of this species once ingested by the New Zealand grass grub, *Costelytra zealandica*. In addition, several transposon vectors have been developed for stable chromosomal integration of foreign genes, such as the *gfp* and luciferase (*luxAB*) gene (Albertson *et al.*, 1996; Herrero *et al.*, 1990; De Lorenzo *et al.*, 1990; Matthysse *et al.*, 1996; Sohaskey *et al.*, 1992). Two important features of these transposon vectors are the inability of the suicide plasmid to replicate in the absence of the π protein (λpir) and that the

resulting mutants are stable because the transposase gene is located on the suicide delivery plasmid and not within the transposon, thus preventing additional rounds of transposition. In fact, these vectors have been successfully used for tagging Gram-positive and -negative bacteria for monitoring gene expression, location and viability of bacteria (Albertson *et al.*, 1996; Ferguson *et al.*, 1998; Normander *et al.*, 1999; Scott *et al.*, 2000; Stretton *et al.*, 1998). Scott *et al.* (2000) chromosomally-marked *Lactococcus lactis* and *Enterococcus faecalis* with the *gfp* gene and monitored the survival of the two strains under conditions designed to simulate the human colon. Conversely, Ferguson *et al.* (1995) chromosomally-marked the fish pathogen *Aeromonas salmonicida* with the *luxAB* gene and monitored the colonization and transmission of this strain following challenge by cohabitation (Ferguson *et al.*, 1998).

We have clearly shown that *Haliotis midae* continuously fed a diet supplemented with *Vibrio midae* SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1 have an improved survival and growth rate compared to animals not fed probiotics (Macey and Coyne, 2005). In an endeavor to determine whether the three strains are capable of colonizing the digestive tract of *H. midae*, survival of the three strains will be monitored in the digestive tract of abalone following cessation of feeding with a probiotic-supplemented feed by plating serial dilutions of homogenized digestive tract tissue onto selective solid media. In conjunction with this technique, 16S and 18S rRNA gene sequencing will be done to confirm the identity of the presumptive probiotic cells growing on selective agar. Furthermore, the probiont *V. midae* SY9 will be chromosomally-tagged with the *gfp* gene in order to monitor the survival of this strain in the digestive tract of *H. midae*.

5.3 Materials and methods

All media and solutions used in this study are listed in appendix A.

5.3.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 5.1. The plasmid pLOFKm*gfp* and the *gfp* reporter transposon construct are shown in Fig. 5.1.

5.3.2 Media and culture conditions

E. coli SM10 harboring the pLOFKm*gfp* plasmid, obtained from Molecular Biology Vectors (ATCC 87711), was either grown in LB10 broth (Appendix A.1.6) or on LB10 agar (Appendix A.1.7) containing 85 μg ml^{-1} kanamycin (Km) (Appendix A.2.1) and 100 μg ml^{-1} ampicillin (Amp) (Appendix A.2.1) at 37 °C. Wild-type *V. midae* SY9, *Cryptococcus* sp. SS1 and *D. hansenii* AY1 were grown at 22 °C as described in Chapter 2. *V. midae* SY9.1 was either grown in VNSS broth (Appendix A.1.10) or on VNSS agar (Appendix A.1.11) containing 120 μg ml^{-1} streptomycin (Sm) (Appendix A.2.1) at 30 °C, unless otherwise stated. The mutant *V. midae* SY9.8 was either grown in MB (Appendix A.1.1) or on VNSS agar containing 400 μg ml^{-1} Km.

5.3.3 Transposon mutagenesis of *V. midae* SY9

In order to chromosomally-tag *V. midae* SY9 with a gene encoding green fluorescent protein (GFP), transposon mutagenesis using a mini-Tn10-based reporter transposon containing promoterless *gfp* (mut2) was used (Fig. 5.1b). Streptomycin resistant *V. midae* SY9 was generated by spread-plating an O/N culture of *V. midae* SY9 onto a MA/Sm gradient plate, containing Sm ranging in concentration from 0 to 120 μg ml^{-1}. Plates were incubated at room temperature for 24 hrs. Streptomycin-resistant colonies were routinely streaked onto fresh MA/Sm gradient plates until single colonies, capable of growing on Sm at a concentration of 120 μg ml^{-1}, were isolated. Streptomycin-resistant *V. midae* SY9 was designated *V. midae* SY9.1.

Table 5.1. Strains and plasmids used in this study

Strain/plasmid	Genotype/relevant characteristic(s)[a]	Reference
Strains		
Vibrio midae SY9	*H. midae*, South Africa	This study
Vibrio midae SY9.1	Sm^r	This study
Vibrio midae SY9.8	SY9.1::mini-Tn10-*gfp-kan*, Sm^r Km^r GFP^+	This study
Cryptococcus sp. SS1	*H. midae*, South Africa	This study
Debaryomyces sp. AY1	*H. midae*, South Africa	This study
E. coli SM10	*thi thr leu ton*A *lac*Y *sup*E (λpir) *rec*A::RP4-2-Tc::Mu Km^r	Miller and Mekalanos, 1988
Plasmids		
pLOFKm*gfp*	pLOFKm with promoterless *gfp* cloned upstream of *kan*	Stretton *et al.*, 1998

[a] Sm^r, streptomycin resistant; Km^r, kanamycin resistant.

Plasmid pLOFKm*gfp* was transferred from *E. coli* SM10 into *V. midae* SY9.1 by mobilization with the filter-mating technique described by Egan *et al.* (2002) with the following modifications. Five-milliliter overnight cultures of both donor *E. coli* SM10 (pLOFKm*gfp*) and the streptomycin-resistant recipient *V. midae* SY9.1 were prepared. Donor and recipient cultures were mixed at a volume ratio of 1:10 (100 µl *E. coli* SM10 + 1000 µl *V. midae* SY9.1) in 5 ml of wash solution (50% NSS (Appendix A.2.3): 50% 10mM $MgSO_4$ (Appendix A.2.4)) and gently mixed by inversion. The donor and recipient mixture was filtered through a 0.22 µm Whatman filter (2.5 cm in diameter) and washed with another 5 ml of wash solution. The filters were then placed cell-side up onto LB15 agar (Appendix A.1.9) containing 3 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated for 4 hrs at 30 °C.

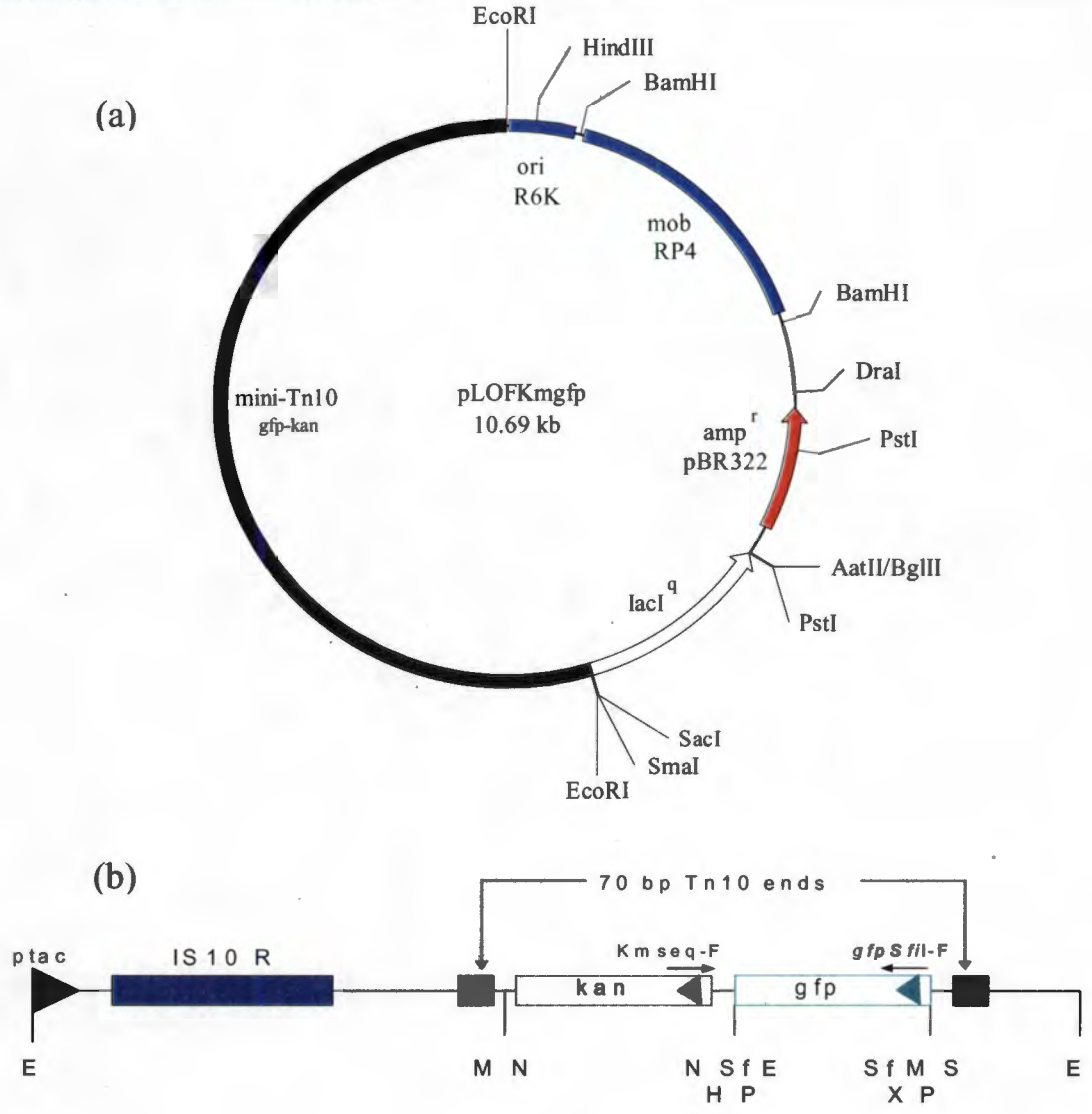


Figure 5.1. Diagrammatic representation of pLOFKm*gfp* (a) and mini-Tn10-*gfp-kan* (b) used for transposon mutagenesis of *V. midae* SY9. Plasmid pLOFKm*gfp* is a derivative of pGP704 and contains the *amp*^{r} gene from plasmid pBR322, a mobilization fragment from plasmid RP4, the *ori* gene from plasmid R6K, and the *lacI*^{q} gene from plasmid pMJR1560. In mini-Tn10-*gfp-kan,* the transposase gene of $IS10_R$ is located outside of the inverted repeats (filled black-boxes) of the mobile element and downstream of the p*tac* promoter. Primers used for PCR amplification of the 1.3 kb *gfp-kan* fragment are indicated above the open boxes depicting the genes in mini-Tn10-*gfp-kan*. Relevant restriction enzyme sites are indicated. Abbreviations: E, *Eco*RI; H, *Hind*III; M, *Mlu*I; N, *Not*I; P, *Pst*I; S, *Sal*I; Sf, *Sfi*I; X, *Xba*I. The diagram of pLOFKm*gfp* was adapted from Herrero *et al.* (1990) and mini-Tn10-*gfp-kan* from Stretton *et al.* (1998). Both diagrams are not to scale.

Following incubation, the filters were placed into sterile steriline tubes containing 1 ml of NSS and vortexed to release the bacteria. One hundred microlitre aliquots of cells were then spread-plated onto VNSS agar containing 400 μg ml^{-1} Km and 120 μg ml^{-1} Sm and incubated at 30 °C for 48 hrs to select for recipient *V. midae* SY9.1 strains carrying the mini-Tn10 transposon. Controls of each strain were spotted separately and consisted of 100 μl aliquots of the donor or recipient strain plated onto either VNSS agar or VNSS agar containing 400 μg ml^{-1} Km and 120 μg ml^{-1} Sm.

5.3.4 Screening of recombinant strains

A hundred kanamycin-resistant mutants were randomly selected and screened for *gfp*-expression. Each strain was inoculated into MB containing 400 μg ml^{-1} Km and 120 μg ml^{-1} Sm and grown at 30 °C for 24 hrs. Following incubation, cells were viewed under an Olympus fluorescent microscope equipped with a 488 nm emission filter and scored for fluorescence. Six mutants displaying the highest relative fluorescence were then selected and compared to wild-type *V. midae* SY9 by determination of the growth rates and *gfp*-expression at 30 °C in MB. For growth rate determinations, the wild-type and each mutant strain were grown in 5 ml of MB at 30 °C on an orbital shaker at 100 rpm for 12 hrs. Subsequently, 500 μl of each culture was transferred into 50 ml of fresh media and incubated for a further 12 hrs before inoculating two 5 litre conical flasks, each containing 500 ml of fresh media, so as to achieve a final absorbancy at 600 nm of 0.01. The cultures were incubated at 30 °C on an orbital shaker at 100 rpm, with triplicate 1 ml samples taken from each culture on an hourly basis for determination of the optical density at 600 nm and *gfp*-expression. For the determination of *gfp*-expression, each 1 ml sample was centrifuged at 12 000 x g for 5 min to pellet the cells. The cells were then washed twice and resuspended in an equal volume of PBS (Appendix A.2.4) before transferring 100 μl of each sample to separate wells of a microtiter plate. Each plate was read on a Scan-Analyst Gel Viewer (Bio-Rad) set to measure green fluorescence. The relative fluorescence (*gfp*-expression) of each mutant strain was calculated by subtracting the background fluorescence of the wild-type strain at each time point.

5.3.5 Southern hybridization

Southern hybridization analysis was preformed to determine whether, and to what extent, the mini-Tn10-*gfp-kan* cassette integrated into the chromosome of *V. midae* SY9.1. Genomic DNA was extracted from both *V. midae* SY9 and *V. midae* SY9.8 as described in Appendix B.1. Large scale preparation of pLOFKm*gfp* was carried out using a Midi plasmid isolation kit (Qaigene) according to the manufacturer's instructions. The resultant plasmid DNA was used as a template for PCR amplification of a 1.3 kb *gfp-kan* fragment (Fig. 5.1b) using the primer pair *gfpSfi*I-F and Kmseq-F (Appendix B.8.1) described by Stretton *et al.* (1998). The PCR was conducted as described in Appendix B.8.2. The amplified PCR product (10 µl) was analyzed by agarose gel electrophoresis (Appendix B.3) to verify reaction specificity and fragment size before being purified using a PCR product purification kit (Roche). The purified DNA fragment was then radioactively labeled (Appendix B.6.2) and used as a probe against equal amounts (10 µg) of both *V. midae* SY9 and *V. midae* SY9.8 genomic DNA that had been digested with the restriction enzymes *Hind*III, *Eco*RI, *Sal*I, *Pst*I and *Xba*II (Appendix B.2) and separated on a 0.8% TAE agarose gel. The Southern hybridization procedure that was followed is described in Appendix B.6.

5.3.6 Marker stability

The stability of the *gfp* gene was monitored by repeatedly sub-culturing *V. midae* SY9.8 in MB lacking antibiotics. A 1 ml sample of *V. midae* SY9.8 from an overnight culture in MB containing 400 µg ml^{-1} Km was centrifuged at 12 000 x g for 5 min. The cells were then washed twice and resuspended in an equal volume of PBS before being added to 20 ml of MB. Cells were incubated for 48 hrs at room temperature on an orbital shaker at 100 rpm. Following incubation, a serial dilution was made of the sample from which aliquots of 100 µl were spread plated onto Petri dishes containing MA (Appendix A.1.2). All plates were incubated at room temperature for 24 hrs. Subsequently, 100 colonies were transferred to MA plates with or without 400 µg ml^{-1} Km and incubated at room temperature for 24 hrs. The proportion of colonies retaining kanamycin-resistance was then calculated by comparing the ability of

randomly selected colonies grown without antibiotic selection to subsequently grow in the presence and absence of kanamycin.

5.3.7 Preparation of feed

The basal 'kelp-cake' diet utilized in this study consisted of 40% pre-swollen kelp (~0.1 cm^3 pieces of dried kelp, supplied by Kelpak, soaked O/N in a 3% (w/v) NaCl solution) and 2% Bacteriological agar (Biolab) made up in artificial seawater (ASW, Appendix A.2.2). The mixture was autoclaved at 120 °C for 20 min, allowed to cool to approximately 45 °C and then poured into the bottom of sterile Petri-dishes. The probiotic-supplemented diet consisted of the basal diet supplemented with a mixture of the three probiotic strains, *V. midae* SY9.8, *Cryptococcus* sp. SS1 and *D. hansenii* AY1. Each probiont was added to the feed, once it had cooled to approximately 45 °C, to achieve a final concentration of approximately 1.0 x 10^7 culturable cells g^{-1} kelp-cake. Once cooled, plates were stored at 4 °C until used. The number of culturable probiotic cells in the feed was determined as described in section 3.2.2.

5.3.8 Colonization experiment

A colonization experiment was conducted in order to determine whether the three probiotic strains (SY9.8, SS1 and AY1) are able to survive in the digestive tract of *H. midae*. Animals were maintained in two large polyethylene tanks containing 98 l of aerated and continuously flowing (330 L h^{-1}) natural seawater at 15 – 18 °C. Each tank was stocked with 30 abalone, which were acclimatized for two weeks and fed the basal feed. Animals were fed every second day after first removing the uneaten feed from each tank and each tank was thoroughly cleaned once a week. Three weeks before the start of the experiment, and including the first day of the experiment (T = 0), animals in one tank were fed the probiotic-supplemented feed, while the animals in the remaining tank were continually fed the basal feed. Subsequently, animals in both tanks were fed the basal feed on the following days: T = 2, 4, 7, 9 and 11. On day one (T = 1), three abalone were removed from each tank and immediately sacrificed. Thereafter, three animals were sacrificed from the probiotic-treatment tank only on the following days: T = 2, 3, 4, 8 and 15.

5.3.8.1 Isolation of genomic DNA

Initially, the entire digestive tract from each abalone was aseptically removed and placed on ice. The crop/stomach and the intestine were then carefully dissected (Fig. 3.2) so as to prevent cross-contamination between tissue fluids. Approximately 50 mg of crop/stomach and intestinal tissue from each animal was rapidly transferred to pre-weighed eppendorf tubes, flash-frozen in liquid nitrogen and stored at -70 °C so as to prevent degradation of genomic DNA. The frozen samples were subsequently ground in liquid nitrogen with a mortar and pestle before transferring the ground tissue to a new pre-weighed eppendorf tube. Care was taken to keep samples frozen throughout this process. The eppendorf tubes were weighed to the nearest 0.01 mg before extracting total cellular DNA, which included both abalone and bacterial DNA, using a Genomic DNA isolation kit (Fermentas, #K0512) according to the manufacturer's instructions. The isolated DNA was quantified with a spectrophotometer and DNA quality was tested by agarose gel electrophoresis. Isolated DNA was stored at -20 °C until needed.

5.3.8.2 *In situ* enzyme assays and enumeration of culturable probiotic cells

A portion of the remaining dissected tissue from the crop/stomach and intestine was transferred into pre-weighed eppendorf tubes and weighed to the nearest 0.01 mg before being resuspended in 2 ml of sterile PBS. Each tube was vortexed for approximately 1 min before making serial dilutions and spread-plating onto VNSS agar, containing 400 μg ml^{-1} Km, and potato dextrose agar (PDA) (Difco), containing 250 μg ml^{-1} chloramphenicol (Cm) (Appendix A.2.1), to culture the bacterial and yeast strains respectively. Cell numbers were recorded after incubating the VNSS agar plates for 18 hrs and PDA plates for 3 to 4 days and expressed as the number of cfu g^{-1} gut tissue. Subsequently, each eppendorf tube was centrifuged at 12 000 x g for 10 min at 4 °C and the supernatant transferred to a new eppendorf tube. Protease and amylase activity of the supernatants from the intestine and crop/stomach regions of *H. midae* was determined as described in sections 2.3.3.1 and 2.3.3.2 and expressed as units of activity g^{-1} of gut tissue.

5.3.8.3 Dot blot hybridization

To further establish whether *V. midae* SY9.8 is capable of colonizing the digestive tract of *H. midae*, a dot blot analysis was preformed. Equal amounts of genomic DNA (12 µg) isolated from the crop/stomach and intestinal tissue from each animal was transferred to a Hybond N+ nylon membrane (Amersham) using the dot blot procedure described in Appendix B.7. A positive control consisting of a dilution series of known quantities of *V. midae* SY9.8 genomic DNA and a negative control consisting of a dilution series of known quantities of wild-type *V. midae* SY9 genomic DNA were also transferred to each membrane. Genomic DNA from abalone fed the basal diet served as an additional negative control. The 1.3 kb *gfp-kan* fragment used for the Southern hybridization analysis described in section 5.3.5 was radioactively-labeled (Appendix B.6.2) and used as a probe against wild-type *V. midae* SY9, *V. midae* SY9.8, crop/stomach and intestinal genomic DNA. The Southern hybridization procedure that was subsequently followed is described in Appendix B.6.3 and B.6.4.

The membranes were exposed to a phosphor screen and the hybridization signals quantified with a Personal Molecular Images FX phosphor imager (Bio-Rad) equipped with Quantity-One® quantitation software. The amount of DNA detected in the crop/stomach and intestinal samples was determined by comparison to hybridization signals obtained with the DNA standards (dilution series of *V. midae* SY9.8 DNA) included on each membrane and expressed as micrograms of DNA per gram of gut tissue. As in Hermansson and Lindgren (2001), estimation of bacterial cell numbers was based on the following assumptions: the genome size of *V. midae* SY9.8 is similar to that of *V. parahaemolyticus* (5.2 Mb); there is only 1 genome/cell; 1 kb of dsDNA is approximately equal to 0.662×10^6 Daltons (Coyne *et al.*, 1996); and 1 Dalton is equal to 1.67×10^{-24} grams (Garrett and Grisham, 1995). The amount of DNA obtained per gram of gut tissue was then divided by the estimated amount of DNA per cell to determine the number of cells per gram of gut tissue.

5.3.8.4 16S and 18S rRNA gene sequencing of presumptive probiotic strains

In order to establish whether the cells that grew on the selective plates in section 5.3.8.2 are indeed the probiotic strains that were initially added to the probiotic-supplemented feed, 16S and/or 18S rRNA gene sequencing was preformed. A single colony of each strain growing on the selective plates was inoculated into 5 ml of either MB or YPD broth. Genomic DNA was then extracted from each strain as described in Appendix B.1 and used as a template for the isolation of the 16S and/or 18S rRNA gene by PCR as described in sections 2.3.5 and 2.3.10 respectively. Each sequence was edited and assembled using DNAMAN version 4.13 (Lynnon BioSoft). Homology searches were carried out using the BLASTN algorithm (Altschul *et al.*, 1989) provided by the internet service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nlm.nih.gov/BLAST/).

5.3.9 Statistical analysis

All data is presented as means and standard errors. For comparisons of two means, an unpaired student t-test was used. For multiple comparisons, data was analyzed by one-way or two-way ANOVA. When the effects of the ANOVA were significant, the Tukey test was used to test for significant differences between sample means. Pearson product movement correlations were used to determine the strengths of the association between enzyme activities and probiotic cell numbers. Prior to statistical analysis, the cell count data underwent natural log transformations. Significant differences were established at $P<0.05$.

5.4 Results

5.4.1 Transposon mutagenesis of *V. midae* SY9

The mini-Tn10-*gfp-kan* cassette delivered from pLOFKm*gfp* transposed at a high frequency to the recipient strain *V. midae* SY9.1. Transconjugants grew on VNSS agar plates containing Km and Sm at a concentration of 5.0 x 10^3 cfu ml^{-1} of conjugation mix. Unfortunately, detection of GFP in SY9.1 transconjugant colonies was not possible by eye using a hand-held BlakRay UV light (UVP model UVL-56) emitting long-wavelength UV at 366 nm. As a result, 100 randomly selected transconjugants were screened by epifluorescence microscopy to identify transconjugants in which the mini-Tn10-*gfp-kan* cassette had inserted downstream from a strongly expressed constitutive promoter. Most displayed weak fluorescence and only six transconjugants were identified as possibly having strongly expressed promoter-*gfp* fusions. Furthermore, the amount of GFP-production was the same when mutant cells were grown in either MB, TSB or VNSS broth (data not shown).

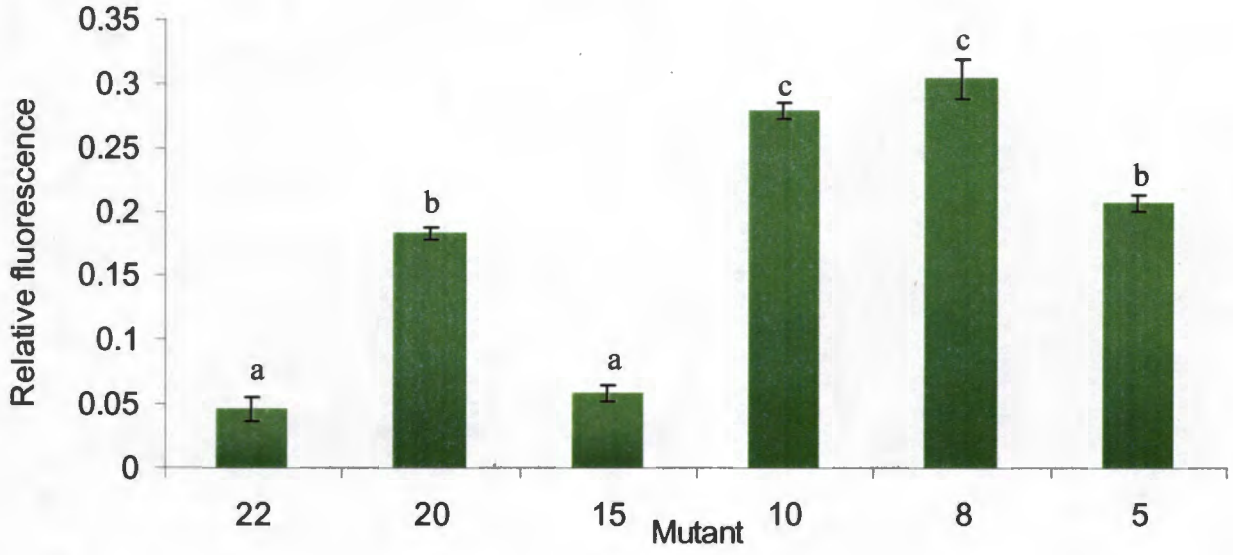


Figure 5.2. GFP-synthesis in six mutant *V. midae* SY9 strains expressed as fluorescence relative to the wild-type *V. midae* SY9. All data represents the mean (± S.E.) of at least three readings. Different subscripts represent a significant difference ($P<0.05$; one-way ANOVA) between values.

A comparative analysis of *gfp*-expression in these six mutants identified two (mutants 8 and 10) as having the highest relative fluorescence (Fig. 5.2). However, after comparing the growth rate of each mutant strain to that of the wild-type strain, mutant 8, designated *V. midae* SY9.8, was identified as the best candidate for further analysis. There was no difference in the growth rate of the mutant *V. midae* SY9.8 and the wild-type *V. midae* SY9 (Fig. 5.3), although the mutant did have a slightly lower maximum biomass yield compared to the wild-type. Furthermore, the amount of *gfp*-expression appeared to be directly correlated to cell-density and tended to decline once the cells reached stationary phase of growth. However, when the cells were viewed under a fluorescent microscope, the relative fluorescence exhibited by SY9.8 appeared to be too low for *in situ* detection of the mutant strain in the digestive tract of *H. midae*. Indeed, at an absorbancy of 1, which corresponds to a cell density of approximately 1.0 x 10^8 cfu ml^{-1} (Fig. 2.11a), the relative fluorescence was only 0.1.

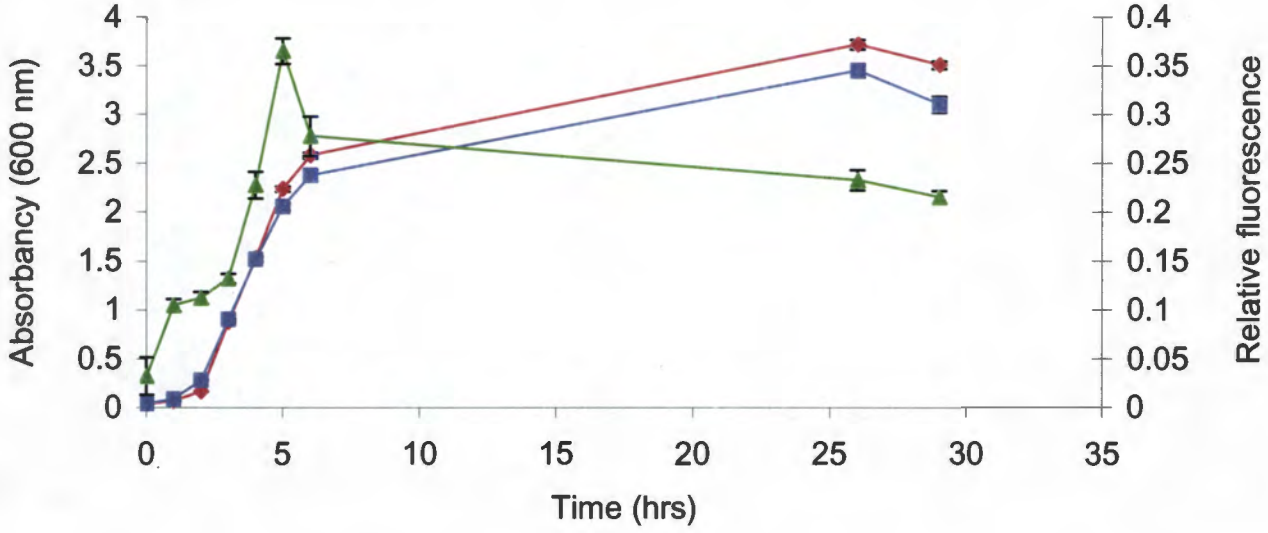


Figure 5.3. The growth profiles of *V. midae* SY9 (♦) and *V. midae* SY9.8 (■) during shake-flask cultivation in MB at 30 °C. The relationship between *gfp*-expression (▲) in *V. midae* SY9.8 and cell-density is indicated. All data represents the mean (± S.E.) of at least three readings.

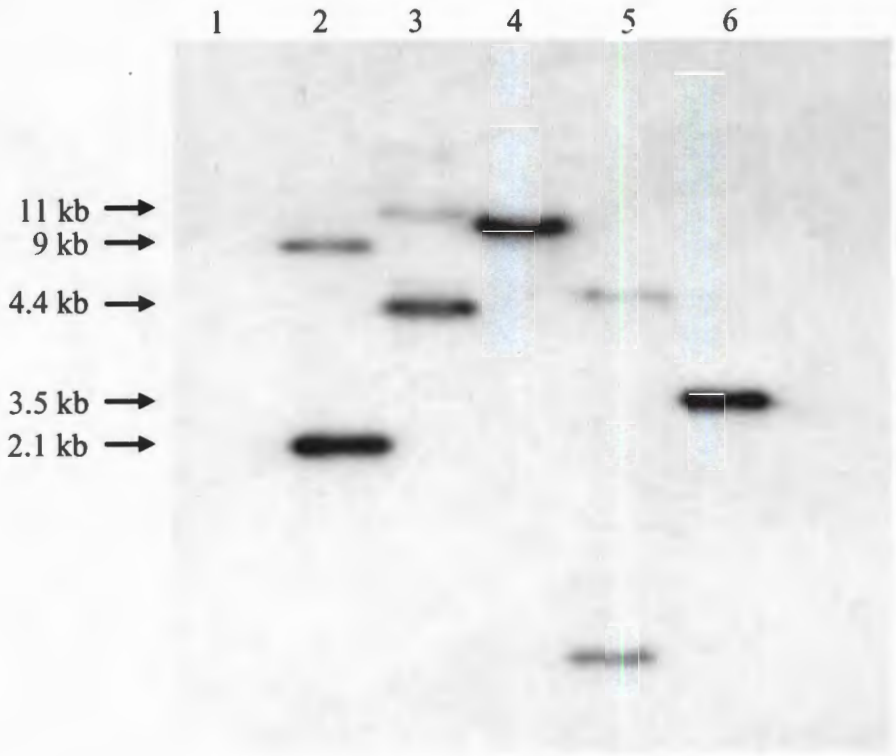


Figure 5.4. Southern hybridization of the 1.3 kb PCR product from pLOFKm*gfp* against *V. midae* SY9 and *V. midae* SY9.8 genomic DNA. *V. midae* SY9 genomic DNA digested with *Hind*III (Lane 1), *V. midae* SY9.8 genomic DNA digested with *Hind*III (Lane 2), *Eco*RI (Lane 3), *Sal*I (Lane 4), *Pst*I (Lane 5) and *Xba*I (Lane 6). The arrows indicate the approximate sizes of the bands in kb.

Southern hybridization of chromosomal DNA purified from the wild-type and the mutant strains to a ^{32}P-labelled *gfp-kan* PCR product confirmed the chromosomal integration of the mini-Tn10-*gfp-kan* cassette (Fig. 5.4). Single hybridization bands were observed when *V. midae* SY9.8 chromosomal DNA was digested with the restrictions enzymes *Sal*I and *Xba*I, which do not cut within the *gfp-kan* gene, and two bands obtained following digestion with the restriction enzymes *Hind*III, *Eco*RI and *Pst*I, which cut once within the *gfp-kan* gene (Fig. 5.1b), show that only one copy of the transposon is present per chromosome. Hybridizing restriction fragments generated by *Eco*RI digestion of chromosomal DNA from nine different transconjugants were different in size, indicating that mini-Tn10-*gfp-kan* inserted randomly (data not shown).

Furthermore, after repeatedly sub-culturing *V. midae* SY9.8 in media lacking antibiotics, we found that all 100 colonies grew in the presence of kanamycin, indicating that the chromosomal marker is stably maintained in this strain in the absence of antibiotics. As a result of the low fluorescence exhibited by *V. midae* SY9.8, the chromosomally integrated *gfp* gene was detected with a ^{32}P-labelled DNA probe in subsequent colonization experiments.

5.4.2 Longevity of probiotic strains in the digestive tract of *H. midae*

The ability of *V. midae* SY9.8, *Cryptococcus* sp. SS1 and *D. hansenii* AY1 to colonize the digestive tract of *H. midae* was assessed by feeding abalone the probiotic-supplemented feed for three weeks before monitoring the survival of each probiont in the crop/stomach and intestine of abalone after cessation of feeding with the probiotic-supplemented feed. Each probiont was added to the feed so as to achieve a final concentration of approximately 1.0×10^7 culturable cells g^{-1} of feed. In fact, when the actual cell numbers were enumerated in prepared probiotic feed, cell counts of 8.0×10^7, 3.0×10^6 and 2.0×10^7 cfu g^{-1} were obtained for SY9.8, SS1 and AY1 respectively.

On the whole, probiotic cell numbers in the crop/stomach (Fig. 5.5a) and intestine (Fig. 5.6a) decreased significantly from day 1 to 2 after cessation of feeding with the probiotic feed, with the exception of the two yeast strains (SS1 & AY1) in the intestine, and tended to decrease from days 2 to 15, where the cell numbers of each probiont were significantly lower ($P<0.05$) compared to day 1. There was no significant difference ($P<0.05$) in SY9.8 cell numbers from days 2 to 8 in the crop/stomach, before decreasing significantly ($P<0.05$) on day 15. Similarly, there was no significant difference ($P<0.05$) in SS1 cell numbers from days 1 to 4 in the intestine, before decreasing significantly on days 8 and 15. The number of probiotic cells had declined substantially fifteen days after feeding with the probiotic feed had been halted. The number of culturable SY9.8, SS1 and AY1 cells were 0, 24 and 570 cfu g^{-1} respectively in the crop/stomach (Fig. 5.5a) and 317, 22 and 450 cfu g^{-1} respectively (Fig. 5.6a) in the intestine on day 15.

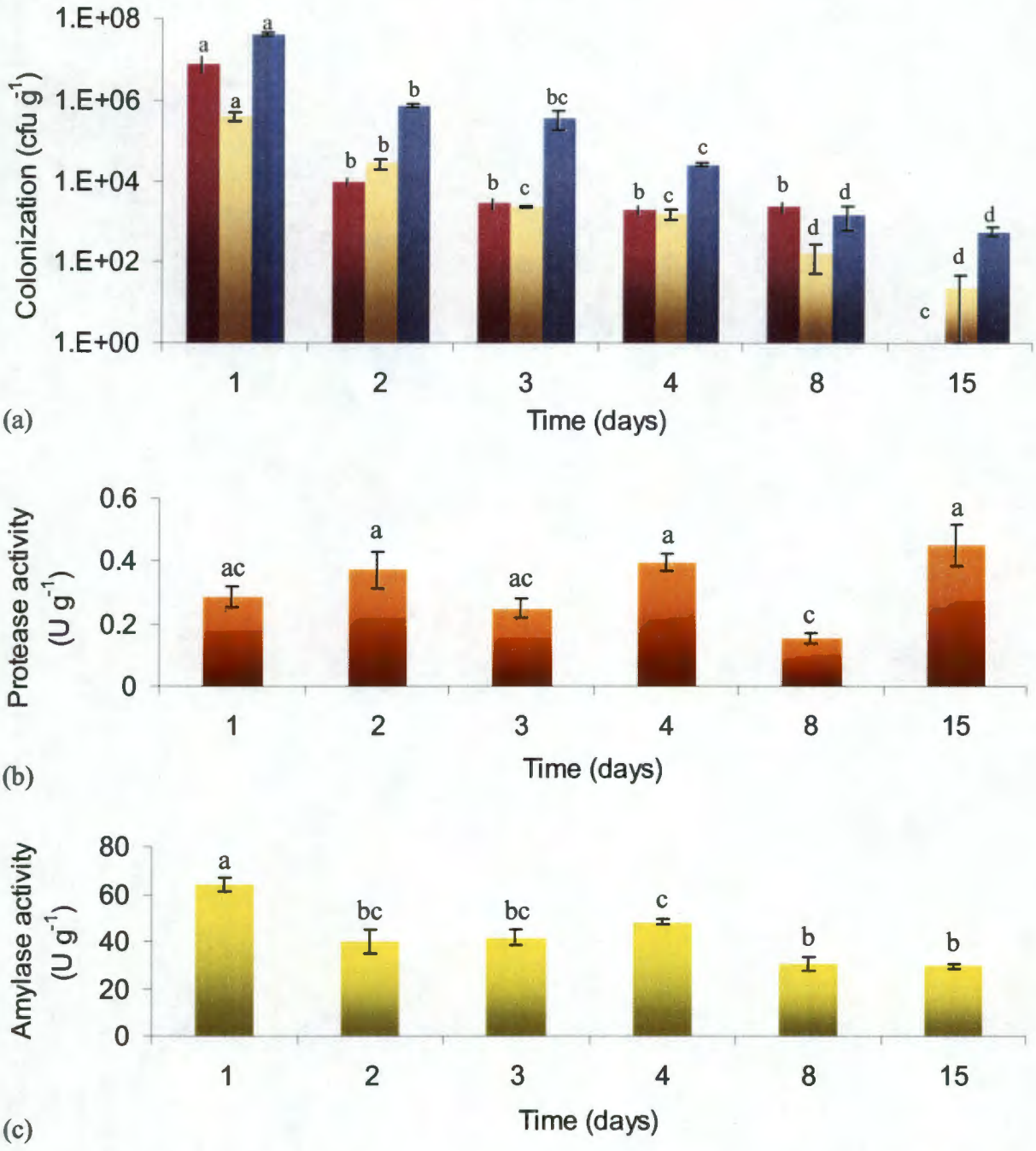


Figure 5.5. (a) Total number of *V. midae* SY9.8 (■), *Cryptococcus* sp. SS1 (■) and *D. hansenii* AY1 (■) cells (cfu g^{-1}) re-isolated from the crop/stomach of *H. midae* 1, 2, 3, 4, 8 and 15 days after being fed a probiotic-supplemented diet for three weeks. Protease (b) and amylase (c) activity (Units g^{-1}) in the crop/stomach on each of these days. All data represents the mean (± S.E.) of triplicate readings from three animals. Different subscripts represent a significant difference ($P<0.05$; one-way ANOVA) between values on different days.

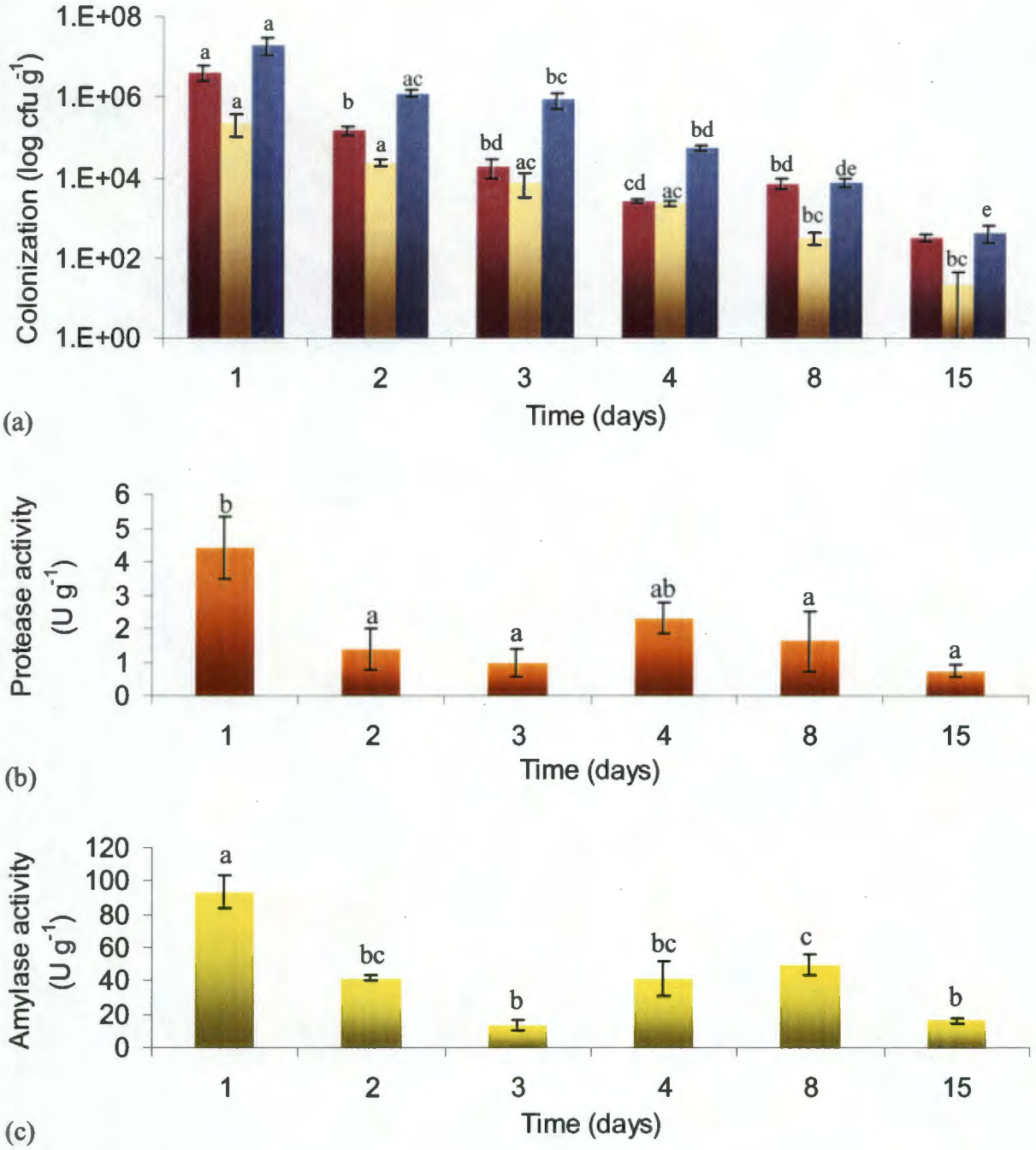


Figure 5.6. (a) Total number of *V. midae* SY9.8 (■), *Cryptococcus* sp. SS1 (■) and *D. hansenii* AY1 (■) cells (cfu g^{-1}) re-isolated from the intestine of *H. midae* 1, 2, 3, 4, 8 and 15 days after being fed a probiotic-supplemented diet for three weeks. Protease (b) and amylase (c) activity (Units g^{-1}) in the intestine on each of these day. All data represents the mean (± S.E.) of triplicate readings from three animals. Different subscripts represent a significant difference ($P<0.05$; one-way ANOVA) between values on different days.

In comparison, control animals previously fed the basal diet that lacked probiotics, did not reveal the presence of any probiotic cells in the crop/stomach or intestine during or after the feeding period. It should also be noted that AY1 cell numbers in the crop/stomach and intestine were generally higher than the other two strains throughout the experiment.

To determine whether the probiotic strains are contributing towards the pool of digestive enzymes within the digestive tract of *H. midae*, the protease and amylase activity of the supernatants from the intestine and crop/stomach of abalone that had previously been fed either the basal or the probiotic feed were compared. The results clearly showed that intestinal protease and amylase activity (Fig. 5.7) was significantly higher ($P<0.05$) in animals previously fed the probiotic feed as opposed to the basal feed on day 1. However, there was no significant difference ($P<0.05$) in protease and amylase activity in the crop/stomach region of abalone previously fed the basal feed or the probiotic feed (Fig. 5.7). These results support the data obtained in Chapter 3.

In order to determine whether a relationship exists between enzyme activity and cell numbers over time, the protease and amylase activity of the supernatants from the intestine and crop/stomach of abalone previously fed the probiotic feed was determined. Crop/stomach amylase activity decrease significantly ($P<0.05$) from days 1 to 2, coinciding with a significant decrease in probiotic cell numbers over the same time period (Fig. 5.5). Subsequently, there was no significant change ($P<0.05$) in crop/stomach amylase activity from days 2 to 15. Conversely, there was no significant difference ($P<0.05$) in crop/stomach protease activity from days 1 to 15, with the exception of day 8. Intestinal protease and amylase activity decreased significantly ($P<0.05$) from days 1 to 2, coinciding with a significant decrease ($P<0.05$) in *V. midae* SY9.8 cell numbers over the same time period (Fig. 5.6). Although *Cryptococcus* sp. SS1 cell numbers also decreased over this time period, the decrease was not significant ($P<0.05$) (Fig. 5.6). Subsequently, there was no significant change ($P<0.05$) in protease and amylase activity from days 2 to 15.

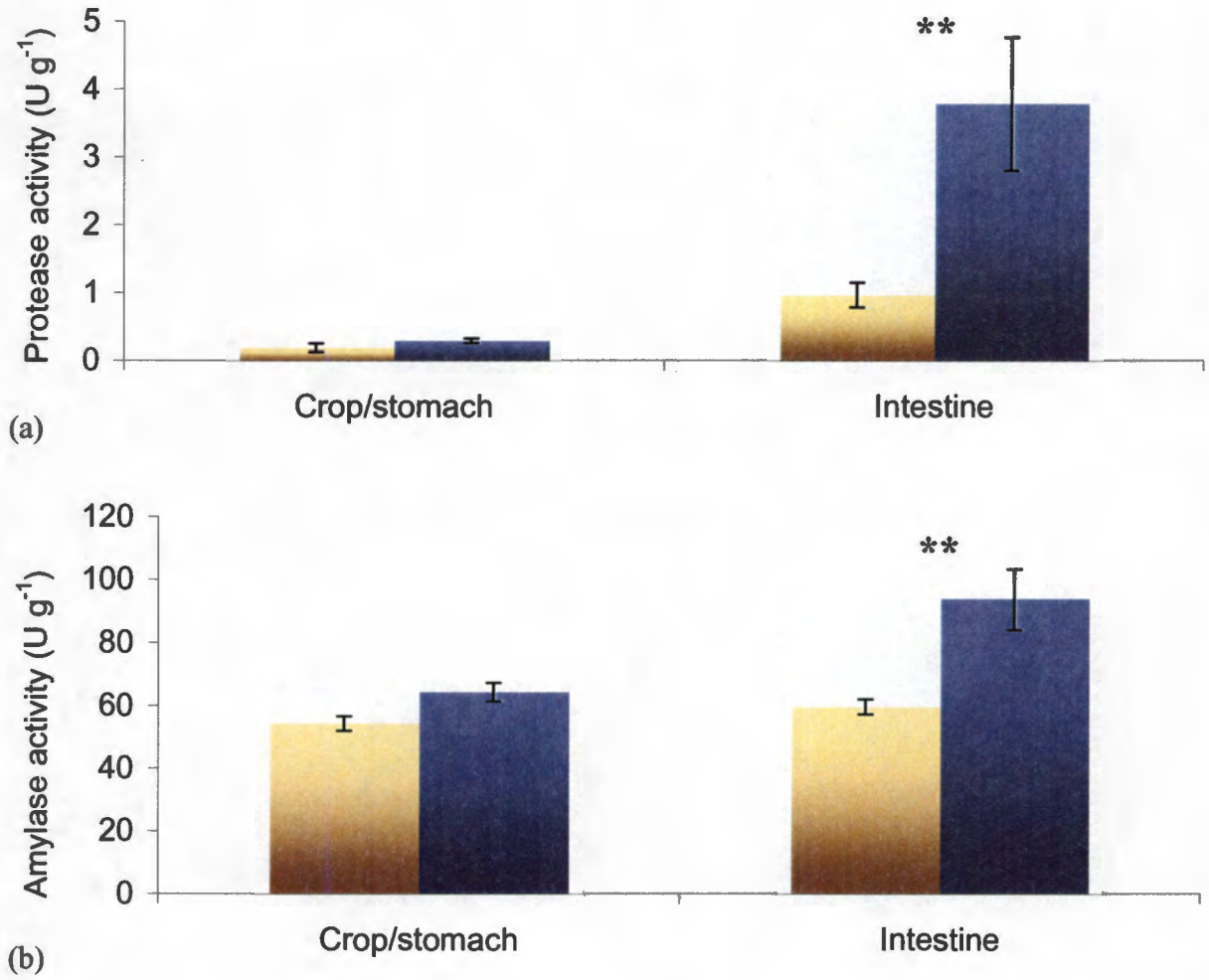


Figure 5.7. A comparison of the *in situ* protease (a) and amylase (b) activity in the crop/stomach and intestine of *H. midae* 24 hrs after being fed either the basal diet (▪) or the probiotic-supplemented diet (■). Each data point represents the mean (± S.E.) of triplicate readings from three animals. **($P<0.05$, Student t-test) represent a significant difference between means.

A correlation analysis was conducted to determine the strength of the association between enzyme activity and biomass of each probiont over the 15 day sampling period. A Pearson product movement correlation between amylase activity and SS1 cell numbers (cfu g^{-1}) in the intestine revealed a significant positive correlation ($r=0.681$, $P<0.05$, $n=17$), suggesting an association between *Cryptococcus* sp. SS1 and amylase activity in the intestine of *H. midae*.

Similarly, a Pearson product movement correlation between protease activity and SY9.8 cell numbers (cfu g^{-1}) in the intestine revealed a significant positive correlation ($r=0.711$, $P<0.05$, $n=17$), suggesting an association between *V. midae* SY9.8 and protease activity in the intestine of *H. midae*. As expected, no correlation was found between AY1 and protease and/or amylase activity. Furthermore, there was no correlation between SS1 and protease activity or between SY9.8 and amylase activity in the digestive tract of *H. midae*.

The nucleotide sequences of the products obtained by PCR amplification of the bacterium SY9.8 16S rRNA gene and the yeast SS1 and yeast AY1 18S rRNA gene were edited and assembled in DNAMAN version 4.13 (Lynnon BioSoft). A BLAST search of the GENBANK database with the assembled sequences revealed that the bacterium SY9.8 16S rRNA gene sequence showed high identity to a number of *Vibrio* species, confirming the results obtained in chapter 2 (Table 2.2). In addition, a BLAST search of the GENBANK database with the assembled yeast sequences confirmed the results obtained for yeast SS1 and AY1 obtained in Chapter 2 (Table 2.5). Furthermore, the assembled sequences of the re-isolated probionts were 100% homologues to the original sequences reported in Chapter 2, indicating that the strains re-isolated from the digestive tract of *H. midae* in the colonization experiment are indeed the probiotic strains that were initially added to the probiotic-supplemented feed.

5.4.4 Longevity of chromosomally marked *V. midae* SY9.8 in the digestive tract of *H. midae*

To further establish whether *V. midae* SY9.8 is capable of colonizing the digestive tract of *H. midae*, a dot blot analysis of a 1.3 kb *gfp*-fragment probed against chromosomal DNA isolated from the crop/stomach and intestine of abalone previously fed either the basal or the probiotic feed was conducted. The ^{32}P-labeled *gfp*-probe did not hybridize to the negative controls, which included chromosomal DNA isolated from abalone previously fed the basal feed and chromosomal DNA isolated from wild-type SY9 (Fig. 5.8a and Fig.5.9a). However, the ^{32}P-labeled *gfp*-probe did hybridize with varying intensities to chromosomal DNA isolated from the crop/stomach and intestine of abalone previously fed the probiotic feed, indicating a variation in SY9.8 cell numbers over time.

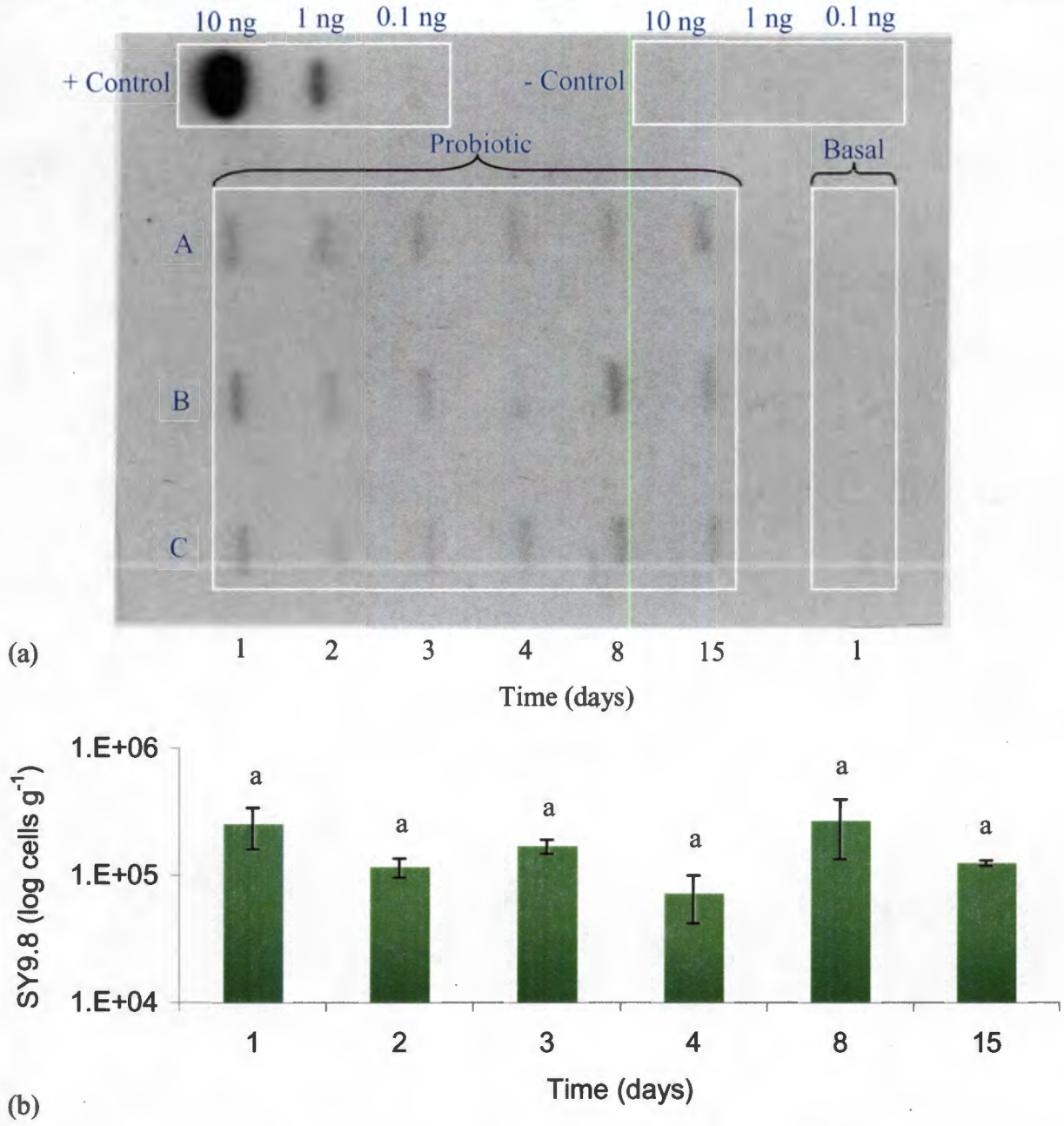


Figure 5.8. (a) Dot blot analysis of the 1.3 kb PCR fragment of pLOFKm*gfp* probed against genomic DNA isolated from the crop/stomach of *H. midae* 1, 2, 3, 4, 8 and 15 days after receiving the probiotic diet and 1 day after receiving the basal diet (negative control). A dilution series of SY9.8 DNA was included as a positive control and a dilution series of SY9 DNA was included as an additional negative control. (b) Estimated number of SY9.8 cells at each time point. Data represents the mean (± S.E.) of values from three abalone (A, B & C) sampled at each time point. Different subscripts represent a significant difference ($P<0.05$; one-way ANOVA) between means.

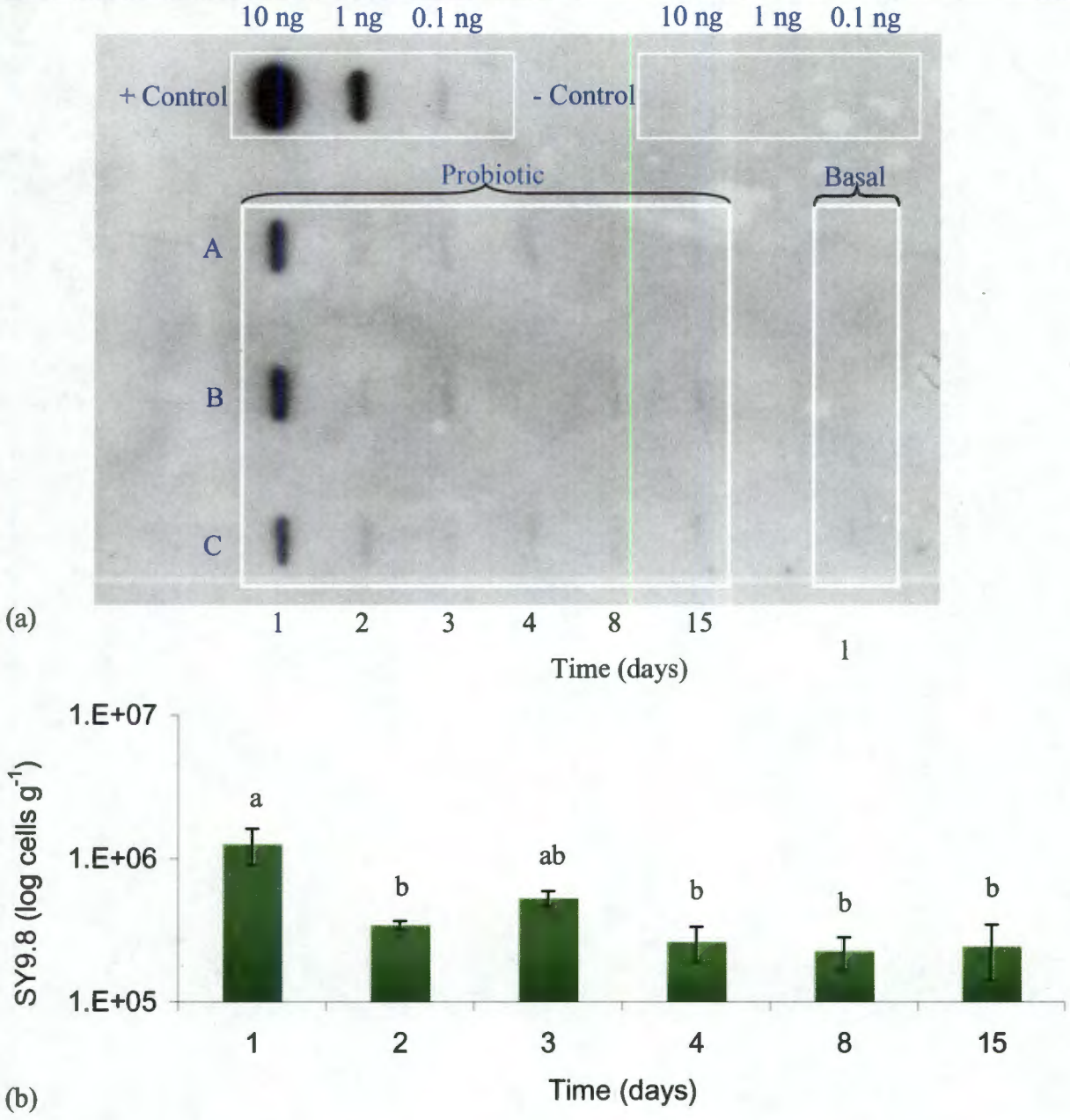


Figure 5.9. (a) Dot blot analysis of the 1.3 kb PCR fragment of pLOFKm*gfp* probed against genomic DNA isolated from the intestine of *H. midae* 1, 2, 3, 4, 8 and 15 days after receiving the probiotic diet and 1 day after receiving the basal diet (negative control). A dilution series of SY9.8 DNA was included as a positive control and a dilution series of SY9 DNA was included as an additional negative control. (b) Estimated number of SY9.8 cells at each time point. Data represents the mean (± S.E.) of values from three abalone (A, B & C) sampled at each time point. Different subscripts represent a significant difference ($P<0.05$; one-way ANOVA) between means.

Table 5.2 The number of *V. midae* SY9.8 cells re-isolated from the crop/stomach and intestine of *H. midae* 1, 2, 3, 4, 8 and 15 days after being fed a probiotic-supplemented diet for three weeks, determined by the plate count method and *in situ* hybridization using a *gfp-kan* DNA probe. Data represents the mean value at each time point.

Time (days)	Region of Digestive Tract			
	Crop/stomach		Intestine	
	Plate count	Hybridization	Plate count	Hybridization
1	7.24E+06*	2.50E+05	4.13E+06	1.27E+06
2	8.95E+03	1.15E+05*	1.30E+05	3.46E+05
3	2.39E+03	1.68E+05*	1.42E+04	5.31E+05*
4	1.96E+03	7.10E+04*	2.60E+03	2.64E+05*
8	1.95E+03	2.64E+05*	7.97E+03	2.26E+05*
15	0	1.24E+05*	3.10E+02	2.45E+05*

*(P<0.05, student t-test) represents a significant difference in *V. midae* SY9.8 cell numbers between the plate count and *in situ* hybridization method at a specific time point.

One day after cessation of feeding with the probiotic-supplemented feed, SY9.8 cell numbers were estimated to be 2.5 x 10^5 cells g^{-1} in the crop/stomach (Fig. 5.8b). In addition, there was no significant change (P<0.05, one-way ANOVA) in SY9.8 cell numbers from days 1 to 15 in the crop/stomach. In the intestine, SY9.8 cell numbers were estimated to be 3.5 x 10^6 cfu g^{-1} one day after cessation of feeding with the probiotic-supplemented feed (Fig. 5.9b), before decreasing significantly (P<0.05) on day 2. Subsequently, there was no significant change (P<0.05) in SY9.8 cell numbers in the intestine (days 2 to 15). A direct comparison between cell counts and *in situ* hybridization showed that plate counts of *V. midae* SY9.8 were significantly higher (P<0.05) in the crop/stomach but not in the intestine on day 1 (Table 5.2). However, *V. midae*

SY9.8 plate counts were lower in the crop/stomach of *H. midae* from day 2 onwards and in the intestine of *H. midae* from day 3 onwards, compared to estimates from *in situ* hybridization.

5.5 Discussion

The use of probiotics in aquaculture worldwide has become increasingly popular, with screening of new probiotic strains based mainly on the ability of the new isolates to produce antibacterial metabolites (Vine *et al.*, 2004). Furthermore, the fate of the putative probiotic cells within the gastrointestinal tract of the host organism is often undetermined (Gatesoupe, 1999). In contrast, we have isolated putative probiotic microorganisms with enhanced enzyme activities and have clearly shown that *Haliotis midae* continuously fed a diet supplemented with *Vibrio midae* SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1 have an improved survival and growth rate compared to animals not fed probiotics (Macey and Coyne, 2005). In addition, this chapter has shown that all three probiotic strains are able to survive in, and colonize, the digestive tract of *H. midae* previously fed probiotics. Although previous studies have demonstrated the ability of putative probiotic microorganisms to colonize the digestive tracts of fish and crustaceans (Gildberg *et al.*, 1997; Gullian *et al.*, 2004; Nikoskelainen *et al.*, 2003; Rengpipat *et al.*, 2000; Ringø and Vadstein, 1998; Ringø, 1999; Robertson *et al.*, 2000), this is the first study to report probiotic colonization of the digestive tract of *H. midae*.

Gildberg *et al.* (1997) demonstrated that continuous feeding of Atlantic cod fry, *Gadus morhua*, with a *Carnobacterium divergens* supplemented feed for three weeks resulted in the intestinal flora of *G. morhua* being dominated by lactic acid bacteria. Rengpipat *et al.* (2000) continually fed the black tiger shrimp, *Penaeus monodon*, a diet supplemented with 10^{10} cfu g^{-1} of *Bacillus* S11 and showed that *Bacillus* S11 reached a maximum concentration of approximately 10^6 cfu g^{-1} of shrimp intestine after 30 days of culture. Similarly, Robertson *et al.* (2000) established that after continually feeding *O. mykiss* for 28 days with a diet containing *Carnobacterium* sp., at a concentration of approximately 4.0×10^6 cfu g^{-1}, a maximum cell concentration of 7.4×10^6 cfu g^{-1} of intestine was reached. However, after cessation of feeding with the probiotic-supplemented feed, the number of probiotic cells isolated from trout intestines declined rapidly, such that *Carnobacterium* sp. could no longer be isolated 6 days later. Similar results were reported by Nikoskelainen *et al.* (2003) after continually feeding *O. mykiss* a *Lactobacillus rhamnosus* supplemented feed for 2 weeks. Following the replacement of the *L. rhamnosus* supplemented feed with a non-supplemented feed, the number of lactobacilli decreased rapidly within a week,

with no lactobacilli detected in the intestines of *O. mykiss* after two weeks. In all of these studies, the concentration of the probiotic cells within the digestive tracts of the host animals were enumerated by plating serial dilutions of homogenized tissue onto selective plates. These studies support the findings of the present study, where the number of culturable probiotic cells ranged from 10^6 to 10^7 cfu g^{-1} of crop/stomach and/or intestinal tissue when abalone were continuously fed the probiotic-supplemented feed, before decreasing significantly ($P<0.05$) after subsequent feeding with the non-supplemented feed.

Unlike Robertson *et al.* (2000) and Nikoskelainen *et al.* (2003), culturable cells of *V. midae* SY9 (3.1×10^2 cfu g^{-1} of tissue) were present in the intestine, but not in the crop/stomach, of *H. midae* two weeks after cessation of feeding with the probiotic-supplemented feed, suggesting that *V. midae* SY9 is able to effectively colonize the intestine of the abalone. Harris *et al.* (1998a) stated that bacteria occur throughout the digestive tract of aquatic invertebrates, but in bivalves the hindgut is the most heavily colonized region. Indeed, Erasmus *et al.* (1997) showed that both the number and diversity of bacteria in the digestive tract of *H. midae* is greatest in the intestine compared to other regions of the digestive tract. The accumulation of bacteria in the hindgut of both bivalves and abalone has been suggested to be due to the extended passage time of food through this region (Harris *et al.*, 1998a). In fact, studies conducted on *Haliotis rubra* indicate that faeces are produced up to seven days after cessation of feeding, indicating that there is ample opportunity for microbial colonization. In addition, the intestine of abalone has numerous folds and grooves that provide a large surface area for bacterial colonization (Harris *et al.*, 1998a). These findings may therefore explain why *V. midae* SY9 effectively colonizes the intestine, but not the crop/stomach, of *H. midae*. In contrast, culturable cells of *Cryptococcus* sp. SS1 (~2.0×10^1 cfu g^{-1}) and *D. hansenii* AY1 (~5.0×10^2 cfu g^{-1}) were present in the crop/stomach and intestine of *H. midae* two weeks after cessation of feeding with the probiotic-supplemented feed, suggesting that both yeast strains can effectively colonize the crop/stomach and intestine of the abalone. Erasmus (1996) observed large coccoid bodies in the stomach of *H. midae* and suggested that they were yeast cells. Indeed, Andlid (1995) identified similar shaped bodies in the gut of fish, which were later identified as yeast cells. The ability of the two yeast strains, originally isolated from *H. midae*, to colonize the crop/stomach of *H. midae* suggests that the coccoid bodies observed by Erasmus (1996) were indeed yeast cells. To my knowledge, this

is also the first study to report on the colonization of yeast cells in the digestive tract of a marine invertebrate.

The use of a Tn10-based transposon for mutagenesis of the marine bacterium *V. midae* SY9 proved to be successful, confirming the results obtained by Stretton *et al.* (1998). In that study, the authors tested both Tn10 and Tn5-based transposons with a number of marine bacteria and found that only the Tn10-based transposons worked. In fact, attempts to chromosomally tag *V. midae* SY9 with a *luxAB-tet* gene by mutagenesis with a mini-Tn5 transposon (Ferguson *et al.*, 1995) failed (data not shown). Stretton *et al.* (1998) found that the mini-Tn10-*gfp-kan* transposon, delivered from pLOFKm*gfp*, transposed at a high frequency (2.4×10^5 cfu ml^{-1}), but only a small percentage (9%) of the transconjugants had strongly expressed promoter-*gfp* fusions. Similarly, *V. midae* SY9 transconjugants grew on selective plates at a concentration of 5.0×10^3 cfu ml^{-1} of conjugation mix and only 6% of the transconjugants appeared to have strongly expressed promoter-*gfp* fusions. Furthermore, the mini-Tn10-*gfp-kan* transposon gave single random chromosomal insertions, confirming data obtained by Albertson *et al.* (1996). However, unlike Stretton *et al.* (1998), the relative fluorescence displayed by transconjugant colonies in this study was insufficient for *in situ* detection in the abalone digestive tract. Cormack *et al.* (1996) (reviewed in Matthysse *et al.*, 1996) found that poor GFP-fluorescence in bacteria was caused by GFP folding incorrectly and precipitating in cells. Subsequently, these authors mutated *gfp* so that the resultant mutant protein folded correctly and remained soluble in cells. However, since the mini-Tn10-*gfp-kan* transposon contains the mutated form of the *gfp* gene, the reason for the poor fluorescence obtained in this study is unknown. It is possible that the promoter, to which the *gfp* gene is fused, is weakly expressed and/or only expressed under certain growth conditions. Indeed, Stretton *et al.* (1998) found that their transconjugant colonies displayed poor fluorescence when grown in enriched media, as apposed to minimal media, and suggested that this could be due to the growth medium.

Indirect methods for quantification of microorganisms in complex environments have become increasingly popular as evidence has accumulated suggesting that a significant fraction of microorganisms are not culturable (Li *et al.*, 1996; Sghir *et al.*, 2000). Many of these methods employ radioactive or non-radioactive probes, targeting specific genes, for the visualization of

microbial cells (Edgcomb *et al.*, 1999; Harmsen *et al.*, 1999; Li *et al.*, 1996; Peccia *et al.*, 2000; Sghir *et al.*, 2000). We have clearly shown that an oligonucleotide probe directed against the *gfp-kan* gene can detect *V. midae* SY9.8 in the digestive tract of *H. midae*. Furthermore, direct comparison of viable cell-counts and *in situ* hybridization using the *gfp-kan* probe, showed that the plate counts of *V. midae* SY9.8 were lower in the crop/stomach and intestine of *H. midae* from days 2 to 15 after cessation of feeding with the probiotic-supplemented feed compared to estimates obtained from *in situ* hybridization. Similar results were reported by Harmsen *et al.* (1999) when comparing viable cell-counts and FISH using rRNA based probes for the quantification of human faecal bacteria. In their study, the authors suggested that most *Lactobacilli* present in the samples were non-viable cells which could only be detected using the FISH method. Therefore, it is possible that a small percentage of *V. midae* SY9.8 cells in the digestive tract of *H. midae* are non-viable cells that are only detected using the *in situ* hybridization method, resulting in the lower estimate of *V. midae* SY9.8 cell numbers using the cell count technique.

Both the viable cell-count and *in situ* hybridization technique clearly showed that there was a significant decrease ($P<0.05$) in the number of probiotic cells in the crop/stomach and intestine of abalone two days after cessation of feeding with the probiotic-supplemented feed. Furthermore, a correlation analysis suggested a positive association between *Cryptococcus* sp. SS1 and amylase activity and *V. midae* SY9.8 and protease activity in the intestine, but not the crop/stomach, of *H. midae*. This data suggests that although the probiotic strains are able to colonize the digestive tract of *H. midae*, they have to be present at a concentration of approximately 10^6 cfu g^{-1} of gut tissue in order to make a significant contribution to the pool of digestive enzymes within the digestive tract of *H. midae*. As one of the proposed functions of the probiotics tested in this study is to contribute towards the pool of digestive enzymes, we suggest that the probiotic containing feed be given to farmed abalone at least every second day to retain the probiotic microorganisms at an effective concentration.

CHAPTER 6

GENERAL DISCUSSION

The successful continuation of *Haliotis midae* mariculture in South Africa depends in part on the development of methods for improving the slow growth rate and disease resistance of farmed *H. midae*. Due to an overwhelming body of evidence suggesting that probiotic microorganisms can significantly improve the growth rate and disease resistance of aquacultured animals, the primary aim of this study was to isolate and identify enteric microorganisms from *H. midae* with probiotic properties. As outlined in the introduction, several possible modes of action of aquatic probiotics exist. Since abalone farmers in South Africa are progressively utilizing formulated feeds that are high in protein and starch, our aim was to isolate and identify putative probionts capable of hydrolyzing the various protein and starch substrates included in these feeds. Upon identification, the ability of the microbes to fulfill the various selection criteria, proposed by Verschuere *et al.* (2000) and Gomez-Gil *et al.* (2000), for selecting aquaculture probiotics would be investigated. In particular, the selected microbes would be tested for their ability to colonize the digestive tract, improve digestion, growth and immunity of farmed *H. midae*.

A number of different microorganisms were isolated from the digestive tract of *H. midae* that are capable of utilizing the protein and starch substrates included in formulated feeds. A comparative enzyme analysis identified two isolates with enhanced enzyme activities. One isolate (isolate SY9) exhibited enhanced proteolytic activity on all of the protein substrates tested and a second isolate (isolate SS1) exhibited enhanced amylase activity on the starch substrates tested. Based on these findings, these two isolates were selected for further analysis. In addition, a second yeast (isolate AY1), previously isolated from *H. midae* in our laboratory, was included in this study as it has been shown that yeasts and yeast extracts have immunostimulatory properties which can confer resistance against a wide range of pathogens (Miles *et al.*, 2001; Suphantharika *et al.*, 2003).

The 16S rRNA gene sequence, together with the physical and the phenotypic characteristics of isolate SY9, suggested that it is a new species of the genus *Vibrio*. Hence, isolate SY9 was designated the species name *midae*, after the abalone from which it was isolated, *H. midae*. However, this is a tentative assignment since the DNA-DNA hybridization data is still outstanding. The 18S rRNA gene sequence, together with the physical and the phenotypic characteristics of isolates SS1 and AY1, suggested that isolate SS1 is a member of the genus *Cryptococcus* and isolate AY1 is a member of the genus *Debaryomyces*. Furthermore, our results suggested that isolate AY1 has significant similarity to other *Debaryomyces hansenii* strains. However, due to the absence of DNA-DNA hybridization data and a lack of adequate phenotypic data published in the literature on these two strains, a more detailed comparison of isolates SS1 and AY1 to other members of the *Cryptococcus* and *Debaryomyces* genera respectively was not possible. Therefore, yeast isolate SS1 was tentatively designated *Cryptococcus* sp. SS1 and yeast isolate AY1 was tentatively designated *Debaryomyces hansenii* AY1.

In an endeavor to investigate the effect of *Vibrio midae* SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1 on the growth and nutrition of farmed *H. midae*, the three putative probionts were successfully incorporated into a local commercial abalone feed. When the probiotic-supplemented feed was fed to abalone in farm-based growth trials, we clearly showed that the growth rate of small (20 mm) and large (67 mm) abalone was significantly improved ($P<0.05$) compared to abalone not fed probiotics. *In situ* protease and amylase assays showed that probiotic treatment significantly increased ($P<0.05$) both protease and amylase activity in the intestinal region of the digestive tract of abalone fed the probiotic-supplemented feed. This correlated with a significant increase ($P<0.05$) in the amount of protein digestion and absorption measured in this region of the abalone gut.

To further investigate the ability of the three probiotic strains to fulfill the selection criteria of aquaculture probiotics, the effect of the three probiotic strains on the health and disease resistance of *H. midae* was investigated. Data collected in this study clearly showed that probiotic administration stimulated the immune system of *H. midae*. Furthermore, enhancement of the immune response in *H. midae* resulted in increased survival following challenge with the pathogenic bacterium, *Vibrio anguillarum*. Improved health of abalone through probiotic

administration was confirmed following histological examination of abalone included in the growth trials. The digestive glands of abalone fed probiotics were found to be bacteria-free, whereas the digestive glands of abalone receiving the non-supplemented feed had a high bacterial load. The absence of bacteria in the digestive glands of healthy *H. midae* has been confirmed by Erasmus (1996).

To address the final aim of this study, viable cell counts and/or *in situ* hybridization was used to monitor the fate of each probiotic strain in the digestive tract of *H. midae* after cessation of feeding with the probiotic-supplemented feed. We showed that all three probiotic strains colonise the digestive tract of *H.* midae, but there is a significant reduction in the number of probiotic cells two days after cessation of feeding with the probiotic-supplemented feed. This correlated with a significant decrease ($P<0.05$) in the *in situ* protease and amylase activity in the digestive tract of abalone previously fed probiotics.

A number of conclusions can be drawn from the results of this study. Firstly, few studies have investigated or reported the use of enteric microorganisms as probiotics for abalone fed an artificial feed. Thus, this is the first study to report on the use of a probiotic-supplemented artificial feed for enhanced growth and disease resistance in farmed *H. midae*. Further studies should be conducted to determine whether the probiotics tested on *H. midae* in this study are applicable to other Haliotid species fed high protein diets. In addition, the results from this study support the initial hypothesis made by Erasmus (1996), which states that 'inoculation of a microorganism(s) with enhanced enzyme activity into *H. midae* could lead to an improvement in the growth rate of this species'. In addition, this study confirms that all three probiotic strains isolated from the digestive tract of *H. midae* fulfil the selection criteria proposed by Verschuere *et al.* (2000) and Gomez-Gil *et al.* (2000) for aquaculture probiotics.

The farm trials conducted in this study proved that inclusion of the probiotics in commercial abalone feed enhances the growth rate of farmed abalone compared to abalone not fed probiotics. From data obtained in the *in situ* protease and amylase assays and protein digestibility analysis, we conclude that the observed enhancement in growth rate is due to the increased intestinal protease and amylase activity generated by the exogenous protease and amylase enzymes

secreted by *V. midae* SY9 and *Cryptococcus* sp. SS1 respectively. We propose that the exogenous enzymes secreted by these two strains enhance the digestion of complex protein and/or starch substrates in the basal diet, thus increasing the rate at which they can be assimilated and absorbed by the host animal. In fact, increased digestion and absorption of protein was observed in the intestinal region of abalone fed probiotics. A similar assessment of starch digestion and absorption in the digestive tract of *H. midae* would help to substantiate this theory. Furthermore, we showed that there was a positive correlation between intestinal protease activity and *V. midae* SY9 cell numbers and intestinal amylase activity and *Cryptococcus* sp. SS1 cells numbers in the digestive tract of *H. midae*, suggesting an association between *V. midae* SY9 and protease activity and *Cryptococcus* sp SS1 and amylase activity in the intestine of *H. midae*, lending further support to the above theory. Finally, we showed that there was a significant reduction in the number of probiotic cells two days after cessation of feeding with the probiotic-supplemented feed. This correlated with a significant decrease in the *in situ* protease and amylase activity in *H. midae*. We therefore propose that the probiotic cells need to be present at a concentration at or above 10^5 cfu g^{-1} of gut in order to make a significant contribution towards digestion of protein and starch in *H. midae*.

Aside from the ability of the probiotic strains to enhance the growth rate of farmed *H. midae*, this study clearly showed that probiotic administration enhances the immune response in *H. midae* as well, resulting in increased survival following infection. We propose that the probiotic strains act as immunogens for abalone immune defense, priming the immune system of *H. midae* so that it may respond more rapidly and aggressively upon infection. This is clearly demonstrated by the enhanced phagocytic rate of the circulating haemocytes from abalone fed probiotics. Furthermore, the fact that the total number, respiratory burst and phagocytic rate of the circulating haemocytes from abalone fed probiotics do not significantly decrease following infection, suggests that the circulating haemocytes have an enhanced capacity to effectively eliminate pathogenic bacteria, thus preventing the further spread of infection, resulting in enhanced survival.

The digestive glands of abalone fed the probiotic-supplemented feed were also shown to be free of bacteria compared to abalone not fed probiotics. As discussed in Chapter 4, stressed abalone

often suffer from a condition called "bloat", caused by the proliferation of bacteria in the gut, which leads to contamination of the digestive gland and possible mortality of the animals. Since the probiotics are able to colonize the gut of *H. midae*, we propose that the probiotic strains help prevent the proliferation of heterotrophic or opportunistic bacteria within the gut of probiotic-fed animals and consequently, these animals have an increased stress tolerance. However, as we have not demonstrated competitive exclusion of bacteria by the probiotics tested in this study, further studies would need to be conducted to substantiate this theory.

Although a number of very important questions were answered in this study, a few questions still remain unanswered and require further investigation. Aside from the suggested topics for further study mentioned above, growth and immunology experiments conducted on abalone fed each probiotic individually would yield valuable information on the exact mode of action of each probiotic. A genetic analysis of immunostimulated abalone could also help determine the effect of probiotic administration on the abalone immune system, increasing our understanding of exactly how probiotic cells enhance the immune response in *H. midae*. This sort of study would also generate valuable and much needed information on the innate immune response of abalone. Furthermore, the effect of probiotics on the growth and survival of abalone larvae, the most susceptible stage of the abalone life-cycle, would yield some interesting and valuable results. Indeed, many of these questions are currently being investigated in our laboratory.

Overall, we were successful in achieving the primary aims of this study. The improved growth rate and disease resistance of farmed abalone fed probiotics would translate directly to an increased turnover rate of abalone on farms, and hence, increased profits for the abalone farmer. Indeed, further development of this concept could help put the South African abalone mariculture industry on the world map as leaders in the production of high quality, fast growing abalone.

APPENDIX A

MEDIA AND SOLUTIONS

CONTENTS

All media were autoclaved at 121 °C for 20 min prior to use, unless otherwise stated.

The water used for making solutions, media and diluting buffers was purified using a Milli-RO Plus (Millipore) water purification system.

A.1 Media

A.1.1 Marine Broth (MB)

NaCl (Saarchem)	30.0 g
$MgCl_2.6H_2O$ (Saarchem)	2.3 g
KCl (Saarchem)	0.3 g
Casamino Acids (Difco)	5.0 g
Yeast Extract (Biolab)	1.0 g
D-glucose (Saarchem)	2.0 g
dH_2O to	1 L

The pH of the media was adjusted to 7.0 with 1 M NaOH.

A.1.2 Marine Agar (MA)

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
Casamino Acids	5.0 g
Yeast extract	1.0 g
D-glucose	2.0 g
Agar (Biolab)	20.0 g
dH_2O to	1 L

The pH of the media was adjusted to 7.0 with 1 M NaOH prior to adding the agar and autoclaving.

A.1.3 Yeast Peptone D-glucose (YPD) broth

Yeast extract	10.0 g
D-glucose	20.0 g
Peptone (Difco)	20.0 g
dH_20 to	1 L

The pH of the media was adjusted to 7.0 with 1 M NaOH.

A.1.4 Yeast Peptone D-glucose (YPD) agar

Yeast extract	10.0 g
D-glucose	20.0 g
Peptone	20.0 g
Agar	20.0 g
dH_20 to	1 L

The pH of the media was adjusted to 7.0 with 1 M NaOH prior to adding the agar and autoclaving.

A.1.5 Alginate Broth

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
Sodium alginate	5.0 g
Peptone	2.5 g
Yeast extract	1.0 g
dH_2O to	1 L

A.1.6 Luria Broth (LB) 10 medium

NaCl	10 g
Tryptone (Biolab)	10 g
Yeast Extract	5 g
dH_2O to	1 L

The pH of the media was adjusted to 7.0 with 1 M NaOH.

A.1.7 Luria Agar (LA) 10 medium

NaCl	10 g
Tryptone	10 g
Yeast Extract	5 g
Agar	15 g
dH_2O to	1 L

The pH of the media was adjusted to 7.0 with 1 M NaOH prior to adding the agar and autoclaving.

A.1.8 Luria Broth (LB) 15 medium

NaCl	15 g
Tryptone	10 g
Yeast Extract	5 g

dH_2O to	1 L

The pH of the media was adjusted to 7.0 with 1 M NaOH.

A.1.9 Luria Agar (LA) 15 medium

NaCl	10 g
Tryptone	10 g
Yeast Extract	5 g
Agar	15 g
dH_2O to	1 L

The pH of the media was adjusted to 7.0 with 1 M NaOH prior to adding the agar and autoclaving.

A.1.10 VNSS Broth

NaCl	17.60 g
Na_2SO_4 (Saarchem)	1.47 g
$NaHCO_3$ (Saarchem)	0.08 g
KCl (Saarchem)	0.25 g
KBr (Saarchem)	0.04 g
$MgCl_2.6H_2O$	1.87 g
$CaCl_2.2H_2O$ (Saarchem)	0.41 g
$SrCl_2.6H_2O$ (Saarchem)	0.008 g
H_3BO_3 (Saarchem)	0.008 g
Peptone	1.0 g
Yeast Extract	0.5 g
D-glucose	0.5 g
$FeSO_4.7H_2O$ (Saarchem)	0.01 g
Na_2HPO_4 (Saarchem)	0.01 g
dH_2O to	1 L

Adjust the pH of the medium to 7.0 with 1 M NaOH.

A.1.11 VNSS Agar

NaCl	17.60 g
Na_2SO_4 (Saarchem)	1.47 g
$NaHCO_3$ (Saarchem)	0.08 g
KCl (Saarchem)	0.25 g
KBr (Saarchem)	0.04 g
$MgCl_2.6H_2O$	1.87 g
$CaCl_2.2H_2O$ (Saarchem)	0.41 g
$SrCl_2.6H_2O$ (Saarchem)	0.008 g

H_3BO_3 (Saarchem)	0.008 g
Peptone	1.0 g
Yeast Extract	0.5 g
D-glucose	0.5 g
$FeSO_4.7H_2O$ (Saarchem)	0.01 g
Na_2HPO_4 (Saarchem)	0.01 g
Agar	15.0 g
dH_2O to	1 L

The pH of the media was adjusted to 7.0 with 1 M NaOH prior to adding the agar and autoclaving.

A.2 Solutions

A.2.1 Antibiotic solutions in growth media

- Ampicillin (Sigma) (100 mg/ml)

  Dissolve 2 g in 20 ml of dH_2O. Filter sterilize and store aliquots at 4 °C.
  Dilute 1:1000 into medium for a final concentration of 100 µg/ml.

- Kanamycin (Sigma) (100 mg/ml)

  Dissolve 2 g in 20 ml of dH_2O. Filter sterilize and store aliquots at 4 °C.
  Dilute 1:250 into medium for a final concentration of 400 µg/ml and 1:1176 into medium for a final concentration of 85 µg/ml.

- Streptomycin (Sigma) (100 mg/ml)

  Dissolve 2 g in 20 ml of dH_2O. Filter sterilize and store aliquots at 4 °C.
  Dilute 1:1000 into medium for a final concentration of 100 µg/ml.

- Chloramphenicol (Sigma) (100 mg/ml)

  Dissolve 2 g in 20 ml of dH_2O. Filter sterilize and store aliquots at 4 °C.
  Dilute 1:400 into medium for a final concentration of 250 µg/ml.

A.2.2 Artificial Sea Water (ASW)

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
dH_2O to	1 L

A.2.3 Nine Salts Solution (NSS)

NaCl	17.60 g
Na_2SO_4	1.47 g
$NaHCO_3$	0.08 g
KCl	0.25 g
KBr	0.04 g
$MgCl_2.6H_2O$	1.87 g
$CaCl_2.2H_2O$	0.41 g
$SrCl_2.6H_2O$	0.008 g
H_3BO_3	0.008 g
dH_2O to	1 L

Adjust the pH of the medium to 7.0 with 1 M NaOH.

A.2.4 General stock solutions

- 0.5 M EDTA

EDTA (Saarchem)	93.05 g
NaOH (Saarchem)	10 g
dH_2O to	500 ml

Dissolve the EDTA and NaOH in 400 ml of dH_2O, adjust the pH to 8.0 and make up to a final volume of 500 ml.

- 1 M Tris base

Tris (Roche)	12.1 g
dH_2O to	100 ml

- 1 M Tris-HCl

Tris	12.1 g
dH_2O to	100 ml

Dissolve the Tris in 80 ml of dH_2O and adjust the pH to the required level with concentrated HCl. Finally, make the volume up to a final volume of 100 ml.

- 10 M NaOH

NaOH	40 g
dH_2O to	100 ml

- 1 M $CaCl_2$

$CaCl_2.2H_2O$	14.7 g
dH_2O to	100 ml

- 10 mM $MgSO_4$

$MgSO_4$	0.12 g
dH_2O to	100 ml

- TE buffer (Tris-EDTA)

1 M Tris-HCl (pH 7.6)	1 ml
0.5 M EDTA	200 µl
dH_2O to	100 ml

- EtBr 10 mg/ml (Ethidium Bromide)

EtBr (Sigma)	0.1 g
dH_2O to	10 ml

Shake well to dissolve and do not autoclave. Caution, this is a powerful mutagen and gloves should be worn at all times when handling the solution.

- 70% EtOH

absolute EtOH (Merck)	70 ml
dH_2O to	100 ml

Do not autoclave. Store at -20 °C

- 10 x Phosphate Buffered Saline (PBS)

NaCl	80 g
KCl	2 g
Na_2PO_4 (Merck)	14.4 g
KH_2PO_4 (Saarchem)	2.4 g
dH_2O to	1 L

Dissolve NaCl, KCl, Na_2PO_4 and KH_2PO_4 in 900 ml of dH_2O. adjust the pH to 7.4 and make up to 1 L with dH_2O.

- 1 x PBS (dilute 10 x PBS 1:10 with dH_2O)

A.2.5 Solutions for Chromosomal DNA extractions

- 10% SDS (Sodium dodecyl sulphate)

SDS (Saarchem)	10 g
dH_2O to	100 ml

Stir on a warm plate to approximately 80 °C to dissolve. Do not overheat and do not autoclave.

- Proteinase K (20 mg/ml)

Proteinase K (Sigma)	20 mg
Sterile dH_2O to	1 ml

Do not autoclave. Store aliquots at -20 °C.

- 5 M NaCl

NaCl	29.22 g
dH_2O to	100 ml

- CTAB/NaCl

NaCl	4.1 g
CTAB (USB)	10.0 g
dH_2O to	100 ml

Dissolve the NaCl in 80 ml of dH_2O and slowly add the CTAB (cetyltrimethylammonium bromide) while heating and stirring. If necessary heat to 65 °C to dissolve. Adjust the final volume to 100 ml with dH_2O. Do not autoclave.

- Chloroform (Merck) / isoamyl alcohol (Merck)
  Mix at a ratio of 24:1

- RNase A (10 mg/ml)

RNase A (Sigma)	0.1 g
1 M Tris-HCl (pH 7.5)	100 µl
5 M NaCl	3 ml
dH_2O to	10 ml

Heat for 15 min at 100 °C before allowing to cool slowly to room temperature. Do not autoclave. Aliquot into eppendorf tubes at store at -20 °C.

A.2.6 Solution for Agarose gel electrophoresis

- 50 X TAE (Tris-acetate buffer)

Tris	242 g
Glacial acetic acid (Saarchem)	57.1 ml
0.5 M EDTA	100 ml
dH_2O to	1 L

A.2.7 Solution for electron microscopy

- 2% Uranyl acetate (pH 5.0) solution

Uranyl acetate (Sigma)	5 g
100% Methanol (Saarchem)	25 ml

Filter sterilize before use. Store in the dark at 4 °C.

A.2.8 Solutions for Azocasein assay for protease

- Azocasein solution

Azocasein (Sigma)	0.05 g
1 M Tris-HCl (pH 9.0)	1 ml
1 M NaCl	4 ml
2 mM $CaCl_2$	20 µl
dH_2O to	10 ml

  Do not autoclave. Store at 4 °C.

- 10% TCA (Trichloroacetic Acid)

TCA (Sigma)	10 ml
dH_2O to	100 ml

  Do not autoclave. Store at 4 °C.

- 0.5 M NaOH

10 M NaOH	10 ml
Sterile dH_2O to	100 ml

A.2.9 Solutions for Dinitrosalicyclic Acid Assay (DNS) for reducing sugars

- 0.5% Starch

Starch	0.5 g
Sterile dH_2O to	100 ml

  Prepare fresh on day of use. Do not autoclave

- DNS reagent

3,5-Dinitrosalicyclic acid (Sigma)	2.65 g
NaOH	4.95 g
NaK-tartrate (Merck)	76.5 g
Phenol (Merck)	1.9 g
Na-metabisulphate (Sigma)	2.075 g
Sterile dH_2O to	354 ml

  Dissolve the 3,5-Dinitrosalicyclic acid, NaOH and NaK-tartrate in dH_2O before adding the other constituents (dissolving them in turn). The phenol is melted at 50 °C. A 3 ml aliquot of the solution must be titrated to an end point with 5-6 ml of 0.1 M HCl using phenolphthalein as an endpoint indicator. If the pH is correct, the pinkish color will be lost after adding 5-6 ml of 0.1 M HCl. If less HCl is required, then solid NaOH must be added to the bulk DNS solution at a rate of 2 mg NaOH for every 1 ml HCl less than the 5 ml limit of titration. The DNS must be stored in a dark bottle under N_2.

A.2.10 Solution for restriction enzyme digestions

- Gel tracking dye

Bromophenol blue	62.5 g
Sucrose (Saarchem)	10.0 g
0.5 M EDTA	1 ml
dH_2O to	25 ml

Do not autoclave.

A.2.11 Solutions for Dot blot and Southern hybridization analysis

- 0.25 M HCl

HCl (Saarchem)	21.35 ml
dH_2O to	1 L

Do not autoclave.

- 1 M NaOH

NaOH	4 g
dH_2O	100 ml

- 0.4 M NaOH

NaOH	16 g
dH_2O	1 L

- 20 x SSC (Sodium chloride tri-sodium citrate)

NaCl	17.50 g
Tri-Na Citrate (Saarchem)	8.82 g
dH_2O to	100 ml

Dissolve NaCl and Tri-Na Citrate in 80 ml of dH_2O, adjust the pH to 7.4 with NaOH and finally make the solution up to 100 ml with dH_2O.

- STE (Sodium chloride-Tris EDTA)

0.1 M NaCl	2.92 g
TE buffer	500 ml

- Sephadex G-50

Sephadex G-50 (Pharmacia)	30 g
TE buffer	250 ml

- Tracking dye

Dextran Blue 2000 (Pharmacia)	0.3 g
NaCl	0.029 g
Orange G (BDH)	0.1 g
dH_2O to	10 ml

- PB stock solution (1 M Na_2HPO_4, pH 7.2)

$Na_2HPO_4.7H_2O$ (Saarchem)	134 g
85% H_3PO_4 (Saarchem)	4 ml
dH_2O to	1 L

- 25% SDS (Sodium dodecyl sulphate)

SDS	250 g
dH_2O to	1 L

Stir on a warm plate to dissolve. Do not overheat and do not autoclave.

- Church pre-Hybridization Buffer (pre CHB)

Non-fat dry milk (elite)	0.5 g
PB stock solution	50 ml
0.5 M EDTA	0.2 ml
25% SDS	28 ml
dH_2O to	100 ml

- Church Hybridization Buffer (CHB)

PB stock solution	50 ml
0.5 M EDTA	0.2 ml
25% SDS	28 ml
dH_2O to	100 ml

- Wash Buffer A (WBA)

PB stock solution	20 ml
0.5 M EDTA	1 ml
25% SDS	100 ml
dH_2O to	500 ml

- Wash Buffer B (WBB)

PB stock solution	40 ml
0.5 M EDTA	2 ml
25% SDS	40 ml
dH_2O to	1 L

A.2.12 Solution for Histology

- Davidson's solution

95% Ethyl alcohol (Saarchem)	330 ml
100% Formalin (Saarchem)	220 ml
Glacial Acetic acid	115 ml
dH_2O to	1 L

Do not autoclave.

A.2.13 Solution for fixing blood cells

- Alsevers solution (pH 7.5)

D-glucose	20.8 g
Sodium Citrate (Saarchem)	8 g
EDTA	3.36 g
NaCl	22.4 g
40% Formalin solution	300 ml
dH_2O to	1 L

A.2.14 Solutions for Phagocytosis assays

- Modified Hank's Balance Salt Solution (MHBSS, pH 7.2)

D-glucose	10.4 g
NaCl	11.2 g
KCl	0.41 g
KH_2PO_4	0.1 g
$CaCl_2$	0.355 g
$MgCl_2$	1.31 g
$MgSO_4$	1.5725 g
EGTA (Sigma)	0.015 g
dH_2O to	500 ml

- 0.1 M $NaHCO_3$ solution (pH 9.0)

$NaHCO_3$ (Saarchem)	0.84 g
dH_2O to	100 ml

- 50% Acetic acid

100% Glacial acetic acid	50 ml
dH_2O to	50 ml

Do not autoclave.

- 1 x PBS (dilute 10 x PBS 1:10 with dH_2O)

- EtBr 10 mg/ml in PBS

EtBr (Sigma)	0.1 g
1 x PBS	10 ml

Shake well to dissolve and do not autoclave. Caution, this is a powerful mutagen and gloves should be worn at all times when handling the solution.

A.2.15 Solutions for the Nitroblue Tetrazolium (NBT) reduction assay

- Tris-HCl buffer containing 2% NaCl (pH 7.6)

Tris	12.1 g
dH_2O to	100 ml

Dissolve the Tris in 80 ml of dH_2O and adjust the pH to the required level with concentrated HCl. Finally, make the volume up to a final volume of 100 ml. Add 2 g solid NaCl prior to autoclaving.

- 50% Methanol

100% Methanol (Merck)	50 ml
dH_2O to	100 ml

Do not autoclave

- 2 M KOH

KOH	11.2 g
dH_2O to	100 ml

A.2.16 Solutions for chromic oxide determinations

- Perchloride reagent

Concentrated HNO_3	200 ml
70% $HClO_4$	200 ml
dH_2O	100 ml

Slowly add the concentrated nitric acid (HNO_3) to the dH_2O. Allow the solution to cool before adding the perchloride ($HClO_4$) reagent. The reagent can be stored in a glass stoppered bottle for 4 to 6 weeks.

APPENDIX B

STANDARD METHODS

CONTENTS

B.1 Large scale preparation of genomic DNA

(Ausubel *et al.*, 1989 unit 2.4)

Grow a 100 ml culture of the strain to saturation. Pellet the cells at 6000 rpm for 10 min and discard the supernatant. Resuspend the cells in 9.5 ml TE buffer (Appendix A.2.4). Add 0.5 ml of 10% SDS and 50 µl of 20 mg/ml proteinase K (Appendix A.2.5), mix and incubate for 1 h at 37 °C. Following incubation, add 1.8 ml of 5 M NaCl (Appendix A.2.5) and mix thoroughly before adding 1.5 ml of CTAB (Appendix A.2.5) solution. Mix and incubate for 20 min at 65 °C. Extract with an equal volume of chloroform/isoamyl alcohol (Appendix A.2.5). Centrifuge at 7000 rpm for 10 min at room temperature to separate the phases. Transfer the upper aqueous phase to a clean tube. Precipitate the DNA by adding 0.6 volumes isopropanol (Saarchem) and mix gently, by inverting the tube several times, until a stringy white DNA pellet precipitates out of the solution. Centrifuge at 15 000 rpm for 15 min at 4 °C. Carefully discard the supernatant and wash the pellet with 70% EtOH (Appendix A.2.4). Centrifuge at 9 000 rpm for 5 min. Decant the supernatant and air-dry the DNA pellet for a few minutes. Resuspend DNA in 1 ml TE buffer with 10 µl RNase A (Appendix A.2.5). Measure the DNA concentration on a spectrophotometer.

B.2 Restriction endonuclease digestions

(Ausubel *et al.*, 1989 unit 3.1)

All restriction enzymes and their respective buffers were obtained from Amersham and Roche. Carefully pipette 0.5 to 10 µg of either plasmid or chromosomal DNA into a clean eppendorf tube. Add 2 µl of the appropriate restriction enzyme buffer. Adjust the volume to 18 µl with sterile dH_2O. Add restriction enzyme nuclease (1 to 5 U μg^{-1} DNA) to a final volume of 20 µl. Pulse tube briefly in a bench-top centrifuge and incubate the reaction mixture for 2 hrs at 37 °C. For restriction enzyme digestion of chromosomal DNA, incubate for at least 4 hrs at 37 °C. Stop the reaction by adding 4 µl gel tracking dye (Appendix A.2.10).

B.3 Agarose gel electrophoresis

(Ausubel *et al.*, 1989 unit 2.5)

Melt agarose (Hispanagar D1 LE) in 1x TAE (Appendix A.2.6) by heating in a microwave. Agarose concentrations can vary from 1% for separating small DNA fragments to 0.8% for separating larger DNA fragments, such as restriction enzyme digested chromosomal DNA. Add Ethidium bromide (Appendix A.2.4) solution to the melted agarose to a final concentration of 0.5 $\mu g\ ml^{-1}$. Allow agarose to cool to approximately 55 °C before pouring into a gel-casting platform. Seal the ends of the gel-casting platform with masking tape if open. Pour the melted agarose and insert the gel comb approximately 1 cm from the top of the gel-casting platform, making sure that no air bubbles are trapped underneath the comb. After the gel has hardened, remove the tape from the gel-casting platform and withdraw the comb. Place the gel-casting platform containing the set gel in an electrophoresis tank. Add sufficient 1x TAE buffer to cover the gel. Load DNA samples into the wells of the gel. Attach leads so that DNA migrates into the gel towards the anode. Run gel at 1 to 10 $V\ cm^{-1}$ until the dye in the loading buffer reaches the end of the gel.

B.4 PCR of the 16S rRNA fragment from *V. midae* SY9 and *V. midae* SY9.8

B.4.1 Primers used for PCR amplification

(Schroeder *et al.*, 2001)

F1 5' CGC CAG GGT TTT CCC AGT CAC GAC AGA GTT TGA TCI TGG CTC AG 3'

F3 5' CGC CAG GGT TTT CCC AGT CAC GAC GCC AGC AGC CGC GGT AAT AC 3'

F5 5' CGC CAG GGT TTT CCC AGT CAC GAC GCA TGG ITG TCG TCA GCT CGT 3'

R1 5' CAG GAA ACA GCT ATG ACG TAT TAC CGC GGC TGC TGG CAC 3'

R3 5' CAG GAA ACA GCT ATG ACC ACG AGC TGA CGA CAI CCA TG 3'

R5 5' CAG GAA ACA GCT ATG ACA CGG ITA CCT TGT TAC GAC TT 3'

I = A, C, G or T

The underlined sequence represents sequence complementary to the highly conserved regions of the 16S rRNA gene.

B.4.2 PCR protocol

The PCR master mix for each bacterial strain was prepared as follows:

Reagents (Roche)	Volume (µl)
Genomic DNA (50 ng μl^{-1})	12
Mg (25 mM)	12
PCR buffer (10x)	30
Taq Polymerase (5 U μl^{-1})	3
dNTP's (25mM)	3
Sterile dH_2O	210
TOTAL	270

Aliquots (45 µl) of the master mix were added into 5 separate PCR eppendorf tubes. The following primer pairs (5 µl of each primer) were separately added to each of the 5 PCR tubes:

Tube number	Primer (10 µM)
1	F1 and R5
2	F1 and R3
3	F1 and R1
4	F3 and R3
5	F3 and R5

The PCR cycle profile was setup as follows:

Temperature (°C)	Time (s)	Cycles
96	120	1
96	45	
55	30	30
72	90	
72	180	1

PCR amplification was achieved using the Hybaid thermal cycler equipped with a heated lid. The PCR products were purified with a High pure PCR product purification kit (Roche).

B.5 PCR of the 18S rRNA fragment from *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1

B.5.1 Primers used for PCR amplification

(White *et al.*, 1990 (reviewed in Li *et al.*, 1996))

NS1 5' GTA GTC ATA TGC TTG TCT 3'
NS3 5' GCA AGT CTG GTG CCA GCA 3'
NS8 5' TCC GCA GGT TCA CCT ACG GA 3'

B.5.2 PCR protocol

The PCR master mix for each yeast strain was prepared as follows:

Reagents (Roche)	Volume (µl)
Genomic DNA (50 ng μl^{-1})	9
Mg (25 mM)	15
PCR buffer (10x)	15
Taq Polymerase (5 U μl^{-1})	1.5

dNTP's (25mM)	1.5
Triton X-100	1.5
Dimethyl Sulfoxide (DMSO)	15
Sterile dH_2O	85.5
TOTAL	144

Aliquots (48 µl) of the master mix were added into 2 separate PCR eppendorf tubes. The following primer pairs (1 µl of each primer) were separately added to each of the PCR tubes:

Tube number	**Primer (10 µM)**
1	NS1 and NS3
2	NS1 and NS8

The PCR cycle profile was setup as follows:

Temperature (°C)	**Time (s)**	**Cycles**
96	360	1
96	60	
55	56	35
72	120	
72	300	1

PCR amplification was achieved using the Hybaid thermal cycler equipped with a heated lid. The PCR products were purified with a High pure PCR product purification kit (Roche).

B.6 Southern hybridization procedure

(Coyne *et al.*, 1996)

B.6.1 Southern transfer of DNA from agarose gel onto nitrocellulose membrane

Soak the agarose gel in 2x volumes of 0.25 M HCl (Appendix A.2.11) with agitation for 5 min at room temperature. Rinse the gel with dH_2O to remove excess HCl. Saturate 10 sheets (25 x 20 cm) of Whatman 3MM paper with 0.4 M NaOH (Appendix A.2.11). Place the sheets on top of an inverted gel-casting tray, which has been placed in a tray covered with Saran wrap. Add 0.4 M NaOH / 1 M NaCl (Appendix A.2.11) to the tray so that the ends of the Whatman paper are submerged. Invert the gel and place it on top of the saturated Whatman paper, making sure that no air bubbles remain trapped. Cut Hybond N^+ nylon membrane (Amersham) to the size of the gel. Wet membrane in water and place on the gel, ensuring that no air bubbles remain trapped. Cover the edges of the membrane with Saran wrap. Cut 3 sheets of Whatman 3MM to the size of the membrane and place over the membrane, followed by a 10 cm stack of dry absorbent towel. Place a glass plate on top of the towels, followed by a 0.2 to 0.4 kg weight. Blot overnight. Following blotting, remove absorbent towel and Whatman paper above membrane. Gently turn the membrane and gel over and mark the wells of the gel on the membrane with a pencil. Rinse the membrane in 2x SSC (Appendix A.2.11) for 5 min at room temperature. Air-dry the membrane on Whatman paper and store between 2 sheets of Whatman 3MM paper.

B.6.2 Labeling DNA by random primed labeling

(Protocol from Roche labelling kit)

All reagents utilized in this protocol are supplied in the Random primed DNA labelling kit (Roche). Transfer 25 ng of DNA to an eppendorf tube and denature fragments by heating for 10 min at 100 °C, before cooling rapidly on ice. Add 3 µl of a dATP, dGTP and dTTP mixture (consisting of 1 µl of each dNTP) and 2 µl of reaction mixture. Add 5 µl 50 µCi [α-^{32}P] dCTP and make up the reaction mixture to 19 µl with sterile dH_2O. Add 1 µl Klenow enzyme and incubate the reaction mixture for 30 min at 37 °C. Separate the labelled DNA from the unincorporated nucleotides using the spin column procedure (Appendix B.4.3).

B.6.3 Separation of radioisotope-labeled DNA from unincorporated nucleotides using the spun column procedure

(Ausubel et al., 1989 unit 3.4)

Plug the bottom of a 1 ml disposable syringe with a small amount of sterile glass wool. Prepare a Sephadex-G50 (Appendix A.2.11) column with a bed volume of 0.9 ml in the syringe by adding Sephadex-G50 to the top of the syringe, placing the syringe in a disposable bench-top centrifuge tube and centrifuging for 1 min at 14 000 rpm. Repeat this process until the required bed volume is obtained. Add 0.1 ml STE buffer (Appendix A.2.11) to the column and spin for 1 min at 14 000 rpm to wash the column. To the labelled DNA sample, add 10 µl tracking dye (Appendix A.2.11) and 70 µl STE buffer. Place an eppendorf tube at the bottom of the disposable centrifuge tube and place the syringe containing the prepared Sephadex column inside the centrifuge tube, so that the syringe will empty inside the eppendorf tube. Load the DNA onto the centre of the column and centrifuge for 4 min at 14 000 rpm. The Blue dextran dye will move with the labeled DNA and will empty into the eppendorf tube, whereas the Orange G will remain with the unincorporated nucleotides in the column. Determine the specific activity of the labeled DNA by counting 1 µl of probe in 2 ml of scintillation fluid. Specific activity is expressed as counts per minute (cpm) per µg DNA.

B.6.4 Pre-hybridization, hybridization and washing of Southern blots

(Church and Gilbert, 1984)

Place the Hybond N^+ membrane containing the transferred DNA in a plastic bag. Add 0.2 ml of Church pre-hybridization buffer (pre CHB, Appendix A.2.11) per cm^2 of membrane, seal the bag and incubate for 30 min at 65 °C with agitation. Denature the labeled probe by heating for 10 min at 100 °C and then cool rapidly by placing directly on ice. Remove the pre CHB from the plastic bag and add 50 µl of fresh Church hybridization buffer (CHB, Appendix A.2.11) per cm^2 of membrane. Add 1.0×10^6 cpm of labeled probe per ml of CHB. Remove bubbles and seal the bag. Incubate O/N at 65 °C with agitation.

Remove the membrane from the bag and place in a tray. Wash the membrane in Wash buffer A (WBA) and then Wash buffer B (WBB) (Appendix A.2.11) at 65 °C for 10 min. Monitor the radioactivity on the membrane after each wash using a Geiger counter. If the radiation on the membrane reaches 200 to 500 cpm, stop washing. Seal the membrane in a new plastic bag and place in an X-ray cassette containing enhancer screens. Expose the membrane to X-ray film (Hyperfilm, Amersham) at -70 °C and develop manually by placing in developer for 30 s, dH_2O for 30 s, fixer for 30 s and finally rinsing in dH_20 for 30 s. Air-dry film for approximately 30 min.

B.7 Dot blot hybridization procedure

(Ausubel *et al.*, 1989 unit 2.9)

B.7.1 Transfer of DNA onto nitrocellulose membrane using dot blot apparatus

Cut Hybond N^+ membrane (8 x 11 cm) (Amersham) and soak for 1 min in dH_2O. Cut a piece of Whatman 3MM paper (8 x 11 cm) to fit the manifold of the dot blot apparatus (Bio-Rad) and wet in dH_2O. Place the Whatman 3MM paper in the manifold and lay the membrane on top of it. Assemble the manifold according to the manufacturer's instructions. Add 1 M NaOH (Appendix A.2.11) to each DNA sample to give a final concentration of 0.4 M NaOH. Denature the DNA fragments for 10 min at 100 °C and cool rapidly by placing directly on ice. Switch on the suction of the manifold devise, apply 500 µl dH_2O to each well and allow to filter through. Apply denatured DNA samples to wells, being careful to avoid touching the membrane with the pipette. Allow the samples to filter through. After applying the samples, rinse each well with 500 µl of 0.4 M NaOH (Appendix A.2.11) and dismantle the manifold. Rinse membrane briefly in 2x SSC (Appendix A.2.11). Air-dry the membrane on Whatman paper and store between two sheets of Whatman 3MM paper.

B.8 PCR of the 1.3 kb *gfp-kan* fragment from pLOFKm*gfp*

B.8.1 Primers used for PCR amplification

(Stretton *et al.*, 1998)

*gfpSfi*I-F 5' CTC CTC GGC CGC CTA GGC CGA TTT CTA GAT TTA AGA AGG 3'

Kmseq-F 5' TAC AAT CGA TAG ATT GTC GC 3'

B.8.2 PCR protocol

Prepare a master mix as follows:

Reagents (Roche)	**Volume (µl)**
pLOFKm*gfp* DNA (50 ng μl^{-1})	4
Mg (25 mM)	8
PCR buffer (10x)	8
Taq Polymerase (5 U μl^{-1})	1
dNTP's (25mM)	1
Sterile dH_2O	74
TOTAL	96

Aliquots (48 µl) of the master mix were added to 2 separate PCR eppendorf tubes. One microlitre (10 pmol) each of primer *gfpSfi*I-F and Kmseq-F was added to each tube.

PCR cycle profile was setup as follows:

Temperature (°C)	Time (s)	Cycles
96	120	1
96	30	
55	47	30
72	120	
72	300	1

PCR amplification was achieved using the Hybaid thermal cycler equipped with a heated lid. The PCR products were purified with a High pure PCR product purification kit (Roche).

LITERATURE CITED

Albertson, N. H., Stretton, S., Pongpattanakitshote, S., Östling, J., Marshal, K. C., Goodman, A. E., Kjelleberg, S. (1996) Construction and use of a new vector/transposon, pLBT::mini-Tn10:*lac*:*kan*, to identify environmentally responsive genes in marine bacteria. *FEMS Microbiology Letters* **140**:287-294.

Altschul, S., Gish, W., Miller, W., Meyers, E., Lipman, D. (1989) Basic local alignment search tool. *Journal of Molecular Biology* **215**:403-410.

Andlid, T. (1995) Ecological physiology of yeasts colonizing the intestine of fish. PhD thesis, Lunberg Institute, Goteberg University, Sweden.

Austin, B., Stuckey, L. F., Bertson, P. A. W., Effendi, I., Griffith, D. R. W. (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *Journal of Fish Disease* **18**:93-96.

Ausubel, J. F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K. (1989) *Current Protocols in Molecular Biology*. Green Publishing Associates and Wiley-Interscience, Harvard Medial School, USA.

Aznar, R., Ludwig, W., Amann, R. I., Schleifer, H. E. (1994) Sequence determination of rRNA genes of pathogenic *Vibrio* species and whole-cell identification of *Vibrio vulnificus* with rRNA-targeted oligonucleotide probes. *International Journal of Systemic Bacteriology* **44(2)**:330-337.

Bachère, E. (2000) Shrimp immunity and disease control. *Aquaculture* **191**:3-11.

Bachère, E. (2003) Anti-infectious immune effectors in marine invertebrates: potential tools for disease control in marine larviculture. *Aquaculture* **227**:427-438.

Bachère, E., Destoumieux, D., Bulet, P. (2000) Paenidins, antimicrobial peptides of shrimp: a comparison with other effectors of innate immunity. *Aquaculture* **191**:71-88.

Bachère, E., Mialhe, E., Noël, D., Boulo, V., Morvan, A., Rodrigues, J. (1995) Knowledge and research prospects in marine mollusc and crustacean immunology. *Aquaculture* **132**:17–32.

Baranyi, J., Pin, C. (1999) Estimating bacterial growth parameters by means of detection times. *Applied and Environmental Microbiology* **65(2)**:732-736.

Bardach, J. E., Ryther, J. H., McLerney, W. O. (1972) *Aquaculture: the farming and husbandry of freshwater and marine organisma*. Wiley-Interscience. New York: Chichester.

Barnett, J. A., Payne, R. W., Yarrow, D. (1983) *Yeasts: Characterisation and identification.* Cambridge University Press, Cambridge.

Baumann, P., Furniss, A. L., Lee, J. V. (1984) Genus *Vibrio*, pp 518-538. In *Bergey's manual of systemic bacteriology.* **(Eds)** Krieg, N. R., Holt, J. G. The Williams & Wilkins Co., Baltimore, Md.

Bayne, C. J. (1990) Phagocytosis and non-self recognition in invertebrates: Phagocytosis appears to be an ancient line of defense. *Bioscience* **40(10)**:723-731.

Bevelander, G. (1988) *Abalone: Gross and fine structure.* The Boxwood Press, Pacific Grove, California.

Bissett, A., Burke, C., Dunstan, G. A., Maguire, G. B. (1998) Bacterial colonization of a formulated abalone diet during extended immersion. *Journal of Shellfish Research* **17(4)**:995-1002.

Bland, E. J., Keshavarz, T., Bucke, C. (2001) Using 2', 7'-Dichlorodihydrofluorescein-diacetate to assess the polysaccharides as immunomodulating agents. *Molecular Biotechnology* **19**:125-131.

Bonar, D. B., Weiner, R. M., Colwell, R. R. (1986) Microbial invertebrate interactions and potential for biotechnology. *Microbial Ecology* **12**:101-110.

Branch, G. M., Griffiths, C. L., Branch, M. L., Beckley, L. E. (1994) *Two Oceans. A guide to the marine life of Southern Africa.* David Philip Publishers, South Africa.

Brenner, D. J., Hickman, F. W., Lee, J. V., Steigerwalt, A. G., Fanning, G. R., Hollis, D. G., Farmer III, J. J., Weaver, R. E., Joseph, S. W., Seidler, R. J. (1983) *Vibrio furnissii* (formally aerogenic biogroup of *Vibrio fluvialis*), a new species isolated from human faeces and the environment. *Journal of Clinical Microbiology* **18(4)**:816-824.

Britz, P. J. (1990) Global status of abalone aquaculture. In *Perlemoen farming in South Africa.* **(Ed)** Cook, P. *Mariculture association of South Africa.* pp 20-26.

Britz, P. J. (1996) Effect of dietary protein level on growth performances of South African abalone, *Haliotis midae*, fed fishmeal based semi-purified diets. *Aquaculture* **140**:55-61.

Britz, P. J., Hecht, T., Knauer, J. (1996) Gastric evacuation time and digestive enzyme activity in abalone *Haliotis midae* fed a formulated diet. *South African journal of Marine Science* **17**:297–303.

Britz, P. J., Hecht, T. (1997) Effect of dietary protein and energy level on growth and body composition of South African abalone, *Haliotis midae. Aquaculture* **156**:195–210.

Cai, J., Roberts, I. N., Collins, M. D. (1996) Phylogenetic relationship among members of the Ascomycetous yeast genera *Brettanomyces*, *Debaryomyces*, *Dickers*, and *Kluyveromyces* deduced by small-subunit rRNA gene sequences. *International Journal of Systemic Bacteriology* **46(2)**:542-549.

Chang, C-F., Su, M-S., Chen, H-Y., Liao, I-C. (2003) Dietary β-1,3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. *Fish & Shellfish Immunol*ogy **15**:297–310.

Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J. A., Bulet, P. (1996) Isolation of several cysteine–rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. *Journal of Biological Chemistry* **271**:21808-21813.

Cheng., W., Chen, J.-C. (2002) The virulence of *Enterococcus* to the freshwater prawn *Macrobrachium rosenbergii* and its immune resistance under ammonia stress. *Fish & shellfish Immunology* **12**:97-109.

Cheng, W., Hsiao, I.-S., Hsu, C.-H., Chen, J.-C. (2004a) Change in water temperature on the immune response of Taiwan abalone *Haliotis diversicolor* supertexta and its susceptibility to *Vibrio parahaemolyticus*. *Fish & Shellfish Immunology* **17**:235-243.

Cheng, W., Hsiao, I.-S., Chen, J.-C. (2004b) Effect of ammonia on the immune response of Taiwan abalone *Haliotis diversicolor* supertexta and its susceptibility to *Vibrio parahaemolyticus*. *Fish & Shellfish immunology* **17**:193-202.

Cheng, W., Juang, F.-M ., Chen, J.-C. (2004c) The immune response of Taiwan abalone *Haliotis diversicolor supertaxa* and its susceptibility to *Vibrio parahaemolyticus* at different salinity levels. *Fish and Shellfish Immunology* **16**:295-306.

Cheng, W., Liu, C.-H., Hsu, J.-P., Chen, J.-C. (2002) Effect of hypoxia on the immune response of the giant freshwater prawn *Machrobrachium rosenbergii* and its susceptibility to pathogen *Enterococcus*. *Fish & Shellfish Immunology* **13**:351-365.

Church, G., Gilbert, W. (1984) Genomic sequencing. *Proceeding of the National Academy of Sciences*, USA. **81**:1991-1995.

Chythanya, R., Karunasagar, I., Karunasagar, I. (2002) Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain. *Aquaculture* **208**:1-10.

Cook, P. J. (1990) Potential of abalone culture in South Africa. In *Perlemoen farming in South Africa*. **(Ed)** Cook, P. *Mariculture association of South Africa*. pp 27-32.

Coles, J. A., Farley, S. R. and Pipe, R. K. (1994) Effects of fluoranthene on the immunocompetence of the common marine mussel, *Mytilus edulis*. *Aquatic Toxicology* **30**:367-379.

Coles, J. A., Farley, S. R. and Pipe, R. K. (1995) Alteration of the immune response of the common marine mussel *Mytilus edulis* resulting from exposure to cadmium. *Diseases of Aquatic Organisms* **22**:59-65.

Coyne, V. E., James, M., Reid, S., Rybicki, E. (1996) *Molecular Biology techniques manual* (4th ed.), University of Cape Town, Cape Town.

Deane, S. M, Robb, F. T., Woods, D. R. (1986) Isolation and characterization of a Vibrio alginolyticus mutant that overproduces extracellular protease. *J. Gen. Microbiol.* **132**:893-898.

Divakaran, S., Obaldo, L. G., Forster, I. P. (2002) Note on the methods for determination of chromic oxide in shrimp feeds. *J. Agric. Food Chem.* **50**:464–467.

De Lorenzo, V., Herrero, M., Jakubzik, U., Timmis, K. N. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative Eubacteria. *Journal of Bacteriology* **172(11)**:6568-6572.

De Schrijver, R., Ollevier, F. (2000) Protein digestion in juvenile turbot (*Scophthalmus maximus*) and effects of dietary administration of *Vibrio proteolyticus*. *Aquaculture* **183**:107–116.

Dorsch, M., Lane, D., Stackebrandt, E. (1992) Towards the phylogeny of the genus *Vibrio* based on 16S rRNA sequences. *International Journal of Systemic Bacteriology* **42(1)**:58-63.

Douillet, P. A., Langdon, C. J. (1994) Use of a probiotic for the culture of larvae of the Pacific oyster (*Crassostrea gigas* Thunberg). *Aquaculture* **119**:25–40.

Edgcomb, V. P., McDonald, J. H., Devereux, R., Smith, D. (1999) Estimation of bacterial cell numbers in humic acid-rich salt marsh sediments with probes directed to 16S ribosomal DNA. *Applied and Environmental Microbiology* **65(4)**:1516-1523.

Erasmus, J. H. (1996) The role of enteric bacteria in the abalone, *Haliotis midae*. *M.Sc. thesis*, University of Cape Town

Erasmus, J. H., Cook, P. A., Coyne, V. E. (1997) The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. *Aquaculture* **155**:377–386.

Egan, S., James, S., Kjelleberg, S. (2002) Identification and characterization of a putative transcriptional regulator controlling the expression of fouling inhibitors in *Pseudoalteromonas tunicate*. *Applied and Environmental Microbiology* **68(1)**:372-378.

Esteban, M. A., Mulero, V., Muños, J., Meseguer, J. (1998) Methodological aspects of assessing phagocytosis of *Vibrio anguillarum* by leukocytes of gilthead seabream (*Sparus aurata* L.) by flow cytometry and electron microscopy. *Cell Tissue Research* **293**:133-141.

Farrelly, V., Rainey, F. A., Stackebrandt, E. (1995) Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and Environmental Microbiology* **61(7)**:2798-2801.

Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**:783-791.

Ferguson, Y., Bricknell, I. R., Glover, L. A., MacGregor, D. M., Prosser, J. I. (1998) Colonization and transmission of *lux*-marked and wild-type *Aeromonas salmonicida* in Atlantic salmon (*Salmo salar* L.). *FEMS Microbiology Ecology* **27**:251-260.

Ferguson, Y., Glover, L. A., McGillivray, D. M., Prosser, J. I. (1995) Survival and activity of *lux*-market *Aeromonas salmonicida* in seawater. *Applied and Environmental Microbiology* **61(9)**:3494-3498.

Furet, J.-P., Quénée, P., Tailleiz, P. (2004) Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. *International Journal of Food Microbiology* **97**:197-207.

Garret, R. H., Grisham, C. M. (1995) *Biochemistry*. Saunders College Publishing, Fort Worth, USA.

Garriques, D., Arevalo, G. (1995) An evaluation of the production and use of a live bacterial isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* postlarvae in Ecuador. **(Eds)** Browdy, C. L., Hopkins, J. S. In *Swimming through troubled water, proceedings of the special session on shrimp farming.* World Aquaculture Society pp 53-59.

Gatesoupe, F. J. (1999) The use of probiotics in aquaculture. *Aquaculture* **180**:147–165.

Gatesoupe, F. J., Zambonino Infante, J.-L., Cahu, C., Quazuguel, P. (1997) Early weaning of seabass larvae, *Dicentrarchus labrax*: the effect on microbiota, with particular attention to iron supply and exoenzymes. *Aquaculture* **158**:117-127.

Genthner, F. J., Volety, A. K., Oliver, L. M., Fisher, W. S. (1999) Factors influencing in vitro killing of bacteria by haemocytes of the eastern oyster (*Crassostrea virginica*). *Applied and Environmental Microbiology* **65(7)**:3015-3020.

Gibson, L. F., Woodworth, J., George, A. M. (1998) Probiotic activity of *Aeromonas media* on the Pacific oyster, *Crassostrea gigas,* when challenged with *Vibrio tubiashii*. *Aquaculture* **168**:111-120.

Gildberg, A., Mikkelsen, H., Sandaker, E., Ringø, E. (1997) Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (*Gadus morhua*). *Hydrobiologia* **352**:279-285.

Gomez-Gil, B., Roque, A., Turnbull, J. F. (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture* **191**:259-270.

Gomez-Gil, B., Roque, A., Velasco-Blanco, G. (2002) Culture of *Vibrio alginolyticus* C7b, a potential probiotic bacterium, with the microalga *Chaetoceros muelleri. Aquaculture* **211**:43-48.

Gordon, H. R. and Cook, P. A. (2001) World abalone supply, markets and pricing: Historical, current and future. *Journal of Shellfish Research* **20(2)**:567-570.

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I., Nielsen, T. F. (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment for fish. *Applied and Environmental Microbiology* **65**:969–973.

Gram, L., Løvold, T., Nielsen, J., Melchiorsen, J., Spanggaard, B. (2001) In vitro antagonism of the probiotic *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furunculosis. *Aquaculture* **199**:1–11.

Gullian, M., Thompson, F., Rodriguez, J. (2004) Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei. Aquaculture* **233**:1-14.

Harmsen, H. J. M., Gibson, G. R., Elfferich, P., Raangs, G. C., Wildeboer-Veloo, A. C. M., Argaiz, A., Roberfroid, M. B., Welling, G. W. (1999) Comparison of viable cell counts and fluorescence in situ hybridization using specific rRNA-based probes for the quantification of human faecal bacteria. *FEMS Microbiology letters* **183**:125-129.

Harris, J. O., Burke, C. M., Maguire, G. B. (1998a) Characterization of the digestive tract of greenlip abalone, *Haliotis laevigata* Donovan. I. Morphology and histology. *Journal of Shellfish Research* **17**:979–988.

Harris, J. O., Burke, C. M., Maguire, G. B. (1998b) Characterization of the digestive tract of greenlip abalone, *Haliotis laevigata* Donovan. II. Microenvironment and bacterial flora. *Journal of Shellfish Research* **17**:989–994.

Hayashi, K., Moriwaki, J., Sawabe, T., Thompson, F. L., Swings, J., Gudkovs, N., Christen, R., Ezura, Y. (2003) *Vibrio superstes* sp. nov., isolated from the gut of Australian abalone *Haliotis laevigata* and *Haliotis rubra. International Journal of Systemic and Evolutionary Microbiology* **53**:1813-1817.

Hayat, M. A. (1993) *Stains and Cytochemical Methods*. Plenum Press. New York and London. pp 63–64.

Hermansson, A., Lindgren, P.-E. (2001) Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. *Applied and Environmental Microbiology* **67(2)**:972-976.

Herrero, M., De Lorenzo, V., Timmis, K. N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *Journal of Bacteriology* **172(11)**:6557-6567.

Hilborn, R., Branch, T. A., Ernst, B., Magnusson, A., Minte-Vera, C. V., Scheuerell, M. D., Valero, J. L. (2003) State of the world's fisheries. *Annu. Rev. Environ. Resour.* **28**:359-399.

Holman, J. D., Burnett, K. G., Burnett, L. E. (2004) Effects of hypercapnic hypoxia on the clearance of *Vibrio campbellii* in the Atlantic blue crab, *Callinectes sapidus* Rathbun. *Biol. Bull.* **206**:188-196.

Hubert, F., Noël, T., Roch, P. (1996) A member of the arthropod defensin family from edible Mediterranean mussels (*Mytilus galloprovincialis*). *European Journal of Biochemistry* **240**:302-306.

Hurst, M. R. H., Jackson, T. A. (2002) Use of green fluorescent protein to monitor the fate of *Serratia entomophila* causing amber disease in the New Zealand grass grub, *Costelytra zealandica*. *Journal of Microbiological Methods* **50**:1-8.

Ino, T. (1952) Biological studies on the propagation of Japanese abalone genus *Haliotis*. *Tokai-Ku Sisan Kenkyuto Hokuku*. **5**:1-102. (English translation in possession of Stanford University).

Itami, T., Asano, M., Tokushige, K., Kubono, K., Nakagawa, A., Takeno, N., Nishimura, H., Maeda, M., Kondo, M., Takahashi, Y. (1998) Enhancement of disease resistance of Kuruma shrimp, *Penaeus japonicus,* after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. *Aquaculture* **164**:277-288.

Johansson, M. W., Keyser, P., Söderhäll, K. (1994) Purification and cDNA cloning of a four-domain Kazal proteinase inhibitor from crayfish blood cells. *Eur. J. Biol.* **94**:389-394.

Johansson, M. W., Keyser, P., Sritunyalucksana, K., Söderhäll, K. (2000) Crustacean haemocytes and haematopoiesis. *Aquaculture* **191**:45-52.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**:111-120.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, N. (2001) MEGA2: Molecular and Evolutionary Genetics Analysis software. Bioinformatics, in press.

Knauer, J., Britz, P. J., Hecht, T. (1996) Comparative growth performance and digestive activity of juvenile South African abalone, *Haliotis midae*, fed diatoms and a practical diet. *Aquaculture* **140**:75–85.

Lacoste, A., Jalabert, F., Malham, S. K., Gélébart, F., Cueff, A., Poulet, S. A. (2001) Stress and stress-induced neuroendocrine changes increase the susceptibility of juvenile oysters (*Crassostrea gigas*) to *Vibrio splendidus*. *Applied and Environmental Microbiology* **67(5)**:2304-2309.

Lacoste, A., Malham, S. K., Gélébart, F., Cueff, A., Poulet, S. A. (2002) Stress-induced immune changes in the oyster *Crassostrea gigas*. *Developmental and Comparative Immunology* **26**:1–9.

Lara-Flores, M., Olvera-Novoa, M. A., Guzmán-Méndez, B. E., López-Madrid, W. (2003) Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisae* as growth promoters in Nile Tilapia (*Oreochromis niloticus*). *Aquaculture* **216**:193-201.

Lee, J., Lee, S. Y., Park, S., Middelberg, A. P. J. (1999) Research review paper: Control of fed-batch fermentations. *Biotechnology Advances* **17**:29-48.

Li, S., Cullen, D., Hjort, M., Spear, R., Andrews, J. H. (1996) Development of an oligonucleotide probe for *Aureobasidium pullulans* based on the small-subunit rRNA gene. *Applied and Environmental Microbiology* **62(5)**:1514-1518.

Li, S., Spear, R. N., Andrews, J. H. (1997) Quantitative fluorescence *in situ* hybridization of *Aureobasidium pillans* on microscope slides and leaf surfaces. *Applied and Environmental Microbiology* **63(8)**:3261-3267.

Liu, P. C., Chen, Y. C., Lee, K. K. (2001) Pathogenicity of *Vibrio alginolyticus* isolated from diseased small abalone *Haliotis diversicolor* supertaxa. *Microbios*. **104**:71–77.

Macey, B. M., Coyne, V. E. (2005) Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. *Aquaculture* **242**:249-261.

Macián, M. C., Ludwig, W., Schleifer, K. H., Pujalte, M. J. (2001) *Vibrio agarivorans* sp. nov., a novel agarolytic marine bacterium. *International Journal of Systemic and Evolutionary Microbiology* **51**: 2031-2036.

Maguire, G. B., Wee, K. L., Hindrum, S. M. (1993) Digestibility studies – The "ins" and "outs" of abalone guts. *Austasia Aquaculture* 7:42-46.

Mah, S. A., Moy, G. W., Swanson, W. J., Vacquier, V. D. (2004) A perforin-like protein from a marine mollusk. *Biochemical and Biophysical Research Communications* **316**:468-475.

Malham, S. K., Lactose, A., Gélébart, F., Cueff, A., Poulet, S. A. (2003) Evidence for a direct link between stress and immunity in the mollusk *Haliotis tuberculata*. *J. Exp. Biol.* **295A**:136–144.

Maramorosch, K., Shope, R. E. (1975) *Invertebrate immunology: mechanisms of invertebrate vector-parasite relations*. Academic Press, New York pp 137–145.

Marques, M. R. F., Barracco, M. A. (2000) Lectins, as non-self recognition factors, in crustaceans. *Aquaculture* **191**:23-44.

Martello, L. B., Friedman, C. S., Tjeerdema, R. S. (2000) Combined effects of pentachlorophenol and salinity on the phagocytic and chemotactic function of two species of abalone. *Aquatic Toxicology* **49**:213-225.

Martello, L. B., Tjeerdema, R. S. (2001) Combined effects of pentachlorophenol and salinity stress on chemiluminescence activity in two species of abalone. *Aquatic Toxicology* **51**:351-362

Matthysse, A. G., Stretton, S., Dandie, C., McClure, N. C., Goodman, A. E. (1996) Construction of GFP vectors for use in Gram-negative bacteria other than *Escherichia coli*. *FEMS Microbiology Letters* **145**:87-94.

Meunopol, O., Lopinyosiri, K., Menasveta, P. (2003) The effects of ozone and probiotics on the survival of the black tiger shrimp (*Penaeus monodon*). *Aquaculture* **220**:437-448.

Mialhe, E., Bachére, E., Boulo, V., Cadoret, J. P. (1995) Strategy for research and international cooperation in marine invertebrate pathology, immunology and genetics. *Aquaculture* **132**:33–41.

Miles, D. J. C., Polchana, J., Lilley, J. H., Kanchanakhan, S., Thompson, K.D., Adams, A. (2001) Immunostimulation of striped snakehead *Channa striata* against epizootic ulcerative syndrome. *Aquaculture* **195**:1-15.

Miller, G. L. (1959) The use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Anal. Chemistry* **31**:426-428.

Miller, V. L., Mekalanos, J. J. (1988) A novel suicide vector and its use in the construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *V. cholera* requires ToxR. *Journal of Bacteriology* **170**:2575-2583.

Moore, J. D., Robbins, T. T., Hedrick, R. P., Friedman, C. S. (2001) Transmission of the rickettsiales-like prokaryote "*Candidatus Xenohaliotis* californiensis" and its role in withering syndrome of Californian abalone, *Haliotis* spp. *Journal of Shellfish Research* **20(2)**:867-874.

Murray, R. G .E., Brenner, D. J., Colwell, R. R., De Vos, P., Goodfellow, M., Grimont, P. A. D., Pfennig, N., Stackebrandt, E., Zavarzin, G. A. (1990) Report of the ad hoc committee on approaches to taxonomy within the Proteobacteria. *International Journal of Systematic Bacteriology* **40**:213 – 215.

Nikoskelainen, S., Salminen, S., Bylund, G., Ouwehand, A. C. (2001) Characterization of the properties of human- and dairy-derived probiotics for prevention of infectious diseases in fish. *Applied and Environmental Microbiology* **67(6)**:2430-2435.

Nikoskelainen, S., Ouwehand, A. C., Bylund, G., Salminen, S., Lilius, E-M. (2003) Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish & Shellfish Immunology* **15**:443–452.

Nogami, K., Meada, M. (1992) Bacteria as biocontrol agents for rearing larvae of the crab *Portunus trituber Culatus. Canadian journal of fisheries and aquatic sciences* **49**:2373–2376.

Normander, B., Hendriksen, N. B., Nybroe, O. (1999) Green fluorescent protein-marked *Pseudomonas fluorescence*: localization, viability, and activity in the natural barley rhizospere. *Applied and Environmental Microbiology* **65(10)**:4646-4651.

Olafsen, J. A. (2001) Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture* **200**:223–247.

Ordás, M. C., Novoa, B., Figueras, A. (2000a) Modulation of the chemiluminescence response of Mediterranean mussel (*Mytilus galloprovincialis*) haemocytes. *Fish & Shellfish Immunology* **10**:611–622.

Ordás, M. C., Ordás, A., Beloso, C., Figueras, A. (2000b) Immune parameters in carpet shell clams naturally infected with *Perkinsus atlanticus. Fish and Shellfish Immunology* **10**:597-609.

Peccia, J., Marchand, E. A., Silverstein, J., Hernandez, M. (2000) Development and application of small-subunit rRNA probes for assessment of selected *Thiobacillus* species and members of the genus *Acidiphilium. Applied and Environmental Microbiology* **66(7)**:3065-3072.

Pillay, T. V. R. (1992) *Aquaculture and the environment.* Fishing News Books. University Press, Cambridge. pp 89.

Pipe, R. K. (1992) Generation of reactive oxygen metabolites by the haemocytes of the mussel *Mytilus edulis. Developmental and Comparative Immunology* **16**:111-122.

Pipe, R. K., Farley, S. R., Coles, J. A. (1997) The separation and characterisation of haemocytes from the mussel *Mytilus edulis. Cell Tissue Research* **289**:537-545.

Pipe, K., Coles, J. A., Farley, S. R. (1995) Assays for measuring immune response in mussel Mytilus *edulis. Techniques in Fish Immunology* **4**:93-100.

Press, J., Ashwell, G. (1962) Alginic acid metabolism in bacteria. *Journal of Biological Chemistry* **237(2)**:309-316.

Prescott, L. M., Harley, J. P., Klein, D. A. (1993) *Microbiology: Second addition.* Wm. C. Brown Publishers. Dubuque, Iowa.

Raguénès, G., Christen, R., Guezennec, J., Pignet, P., Barbier, G. (1997) *Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*. *International Journal of Systemic Bacteriology* **47(4)**:989-995.

Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., Menasveta, P. (1998) Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* **167**:301-313.

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasveta, P. (2000) Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiotic bacterium (*Bacillus* S11). *Aquaculture* **191**:271–288.

Riesenberg, D., Guthke, R. (1999) A mini-review: High-cell-density cultivation of microorganisms. *Applied Microbiology and Biotechnology* **51**:422-430.

Ringø, E. (1999) Short communication. Does *Carnobacterium divergens* isolated from Atlantis salmon, *Salmo salar* L., colonize the gut of early developing turbot, *Scophthalmus maximus* L., larvae. *Aquaculture research* **30**:229-232.

Ringø, E., Bendiksen, H. R., Gausen, S. J., Sundsfjord, A., Olsen, R. E. (1998) The effect of dietary fatty acids on lactic acid bacteria associated with the epithelial mucosa and from faecalia of Artic charr, *Salvelinus alpinus* (L). *Journal of Applied Microbiology* **85**:855-864.

Ringø, E., Birkbeck, T. H. (1999) Intestinal microflora of fish larvae and fry. *Aquaculture research* **30**:73–93.

Ringø, E., Birkbeck, T. H., Munro, P. D., Vadstein, O., Hjelmeland, K. (1996) The effect of early exposure to *Vibrio pelagius* on the aerobic bacterial flora of turbot, *Scophthalmus maximus* (L). *Journal of Applied Bacteriology* **81**:207-211.

Ringø, E., Gatesoupe, F.J. (1998) Lactic acid bacteria in fish: a review. *Aquaculture* **160**:177-203.

Ringø, E., Olson, R. E. (1999) The effect of diet on aerobic bacterial flora associated with intestine of Artic charr, (*Salvelinus alpinus* L.). *Journal of Applied Microbiology* **86**:22-28.

Ringø, E., Strøm, E., Tabachek, J-A. (1995) Intestinal microflora of salmonids: a review. *Aquaculture research* **26**:773-789.

Ringø, E., Vadstein, O. (1998) Colonization of *Vibrio pelagius* and *Aeromonas caviae* in early developing turbot (*Scophthalmus maximus* L.) larvae. *Journal of Applied Microbiology* **84**:227-233.

Riquelme, C., Araya, R., Vergara, N., Rojas, A., Guaita, M., Candia, M. (1997) Potential probiotic strains in the culture of the Chilean scallop *Argopecten purpuratus* (Lamarck, 1819). *Aquaculture* **154**:17-26.

Riquelme, C., Araya, R., Escribano, R. (2000) Selective incorporation of bacteria by *Argopecten purpuratus* larvae: implications for the use of probiotics in culturing systems of the Chilean scallop. *Aquaculture* **181**:25-36.

Robertson, P. A. W., O'Dowd, C., Burrels, C., Williams, P., Austin, B. (2000) Use of *Carnobacterium* sp. as a probiotic for atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Aquaculture* **185**:235–243.

Roch, P. (1999) Defence mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture* **172**:125–145.

Romo-Figueroa, M. G., Vargas-Requena, C., Sotelo-Mundo, R. R., Vargas-Albores, F., Higuera-Ciapara, I., Söderhäll, K., Yepiz-Plascencia, G. (2004) Molecular cloning of a β-glucan pattern-recognition lipoprotein from the white shrimp *Penaeus (Litopenaeus) vannamei*: correlations between the deduced amino acid sequence and the native protein structure. *Developmental and Comparative Immunology* **28:**713–726.

Ruimy, R., Breittmayer, V., Elbaze, P., Lafay, B., Boussemart, O., Gauthier, M., Christen, R. (1994) Phylogenetic analysis and assessment of the genera Vibrio, Photobacterium, Aeromonas, and Plesiomonas deduced from small-subunit rRNA sequences. *International Journal of Systemic Bacteriology* **44(3)**:416-426.

Saitou, N., Nei, M. (1987) The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**:406-425.

Sales, J., Britz, P. J. (2001) Research on abalone (*Haliotis midae* L.) cultivation in South Africa. *Aquaculture Research* **32**:863-874.

Sales, J., Britz, P. J. (2002) Evaluation of the reference diet substitution method for determination of apparent nutrient digestibility coefficients of feed ingredients for South African abalone (*Haliotis midae* L.). *Aquaculture* **207**:113-123.

Sawabe, T., Hayashi, K., Moriwaki, J., Thompson, F. L., Swings, J., Potin, P., Christen, R., Ezura, Y. (2004) *Vibrio gallicus* sp. Nov., isolated from the gut of the French abalone *Haliotis tuberculata*. *International Journal of Systemic and Evolutionary Microbiology* **54**:843-846.

Sawabe, T., Setoguchi, N., Inoue, S., Tanaka, R., Ootsubo, M., Yoshimizu, M., Ezura, Y. (2003) Acetic acid production of *Vibrio halioticoli* from alginate: a possible role for establishment of abalone–*V. halioticoli* association. *Aquaculture* **219**:671-679.

Sawabe, T., Sugimura, I., Ohtsuka, M., Nakano, K., Tajima, K., Ezura, Y., Christen, R. (1998) *Vibrio halioticoli* sp. nov., a non-motile alginolytic marine bacterium isolated from the gut of the abalone *Haliotis discus hannai*. *International Journal of Systemic Bacteriology* **48**:573-580.

Schroeder, D. C. (2001) Isolation and characterization of a β(1-4) agarase of an epiphytic bacterial pathogen, *Pseudoalteromonas gracilis* B9, of the red alga, *Gracilaria gracilis*. *PhD thesis*, University of Cape Town.

Schroeder, D. C., Jaffer, M. A., Coyne, V. E. (2003) Investigation of the role of a beta(1-4) agarase produced by *Pseudoalteromonas gracilis* B9 in eliciting disease symptoms in the red alga *Gracilaria gracilis*. *Microbiology* **149(10)**:2919-2929.

Scott, K. P., Mercer, D. K., Richardson, A. J., Melville, C. M., Glover, L. A., Flint, H. J. (2000) Chromosomal integration of the green fluorescent protein gene in lactic acid bacteria and the survival of marked strains in human gut simulations. *FEMS Microbiology Letters* **182**:23-27.

Serviere-Zaragoza, E., Navarrete del Toro, M. A., García-Carreño, F. L. (1997) Protein-hydrolyzing enzymes in the digestive tract of the Mexican blue abalone, *Haliotis fulgens* (Gastropoda). *Aquaculture* **157**:325-336.

Sghir, A., Gramet, G., Suau, A., Rochet, V., Pochart, P., Dore, J. (2000) Quantification of bacterial groups within human faecal flora by oligonucelotide probe hybridization. *Applied and Environmental Microbiology* **66(5)**:2263-2266.

Shipton, T. A., Britz, P. J. (2001a) An assessment of the use of chromic oxide as a marker in protein digestibility studies with *Haliotis midae* L. *Aquaculture* **203**:69-83.

Shipton, T. A., Britz, P. J. (2001b) The effect of animal size on the ability of Haliotis midae L. to utilize selected dietary protein sources. *Aquaculture Research* **32**:393-403.

Shieh, W.-Y., Chen, Y.-W., Chaw, S.-M., Chiu, H.-H. (2003) *Vibrio ruber* sp. Nov., a red, facultatively anaerobic, marine bacterium isolated from sea water. *International Journal of systemic and Evolutionary Microbiology* **53**:479-484.

Simpson, B. J. A. (1994) An investigation of diet management strategies for culture of South African abalone, *Haliotis midae*. *M.Sc. thesis*, University of Cape Town.

Skjermo, J., Vadstein, O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture* **177**:333-343.

Smibert, R.M., Krieg, N.R. (1994) Phenotypic Characterisation. In *Methods for General and Molecular Bacteriology* **(Eds)** Gerhardt, P. R., Murray, R. G. E., Wood, W. A., Krieg, N. R. American Society for Bacteriology, Washington D. C. pp 607-654.

Smith, V. J., Brown, J. H., Hauton, C. (2003) Immunostimulation in crustaceans: does it really protect against infection? *Fish and Shellfish Immunology* **15**:71-90.

Sohaskey, C. D., Im, H., Schauer, A. T. (1992) Construction and application of plasmid- and transposon-based promoter-probe vectors for *Streptomyces* spp. that employ a *Vibrio harveyii* luciferase reporter cassette. *Journal of Bacteriology* **174(2)**:367-376.

Stackebrandt, E., Liesack, W., Witt, D. (1992) Ribosomal RNA and rDNA sequence analyses. *Gene* **115**:255-260.

Stanford, J. (2004) Aquaculture ambition: South African aquaculture industry is heading for an export boom. *Eng. News*, **August 6-12**:16-17.

Stretton, S., Techkarnjanaruk, S., McLennan, A. M., Goodman, A. E. (1998) Use of green fluorescent protein to tag and investigate gene expression in marine bacteria. *Applied and Environmental Microbiology* **64(7)**:2554-2559.

Suh, S.-S., Takashima, M., Hamamoto, M., Nakase, T. (1996) Molecular phylogeny of the ballistoconidium-forming anamorphic yeast genus *Bullera* and related taxa based on small subunit ribosomal DNA sequences. *Journal of General and Applied Microbiology* **42**:501-509.

Suphantharika, M., Khunrae, P., Thanardkit, P., Verduyn, C. (2003) Preparation of spent brewer's yeast β-glucans with a potential application as an immunostimulant for black tiger shrimp, *Peneaus monodon*. *Bioresource Technology* **88**:55-60.

Supungul, P., Klinbunga, S., Pichyangkura, R, Jitrapakdee, S. (2002) Identification of immune-related genes in haemocytes of black tiger shrimp (*Penaeus monodon*). *Marine Biotechnology* **4**: 487-494.

Takashima, M., Sugita, T., Shinoda, T., Nakase, T. (2001) Reclassification of the *Cryptococcus humicola* complex. *International Journal of Systemic and Evolutionary Microbiology* **51**:2199-2210.

Takashima, M., Sugita, T., Shinoda, T., Nakase, T. (2003) Three new combinations from the *Cryptococcus laurentii* complex: *Cryptococcus aureus*, *Cryptococcus carnescens* and *Cryptococcus peneaus*. *International Journal of Systemic and Evolutionary Microbiology* **53**:1187-1194.

Tarr, R. (1992) The abalone fishery of South Africa. In *Abalone of the world: Biology, fisheries and culture*. **(Ed).** Shepard, S. A., Tegner, M. J., del Preo, G. Blackwell Scientific Publication, Oxford. pp 438-447.

Tovar, D., Zambonino, J., Cahu, C., Gatesoupe, F. J., Vázquez-Juárez, R., Lèsel, R. (2002) Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* **204**:113-123.

Ulitzur, S. (1972) Rapid determination of DNA base composition by ultraviolet spectroscopy. *Biochim. Biophys. Acta.* **272**:1–11.

Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* **64(4)**:655-671.

Vine, N. G., Leukes, W. D., Kaiser, H. (2004) In vitro growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish mucus. *FEMS Microbiology letters* **231**:145-152.

Vitalis, T. Z., Spence, M. J., Carefoot, T. H. (1988) The possible role of gut bacteria in nutrition and growth of the sea hare *Aplysia. The Veliger* **30**:333-341.

Volety, A. K., Oliver, L. M., Genthner, F. J., Fisher, W. S. (1999) A rapid tetrazolium dye reduction assay to assess the bactericidal activity of oyster (*Crassostrea virginica*) haemocytes against *Vibrio parahaemolyticus. Aquaculture* **172**:205-222.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P., Truper, H. G. (1987) Report of the Ad Hoc committee on reconciliation of approaches to bacterial systematics. *International Journal of Systemic Bacteriology* **37(4)**:463-464.

Withers, P. C. (1992) *Comparative animal physiology*. Saunders College Publishing. USA.

Woes, C. R., Weisburg, W. G., Hahn, C. M., Paster, B. J., Zablen, L. B., Lewis, B. J., Macke, T. J., Ludwig, W., Stackebrandt, E. (1985) The phylogeny of the purple bacteria: the gamma subdivision. *Systemic and Applied Microbiology* **6**:25-33.

Zizhou, N. (2001) Studies on fed-batch propagation of brewer's yeast in high gravity wort. *M.Sc. thesis*, University of Cape Town.

Zwietering, M. H., Jongenburger, I., Rombouts, F. M., van 'T Riet, K. (1990) Modeling of the bacterial growth curve. *Applied and Environmental Microbiology* **56(6)**:1875-1881.