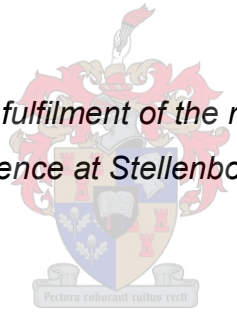
Isolation of potential probiotic and carotenoid producing bacteria and their application in aquaculture

by

Anneke de Bruyn



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Declaration

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December 2012 Anneke de Bruyn

Summary

The ocean's fish resources are declining mainly because of irresponsible exploitation. Fish is a vital source of protein for humans and growing world populations are threatening the sustainability of commercial fisheries. This has led to the rapid growth of aquaculture worldwide. In South Africa, aquaculture of both fresh and marine species is expanding and is now practised in all nine provinces of the country.

One of the major problems in aquaculture is the economic losses as a result of diseases. Viruses, bacteria, fungi and parasites are well known to infect fish, with bacteria causing the majority of diseases. Antibiotics were commonly used to control diseases, however, due to their negative impact on the environment, the use of these agents is questioned. This has led to the search for probiotics as an alternative way to control bacterial diseases in aquaculture. Probiotics used in aquatic environments can be defined as live microbial supplements which have beneficial effects on the host by altering the microbial communities associated with the host and the immediate environment. Probiotics have a variety of different mechanisms of action, including competition with pathogens, production of beneficial compounds, enhancement of host immune response and antiviral effects.

This study aimed to isolate potential probiotic bacteria from the gastrointestinal tract (GIT) of the South African abalone (*Haliotis midae*). Nine different bacterial species were isolated and identified as *Corynebacterium variabilei*, *Staphylococcus carnosus, Staphylococcus equorum, Staphylococcus cohniii, Vibrio aestuarianus*, *Vibrio nigripulchritudo, Vibrio cyclitrophicus, Photobacterium leiognathi*, and *Paracoccus marcusii* (Chapter 2). One of these isolates, *P. marcusii* (isolate 6.15), showed promising probiotic properties together with the potential to be used as a pigmentation source due to its production of the carotenoid astaxanthin. Aquatic animals are not able to synthesize astaxanthin and under aquaculture conditions do not come into contact with natural pigment sources. This results in dark grey meat which is unappealing for consumers. Therefore, astaxanthin is included in the feed of a variety of aquaculture species such as salmon, trout, red see bream and shrimp to give the meat a pink/orange colour. Astaxanthin also plays an important role in other essential biological functions of fish such as increasing the defence potential against oxidative stress and enhancing sexual maturity, embryo development, and egg survival.

Mozambique tilapia (*Oreochromis mossambicus*) and rainbow trout (*Oncorhynchus mykiss*), two important aquaculture species in South Africa, were used to evaluate the probiotic and pigmentation effect of *P. marcusii* (isolate 6.15). Fish feed was coated with freeze dried bacterial cells (10^7 CFU/kg feed) and administrated to tilapia and trout. Because tilapia cannot incorporate astaxanthin into their meat, no pigmentation effect of *P. marcusii* (isolate 6.15) was evaluated for this species. However, tilapia showed significant improvement in growth and immune parameters. Fish supplemented with *P. marcusii* (isolate 6.15) had a higher percentage increase in body weight and a better feed conversion ratio for the duration of the trial. Enhanced lysozyme activity in the blood serum of the fish was also seen (Chapter 3). In contrast, *P. marcusii* (isolate 6.15) did not have any probiotic or pigmentation effect on rainbow trout. A possible reason for this may be that the concentration of *P. marcusii* (isolate 6.15) added to the feed was too low. More probably, it is suspected that no pigmentation was observed due to the destruction of the astaxanthin before being ingested by the trout, because astaxanthin is a very unstable molecule. Furthermore, the GIT microbial communities of trout were investigated over the duration of the trial for the different treatments. No similarities in community structures were observed betwee the different treatments, however, bacterial communities in the GIT of fish sampled at the same time were very similar (Chapter 4).

Opsomming

Die oseaan se vis hulpbronne is besig om af te neem as gevolg van die onverantwoordelike gebruik daarvan. Vis is ‘n belangrike bron van proteïene vir mense en die toenemende wêreld populasie bedreig die volhoubaarheid van kommersiële visserye. As gevolg hiervan is daar ‘n drastiese toename in die akwakultuur industrie wêreldwyd. Ook in Suid Afrika brei die akwakultuur van beide vars water en mariene vis spesies uit.

Een van die grootste probleme in akwakultuur is ekonomiese verliese as gevolg van siektes wat veroorsaak word deur virusse, bakterieë, fungi en parasiete. Bakterieë veroorsaak die meerderheid van die siektes en antibiotika word algemeen gebruik vir die beheer van bakteriële siektes. Die gebruik van antibiotika word egter bevraagteken omdat dit verskeie negatiewe implikasies vir die omgewing inhou Daarom word probiotika oorweeg as ‘n alternatief tot antibiotika om bakteriële siektes te voorkom en te behandel. Probiotika wat in akwatiese omgewings toegedien word kan gedefinieer word as a lewende mikrobiese aanvulling wat ‘n positiewe effek op die gasheer het, deur die mikrobiese gemeenskappe geassosieer met die gasheer en die ommidellike omgewing te verander. Hierdie mikrobiese aanvulling verbeter die gesondheid van die visse deur verskeie meganismes wat insluit kompetisie met patogene, produksie van voordelige chemiese verbindings, verhoging van die gasheer se immuniteit en antivirale effekte.

Die doel van hierdie studie was om potensiële probiotika te isoleer uit die spysverterings kanaal (SVK) van die Suid Afrikaanse perlemoen spesie, *Haliotis midae*. Tydens die studie is daar nege verskillende bakteriële spesies geïsoleer en geidentifiseer as stamme verteenwoordegend van *Corynebacterium variabilei*, *Staphylococcus carnosus, Staphylococcus equorum, Staphylococcus cohniii, Vibrio aestuarianus*, *Vibrio nigripulchritudo, Vibrio cyclitrophicus, Photobacterium leiognathi* en *Paracoccus marcusii* (Hoofstuk 2). Een van die isolate, *P. marcusii,* het belowende probiotika en potensiële pigmentering eienskappe getoon a.g.v. die produksie van die karotenoïed astazantien. Akwatiese diere is nie daartoe instaat om hierdie pigment te produseer nie en onder akwakultuur toestande kom die visse ook nie in kontak met natuurlike bronne van hierdie pigment nie. Dit lei daartoe dat die vleis van visspesies soos forel en salm grys word wat dit onaantreklik vir verbruikers maak. Daarom word astazantien bygevoeg by visvoer om

sodoende ‘n pienk/oranje kleur te verseker.Daar benewens speel astazantien ook ‘n rol in belangrike biologiese funksies van visse. Dit sluit in die verhoging in beskerming teen oksidatiewe stres, bevordering van seksuele volwassenheid, embrio ontwikkeling en eier oorlewing.

Twee belangrike akwakultuur spesies in Suid Afrika, Mosambiek tilapia (*Oreochromis mossambicus*) en reënboog forel (*Oncorhynchus mykiss*), was in hierdie studie gebruik. Die probiotiese en pigmentasie effek van *P. marcusii* op reënboog forel was gëevalueer terwyl slegs die probiotiese effek op tilapia geëvalueer weens die onvermoeë van tilapia om die pigment in hul vleis te inkorpereer. Visvoer korrels was omhul met gevriesdroogde bakteriële selle (10^7 CFU/kg kos) en vir die visse gevoer. Daar was ‘n duidelike verbetering in groei en immuun parameters van tilapia. Visse toegedien met *P. marcusii* het ‘n hoër persentasie vermeerdering in liggaamsgewig en ‘n beter voedsel omsettings verhouding gehad tydens die verloop van die proef in vergelyking met die kontroles (Hoofstuk 3). In kontras hiermee kon daar geen probiotiese of pigmenterings effekte waargeneem word by die reënboog forel nie. ‘n Moontlike rede hiervoor kon wees dat die konsentrasie van *P. marcusii* wat by die kos gevoeg is te laag was. Dit is egter ook moontlik dat die astazantien vernietig was voordat dit deur die forel opgeneem is aangesien astazantien ‘n baie onstabiele molekuul is. Verder het ons die impak van verskillende visvoer behandelings op die mikrobiese gemeenskappe in die spysverteringskanaal (SVK) van forel tydens die verloop van die proef bestudeer. Geen ooreenkomste in mikrobiese gemeenskap strukture in die forel SVK is waargeneem tussen die verskillende voer behandelings nie, maar daar is wel ooreenkomste gevind tussen die mikrobiese gemeenskappe van visse by spesifieke tyd intervalle (Hoofstuk 4).

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Stellenbosch University

Department of Microbiology

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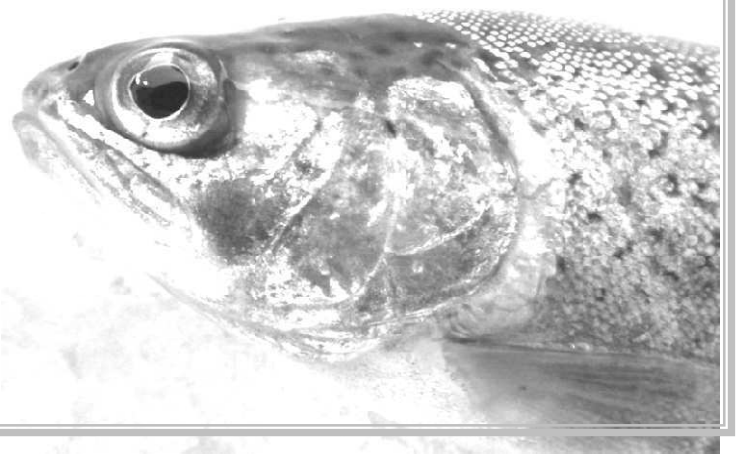
Chapter 1

Literature review



Literature review

1.1 Overview of aquaculture worldwide

Aquaculture species are vital sources of protein for people all over the world (Domingo *et al*., 2007; Sargent, 1997). It is estimated by the Food and Agriculture Organisation (FAO, 2000), that about one billion people rely on these species as their main animal protein source. In North America and Europe 10% of animal protein consumed is fish, 17% in Africa, 22% in China and 26% in Asia (Tidwell and Allan, 2001). Over-exploitation of the ocean's fish resources in the past has led to a global decline in ocean fisheries stock. The increasing demand for protein by the growing world populations, threatens the sustainability of commercial fisheries (Garcia and Rosenberg, 2010; Sargent, 1997). For these reasons there has been a rapid growth in aquaculture globally (Garcia and Rosenberg, 2010).

Aquaculture can be defined as an enclosure of fish, crustaceans, molluscs or aquatic plants in secure systems under optimum growth conditions (Naylor *et al.,* 2000). Other than providing food, aquaculture also helps to preserve aquatic biodiversity, reduces the pressure on fish resources and helps to protect natural habitats and ecosystems (Tidwell and Allan, 2001). Furthermore, there are significant economic advantages to this industry such as job creation and providing foreign income for a country (Balcazar *et al*., 2006; Bostock *et al*., 2010; Wang *et al*., 2008). Various factors play a role in the development of aquaculture including market demand, where good demand and high prices are crucial for development. The availability of environmental resources such as sheltered bays or lagoons with suitable temperature and water quality also play an important role. Other factors include development of technology, investment of local, national or private institutes and profit gained through the industry (Muir, 1998).

The production of farmed fish and shellfish more than doubled in value and weight between 1987 and 1997 (Naylor *et al.,* 2000). The dominating region, Asia, is responsible for 89% by volume and 79% by value of the world's fish food supply (Fig. 1). Other regions include Africa (1.8% by volume and 2% by value), the Americas (4.6% by volume and 9% by value), Europe (4.4% by volume and 9% by value) and Oceania (0.3% by volume and 1% by value) (Bostock *et al*., 2010).

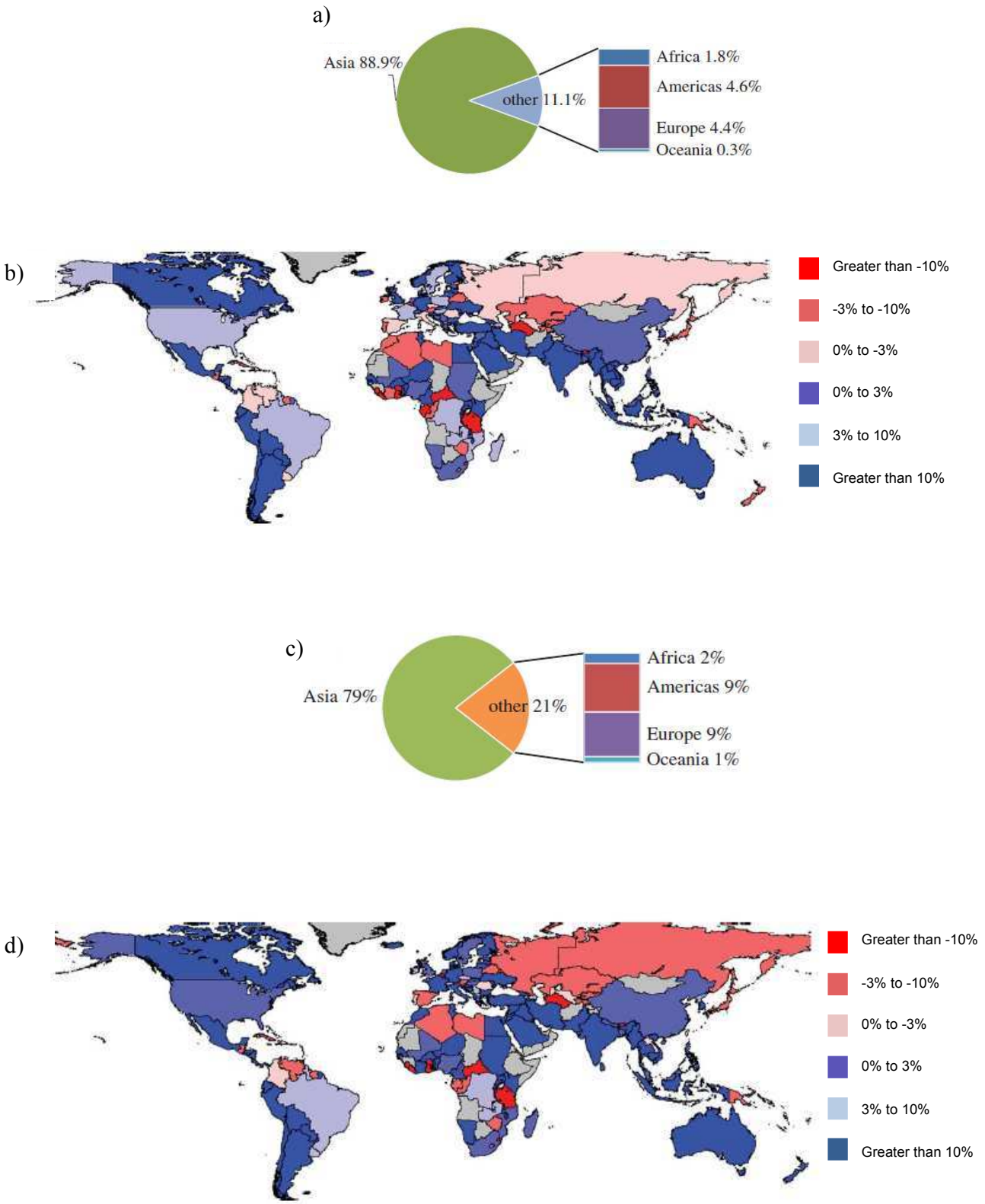


Figure 1 – *a*. Aquaculture production by quantity in 2008 *b.* Average annual growth rate of aquaculture production in terms of quantity. Values were calculated over a 5-year period using mean values from 2000-2002 and 2005-2007. *c.* Aquaculture production by value 2008. *d.* Average annual growth rate of aquaculture production in terms of value. Values were calculated over a 5-year period using mean values from 2000-2002 and 2005-2007 (Modified from Bostock *et al*., 2010).

In South Africa, aquaculture is also becoming very important (Brummett and Williams, 2000; Macey and Coyne, 2005; Ten Doeschate and Coyne, 2008). Aquaculture of both freshwater and marine farming is distributed across all nine provinces of the country (Fig. 2). The Western Cape contributes up to 43,8 % of the aquaculture industry in the country, followed by Mpumalanga, KwaZulu Natal and the Eastern Cape each with 12.5 %, Free State with 6.3 %, Gauteng with 4.7 %, Limpopo with 3.1 % and North West with 1.6 %. A variety of freshwater and marine species (Table 1) are farmed in South Africa. The dominant freshwater species farmed is rainbow trout, *Oncorhynchus mykiss* (Fig. 3a), contributing 44.4 % of freshwater species farmed in South Africa (Botes *et al*., 2006). The dominant marine species farmed is abalone (Fig. 3b) and contributes 68.4% of all marine producers in the country (Botes *et al*., 2006). *Haliotis midae* is the main abalone species farmed in South Africa and the country is currently the largest abalone producer outside Asia (Troell *et al.*, 2006).

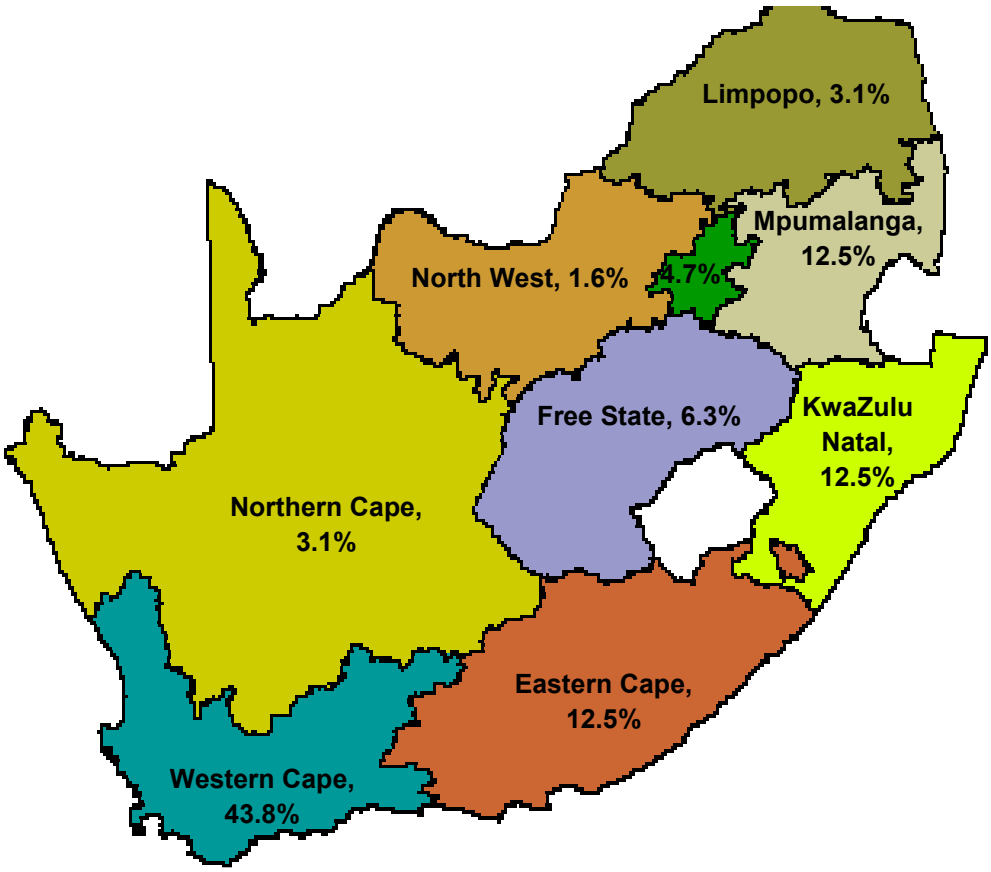


Figure 2 – Distribution of aquaculture (marine and freshwater) across the provinces of South Africa (modified from Botes *et al*., 2006).

Table 1: Aquaculture species farmed in South Africa (Botes *et al.*, 2006)			
Freshwater species	**% of freshwater species farmed**	**Marine species**	**% of marine species farmed**
North African Catfish (*Clarias garipinus*)	13.3	Abalone (*Haliotis midae*)	68.4
Finfish	20		
Carp (*Cyprinus carpio*)	11.1	Finfish	10.5
Goldfish (*Carrasius auratus*)	17.8	Kuruma prawn (*Penaeus japonicus*)	5.3
Ornamental fish (Ornamental fish spp.)	24.4		
Ornamental fish tropicals (Ornamental fish spp)	15.6	Mediterranean mussel (*Mytilus galloprovinvialis*)	5.3
Rainbow Trout (*Oncorhynchus mykiss*)	44.4		
Largemouth black bass (*Micropterus salmoides*)	2.2	Pacific cupped oysters (*Crassostrea gigas*)	26.3
Koi Carp (*Cyprinus carpio*)	24.4		
Marron crayfish (*Cherax tenuimanus*)	11.1	Seaweed (*Gracilaria* spp.)	10.5
Mozambique / Nile Tilapia (*Oreochromis mossambicus*/ (*Oreochromis niloticus*)	17.8		

a)

b)

Figure 3 – a) Dominating freshwater farmed species, rainbow trout (*Oncorhynchus mykiss*). b) Dominating marine farmed species, abalone (*Haliotis midae*).

1.2 Diseases in aquaculture

Significant economic losses often occur in aquaculture due to disease, floods, predation, chemical poisoning, oxygen depletions and theft (Barua and Sarker, 2010). Of these factors, disease causes the biggest economic losses in this industry (Meyer, 1991). Disease control is a multidisciplinary approach because of interactions amongst pathogens, the hosts and their environment. In order to control or prevent disease, understanding the biology of the host, characteristics of potential pathogenic organisms and factors in the environment that will affect these interactions, is important (Toranzo *et al*., 2005).

Diseases can be caused by a variety of different organisms including viruses, bacteria, fungi and parasites (Boudinot *et al.,* 1998; Brunt and Austin, 2005; Muraosa *et al*., 2009; Rimstad, 2011; Robertsen, 2011; Schreier *et al*., 1996; Toranzo *et al*., 2005). Viral diseases in salmon and trout are common and have been studied intensively due to significant economic losses in aquaculture (Boudinot *et al.,* 1998; Rimstad, 2011; Robertsen, 2011). Common viral diseases associated with these two aquaculture species include infectious hematopoietic necrosis, infectious pancreatic necrosis and viral haemorrhagic septicaemia. An effective treatment of viral infections is to quarantine and destroy all infected species to prevent the spreading of viruses. Vaccination is also used against viruses, but is not that effective in fish (Robertsen, 2011). Alternative treatments include probiotics but studies on the control of viral infections with probiotics have had variable results (Balcazar *et al*., 2006).

Fungal pathogens are known to grow on the surface of eggs and larvae of aquatic species, causing mortalities (Schreier *et al*., 1996). These pathogens are also secondary invaders in wounds, abrasions and lesions that are caused by pathogenic bacteria, unfavourable environmental conditions or rough handling (Muraosa *et al*., 2009; Willoughby *et al*., 1995; Willoughby and Pickering, 1997). Only a few parasitic species cause diseases in aquaculture. Protozoans are one of the most abundant parasitic organisms in aquaculture. These parasites are external parasites of finfish, causing wounds through their attachment. These wounds are also likely to be infected by bacterial or fungal parasites (Meyer, 1991; Wright and Colorni, 2002).

Bacterial pathogens cause the majority of diseases in aquaculture. Diseases of finfish are mainly caused by Gram negative bacteria, however, a few Gram positive bacteria are also known to cause diseases (Brunt and Austin, 2005; Egidius, 1987; Hilger *et al*., 1991; Meyer, 1991; Toranzo *et al*., 2005). Systemic diseases usually cause high mortality rates, although fish often have a healthy external appearance. In contrast, some diseases cause significant external lesions such as ulcers, but have a relative low mortality rate (Toranzo *et al*., 2005). Major bacterial diseases in aquaculture include vibriosis, photobacteriosis, "winter ulcer", furunculosis, marine flexibacteriosis, pseudomonadiasis, streptococcosis, bacterial kidney disease, fish tuberculosis (mycobacteriosis) and piscirickettsiosis (Toranzo *et al*., 2005) (Table 2).

Bacterial diseases are usually controlled by the use of antibiotics as a preventive measure. Some commonly used aquaculture antibiotics include ampicillin, chloramphenicol, streptomycin, erythromycin, tetracycline, sulfonamides and enrofloxacin (Heuer *et al*., 2009; Hirsch *et al*., 1999). Antibiotics are usually given as a component of fish food, but can sometimes be injected or added to baths (Cabello, 2006). After antibiotics are ingested by the fish, it moves from the esophagus through the stomach to the gastrointestinal tract (GIT). In the GIT some of the antibiotics are absorbed, metabolized and circulated through the fish body. However, a large percentage of the agent is excreted through faeces and urine (Rigos and Troisi, 2005). It has been estimated that about 75% of antibiotics used in feed are lost to the environment (Lalumera *et al*. 2004). Fish faeces and unconsumed food containing antibiotics reach the sediment at the bottom of the tanks/dams, resulting in the leaching of antibiotics into the sediment. Currents may carry these sediments to other parts of the aquatic environment, creating selective pressure for natural occurring microbial communities to develop antibiotic resistance (Cabello, 2006).

One of the major concerns of spreading antibiotics in aquatic environments is the development of antibiotic resistance in bacteria (Farzanfar, 2006). Studies found that more than 70 % of bacteria found in aquatic environments are resistant to at least one antibiotic (Hirsch *et al*., 1999). Furthermore, many bacteria have been shown to have resistance to multiple antibiotics. A major concern of antibiotic resistant bacteria in aquatic environments is the transfer of resistance between aquatic and terrestrial environments (Sørum, 1998). The spread of antibiotic resistance through aquatic activities, therefore, holds a significant risk to human health. An example of this transfer of antibiotic resistance

genes is from a wellknown aquaculture pathogen *Aeromonas* sp. to a human pathogen *Escherichia coli* (Heuer *et al.,* 2009). The transfer of the antibiotic resistance gene, *tet*(S), from the fish pathogen *Lactococcus garvieae* to the human pathogen *L. monocytogenes* was also demonstrated (*in vitro*) (Gugliemetti *et al*., 2009).

Table 2 - Major bacterial diseases in aquaculture

Disease	Bacterium	Symptoms of host	Reference
Vibriosis	*Vibrio ordalii,* *V. salmonicida,* *V. vulnificus,* *Listonella anguillarum* *(formerly V. anguillarum)*	Haemorrhages, superficial skin lesions, septicaemia, pale gills which reflects anaemia, anorexic fish.	Egidius, 1987; Toranzo *et al.*, 2005
Photobacteriosis	*Photobacterium damselae*	White nodules in the internal viscera, specific spleen and kidney.	Magarinos *et al*., 2000; Toranzo *et al*., 2005
"Winter ulcer"	*V. viscosus,* *V. wodanis*	Skin ulcers on scale-covered parts, mostly on the sides.	Lunder *et al*., 2000 Toranzo *et al.*, 2005
Furunculosis	*Aeromonas salmonicida*	Haemorrhagic septicaemia, liquefactive necrosis and deep ulcerative lesions.	Bast *et al*., 1988; Toranzo *et al.*, 2005
Flexibacteriosis	*Tenacibaculum maritimum* (formerly *Flexibacter maritimus*)	Tail rot, eroded and haemorrhagic mouth, skin lesions, frayed fins.	Hilger *et* al., 1991; Toranzo *et al.,* 2005
Pseudomonadiasis	*Pseudomonas chlororaphis,* *P. anguilliseptica,* *P. anguilliseptica,* *P. fluorescens,* *P. putida,* *P. plecoglossicida*	Abdominal distension, haemorrhagic petechia in the skin and organs.	Toranzo *et al*., 2005
Streptococcosis	*Lactococcus garvieae* (formerly *Enterococcus seriolicida*), *Streptococcus iniae,* *S. parauberis,* *S. phocae*	Hemorrhages in the eye chamber, inside the opercula and at base of fins, pale livers and bloody peritoneal fluid.	Brunt and Austin, 2005; Schoonbee and Smit, 1979; Toranzo *et al*., 2005
Bacterial kidney disease	*Renibacterium salmoninarum*	Exophthalmia, abdominal distension and pethechial haemorrhage.	Toranzo *et al.*, 2005
Fish tuberculosis (mycobacteriosis)	*Mycobacterium marinum*	Scale loss, greyish-white nodules in th liver, spleen and kidney.	Majeed *et* al., 1981; Toranzo *et al*., 2005
Piscirickettsiosis	*Piscirickettsia salmonis*	Lethargy, anorexia, darkening of skin and respiratory distress.	Fryer and Hedrick, 2003; Toranzo *et al.*, 2005

The use of antimicrobial agents in aquaculture is questioned due to damaging effects on animal and human health (Cabello, 2006; Guglielmetti *et al*., 2009; Hirsch *et al*., 1999; Wang *et al*., 2008). This has led to the search for probiotics as an alternative way to control bacterial diseases in aquaculture.

1.3 Probiotics in aquaculture: definition

Probiotics are commonly defined as living microorganisms which have a positive effect on the health of the host by improving the properties of its microflora (Farzanfar, 2006; Verschuere *et al*., 2000). However, the definition of probiotics needs to be modified when referring to aquatic environments. Probiotic bacteria administered to terrestrial animals depend on the relatively moist GIT environment of their host. These probiotic bacteria normally colonize the GIT of the host and when these bacteria are excreted they usually do not re-enter the host (Verschuere *et al*., 2000; Kesarcodi-Watson *et al*., 2008; Harris, 1993). In contrast, probiotic bacteria administrated in aquatic environments are able to survive and sometimes proliferate in the surrounding aqueous environment, independent of the host (Kesarcodi-Watson *et al*., 2008). Thus, the aquatic host is continuously exposed to the probiotic bacteria although the bacteria may not actively colonize the gut. The definition of probiotics used in aquatic environments can, therefore, be modified to be a live microbial supplement which has a beneficial effect on the host by altering the microbial communities associated with the host (microflora) as well as the immediate environment (Verschuere *et al*., 2000).

The use of probiotics in terrestrial vertebrates such as pigs, chickens and humans, have been studied extensively (Heilig *et al.,* 2002; Ishibashi and Yamazaki, 2001; Schiffrin *et al*., 1997;). Probiotics for terrestrial animals have been applied in aquaculture but with variable success (Venkat *et al*., 2004). Studies found that probiotics isolated from environments in which it will be applied, such as aquatic environments, yield better results. In order to obtain potential probiotics for aquaculture, candidate bacteria should ideally be isolated from the GIT of aquatic species (Austin *et al*., 1995; Gram *et al*., 1999; Macey and Coyne, 2006; Vine *et al*., 2004). Research on the use of probiotics in aquaculture is growing rapidly and show promising possibilities (Burr and Gatlin, 2005).

1.4 GIT microflora and interactions with aquatic hosts

The majority of bacteria isolated from the GIT of aquatic animals are facultative anaerobes that include species such as *Vibrio*, *Micrococcus* and *Pseudomonas* (Harris *et al*., 1991). Some studies have also reported the isolation of aerobes (Klug and Kotarski, 1980) and obligate anaerobes (Sugita *et al*., 1987). Isolates included both motile and non-motile species (Harris *et al*., 1991). Gram negative bacteria are commonly found in the GIT of aquatic animals (Harris *et al*., 1991; Klug and Kotarski, 1980), however, some Gram positive bacteria such as *Staphylococcus*, *Lactobacillus, Pediococcus*, *Carnobacterium* and *Bacillus* species have also been isolated (Sugita *et al*., 1987). A variety of associations occur between GIT microflora and their aquatic host (Fig. 4) (Harris, 1993).

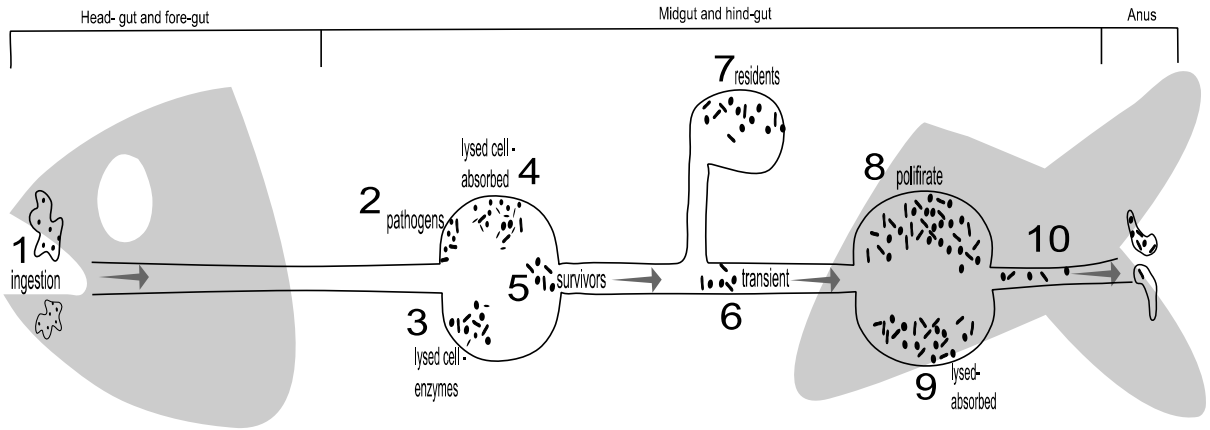


Figure 4 – Schematic diagram of different associations occurring between GIT microflora and their invertebrate hosts. 1 – Ingestion of bacteria, 2 – pathogenic bacteria, 3 – cells lysed and absorbed as nutrients, 4 – cells lysed and enzymes are released that are active in the GIT, 5 – survivors pass through the GIT, 6 – transient bacteria, 7 – resident bacteria which attached to the membrane and form symbiotic relationships, 8 – proliferation of transient bacteria, 9 – transient bacteria lysed and absorbed or enzymes released, 10 – faeces are excreted with either high bacterial numbers or low bacterial numbers (Adapted from Harris, 1993).

Bacteria present in food and the environment are ingested by the host. Some of these ingested bacteria are opportunistic pathogens which can cause disease in the host under the right conditions. Bacteria ingested can also be lysed by lytic enzymes, bile and gastric acids present in the GIT of the host (Seiderer *et al*., 1987). Nutrients of these lysed bacteria can be absorbed by the host and active bacterial enzymes released, can aid in

digestion or defense against pathogens (Anand *et al*., 2009; Erasmus *et al*., 1997; Harris *et al*., 1993; Izvekova, 2005; Izevekova and Komova, 2005). Identifying bacterial enzymes present in the GIT is a challenge due to difficulties in distinguishing between bacterial and host enzymes. One method of identifying bacterial enzymes is to isolate pure bacterial cultures from the GIT and perform plate assays for degradative enzymes (Erasmus *et al*., 1997). Commonly found enzymes include chitinase, protease, lipase, agarase and gelatinase. However, it is uncertain if the isolates are able to produce the enzymes when in their natural environment as GIT microflora (Harris *et al*., 1991).

Bacteria which survive in the GIT can either be transient or resident bacteria (Fig. 4). Transient bacteria can pass through the GIT and are excreted through the faeces or proliferate in the GIT. Proliferating cells can either be lysed and their nutrients absorbed or they can release their active enzymes in the GIT (Anand *et al*., 2009; Izevekova and Komova, 2005). High bacterial counts in the faeces may be the result of transient bacteria which passed through the GIT, where low numbers may be an indication of attachment and proliferation of transient bacteria in the GIT (Harris *et al*., 1991). Bacteria able to attach and colonize the membrane of the GIT to form symbiotic relationships with the host are known as resident bacteria (Harris *et al*., 1991). In most cases, these bacteria are considered potential probiotics (Balcazar *et al*., 2008) because colonization of the GIT allows the bacteria to continue with their probiotic functions (Vine *et al*., 2004).

Many factors influence the presence of bacteria in the GIT of the host (Harris, 1993). An important factor is the GIT structure of the host. In general, the GIT of fish begins at the mouth opening and extents to the anus. The GIT is divided into functional units namely the head-gut, fore-gut, midgut, hind-gut and anus. The head-gut includes the buccal and throat cavity, but there are usually no defined borders between these structures. The esopagus and stomach form part of the fore-gut. The stomach is usually very poorly defined and even absent in some species, while the midgut is the longest portion of the intestine. It usually consists of a series of complicated loops that are unique to a species. This section begins at the pylorus and is often continuous with the hind-gut. The posterior end of the hind-gut forms the anus (Al-Hussaini, 1949; Harder, 1975).

The structure of fish GIT, vary highly between different species and is mostly influences by different feeding habits. Five main feeding habits occur namely carnivory, omnivory, herbivory, macrophagy or microphagy (Stevens and Hume, 1998). The most commonly

farmed fish in South Africa are either carnivores, e.g. rainbow trout, *Oncorhynchus mykiss* (Geurden *et al*., 2007) or herbivores such as tilapia, *Oreochromis mossambicus* (Adams *et al*., 1988). The physiology of the GIT differs greatly between these two groups (Fig. 5) (Buddington *et al*., 1987). Herbivores have a much longer mid- and hind-gut section with a series of loops (Al-Hussaini, 1949). In contrast, carnivores have a shorter and straighter mid- and hind-gut section. Diet and physiology of fish, play an important role in the selection of resident bacterial communities. For instance, longer tracts prevent bacteria from being washed out of the GIT. Furthermore, the cell lining of the GIT also plays a role in the attachment of bacteria. Each section of the tract is lined with different cells types, performing specific functions such as secretion of compounds in the GIT. These compounds together with factors such as water quality can change conditions inside the GIT. Chemical and physical conditions inside the GIT known to affect bacteria include pH, salinity, oxygen levels and available nutrients in the GIT, redox potential and toxic substances produced in the GIT (Harris, 1993; Spanggaard *et al*., 2000).

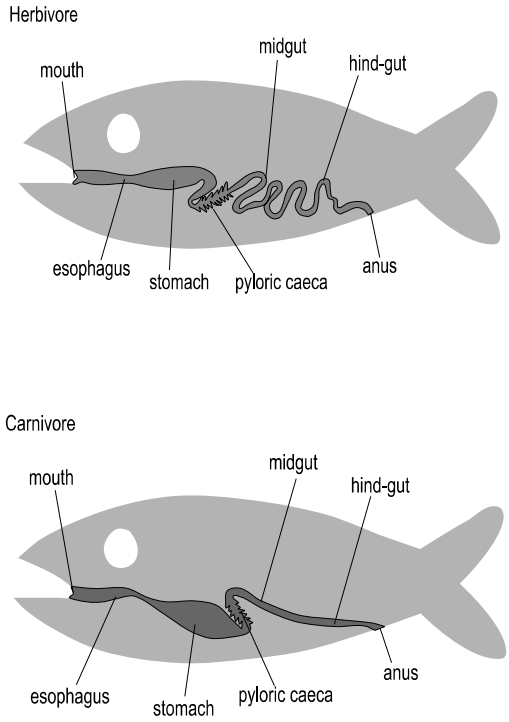


Figure 5 – Differences between herbivous and carnivours fish GITs.

1.5 Mechanisms of probiotic actions

Probiotics have different modes of action, for instance by enhancing the immunity of the host. It can also affect other microorganisms and produce of a variety of different beneficial and antagonistic compounds (Cleveland *et al*., 2001; Kümmerer, 2009; Oelschlaeger *et al*., 2010; Verschuere *et al*., 2000; Wang, 2007). Some of the mechanisms of probiotics are discussed below.

i. Competition with pathogenic bacteria

Probiotic bacteria can eliminate or reduce colonization of the host by pathogenic organisms through the secretion of antagonistic compounds (Balcazar *et al*., 2006; Verschuere *et al*., 2000). These compounds can be defined as chemical substances produced by microorganisms that inhibit or are toxic towards other microorganisms in the surrounding environment. Antagonistic compounds include the production of secondary metabolites such as antibiotics and bacteriocins, which are produced by some bacteria to kill or inhibit the growth of surrounding bacteria, fungi and viruses (Cleveland *et al*., 2001; Kümmerer, 2009).

Bacteriocins are antimicrobial proteins synthesized by the ribosomes. Genes encoding for bacteriocin proteins can be transferred between bacteria through transposons and plasmids. Bacteriocins can be divided into the following three classes (De Vuyst and Leroy, 2007; Garneau *et al*., 2002; Parente and Ricciardi, 1999):

Class I: Lantibiotics, contains amino acids such as lanthionines or β-methyllanthionine.

Class II: Non-lantibiotics that are subdivided into:

a. pediocin-like bacteriocins,
b. two-peptide bacteriocins
c. Circular bacteriocins

Many of the bacteriocins produced by lactic acid bacteria belong to the first two classes, lantibiotics (class I) and non-lantibiotics (class II) (Garneau *et al*., 2002; Parente and Ricciardi, 1999). Class III are large, heat-labile, lytic proteins (De Vuyst and Leroy, 2007; Garneau *et al*., 2002; Parente and Ricciardi, 1999).

Another well known antagonistic mechanism is the production of metabolites that can inhibit the growth of other microorganisms. Species such as the lactic acid bacteria do not utilize the cytochrome system and, therefore, do not reduce oxygen to water. This often

results in the formation of H_2O_2 that kill or inhibit surrounding bacteria, especially bacteria with no or low catalase peroxidases or other H_2O_2-scavenging enzymes (Eschenbach *et al*., 1989). Other bacteria produce organic acids, resulting in a change of pH in the GIT that inhibits or slows the growth of pathogens (Sugita *et al*., 1997).

The production of antagonistic compounds is not the only way probiotic bacteria are able to compete with pathogens. Alternative strategies include better growth characteristics of the probiotic species, such as a shorter lag period and doubling time (Vine *et al*., 2006). Probiotics can also compete with pathogens for adhesion sites where they can proliferate in the GIT and this may also increase the production of antagonistic compounds (Verschuere *et al*., 2000). Probiotics also compete against pathogens for nutrient sources (Vine *et al*., 2006). Competition for nutrients plays an important role in the composition of the GIT microflora of animals. The microflora of aquatic animals usually consists of heterotrophs, competing for organic substrates for energy and carbon (Verschuere *et al*., 2000).

Many studies have demonstrated the inhibitory effect different probiotic species have on common aquaculture pathogens (Austin *et al*, 1995; Austin and Billaud, 1990; Harzevili *et al*, 1998; Rengpipat *et al*., 1998; Ten Doeschate and Coyne, 2008). *Pseudomonas fluorescens* (AH2) inhibits the pathogen *Vibrio anguillarum* in rainbow trout, and the use of the probiotic significantly reduced the mortality rate of fish infected by *V. anguillarum* (Ten Doeschate and Coyne, 2008). It was also shown that *Lactococcus lactis* inhibits *V. anguillarum* (Harzevili *et al*., 1998). In Shrimp, the probiotic *Bacillus* S11, isolated from black tiger shrimps, showed an almost four-fold increase in the survival rate after infection by *V. harveyi*, compared to the control groups that did not receive any probiotic (Rengpipat *et al*., 1998). Other probiotics include *Planococcus*, isolated from sea water, which inhibits an Atlantic salmon pathogen, *Serratia liquefaciens* (Austin and Billaud, 1990). *Vibrio alginolyticus* inhibits *Aeromonas salmonicida*, *Vibrio anguillarum* and *V. ordalii* which are all aquaculture pathogens (Austin *et al*., 1995).

ii. Production of beneficial compounds

Probiotic bacteria produce a variety of different compounds beneficial to the host such as enzymes, vitamins, proteins, short-chain fatty acids and carotenoids. Through the production of extracellular enzymes, probiotic bacteria aid in the digestion processes of the host (Wang, 2007). The most common reported enzymes produced by bacteria isolated

from the GIT of aquatic animals include cellulase, protease, lipase and chitinase. Less commonly isolated enzymes include amylase, endoglucanase, cellulase, agarase, lysozyme and alginase (Harris, 1993). In a study done on Panaeid shrimp, several species of bacteria were isolated and screened for enzymes that are known to aid in the digestion processes inside the host (Dempsey and Kitting, 1987). These species were tested *in vitro* for the production of cellulase, lipase, amylase, gelatinase and chitinase (Table 3). Through the production of these enzymes, it is suggested that these bacteria play an important role in the digestion processes of the host. However, *in vivo* studies still need to be done in order to confirm if these bacterial species also produce these enzymes inside the host (Dempsey and Kitting, 1987).

Table 3 - Enzymes produced by GIT microflora isolated from Panaeid shrimp (modified from Dempsey and Kitting, 1987)

Bacterium	Enzyme produced
Alteromonas	Lipase, amylase, gelatinase
Cytophaga	Cellulase, amylase, gelatinase and chitinase
Flavobacterium	Cellulase, lipase, amylase and gelatinase
Moraxella	Lipase, amylase, gelatinase
Photobacterium	Lipase, gelatinase
Pseudomonas	Lipase, amylase, gelatinase
Vibrio	Lipase, amylase, gelatinase

Probiotic bacteria can also contribute to the well-being of their host through the production of beneficial compounds such as vitamins. Vitamins are known to be produced by some bacteria as secondary metabolites (Baya *et al*., 1981; Croft *et al*., 2005; Merchie *et al*., 1997; Poston, 1964; Sugita *et al*., 1991; Vine *et al*., 2006). This could only be observed in fish fed on a vitamin B_{12} deficient diet (Vine *et al*., 2006). Bacteria able to produce vitamin B_{12} were isolated from rainbow trout, tilapia and catfish (Sugita *et al*., 1991). The production of vitamin K by bacteria isolated from fish is also well known (Poston, 1964). For the production of vitamin C from glucuronic acid, the majority of animals produce the enzyme gulonolactone oxidase. However, fish do not produce this enzyme and need to obtain vitamin C from their diet (Merchie *et al*., 1997). In this case, vitamin C-producing bacteria can potentially be valuable as a dietary supplement and a possible probiotic for fish.

The focus of research on the role of microorganisms in aquaculture is mostly on the use of probiotics and their ability to outcompete pathogens, however, other applications of microorganisms in aquaculture are also possible. Certain fish species, such as trout and salmon are unable to synthesize carotenoids (Martin *et al*., 2008) and obtain the pigments from their natural diets. However, under aquaculture conditions fish do not come in contact with these natural pigments which results in dark gray meat which is unappealing for consumers (Higuera-Ciapara *et al*., 2006). It is, therefore, important to include these carotenoids in the diet of certain aquaculture fish species such as salmonids, red seebream, trout, lobster and shrimp. The more red the meat of the fish, the more attractive it is for consumers, mostly because the fish look fresher, are better flavoured and of higher quality leading to a higher priced fish (Kurnia *et al.,* 2010).

Carotenoids included in the diet of aquaculture fish are usually chemically synthesised. However, studies found that naturally produced carotenoids by bacteria, fungi, algae, mosses and higher plants (Table 4), are more effective in enhancing pigmentation in aquaculture species.

Table 4: Natural sources of astaxanthin

Group	Species	Reference
Plant	*Adonis aestivalis*	Cunningham and Gantt, 2005
Microalgae	*Haematococcus pluvialis*	Ausich, 1997 Lorenz and Cysewski, 2000
	Chlamydomonas nivalis	Ausich, 1997
	Neochloris wimmeri	Ausich, 1997
Yeast	*Phaffia rhodozyma*	Ausich, 1997
Bacteria	*Paracoccus marcusii*	Lee *et al*., 2004
	Paracoccus carotinifaciens	Lee *et al*., 2004
	Paracoccus haeundaensis	Lee *et al*., 2004
	Brevibacterium linens	Guyomarc'h *et al.,* 2000
	Agrobacterium aurantiacum	Yokoyama *et al*., 1995

There are more than 600 known carotenoid pigments known (Olson and Krinsky, 1995). Two of the most important groups are carotenes and xanthophylls (Fig. 6). Carotenes contain only carbon and hydrogen while xanthophylls also contain oxygenated derivatives. Xanthophylls can further be divided three groups. The first group is known as zeaxanthin, where oxygen is present as an OH-group. In the second group, canthaxanthin, oxygen is present as keto-groups (C=O) and in the third group, astaxanthin, both these groups (OH and C=O) are present (Higuera-Ciapara *et al*., 2006; Martin *et al*., 2008; Tapiero *et al*.,

2004). All carotenoids are derived from lycopene which is an open chain hydrocarbon containing two non-conjugated and eleven conjugated double bonds (Ausich, 1997). Each of these double bonds can exist in two configurations known as geometric isomers *cis* and *trans*. In nature, most of the carotenoids are *trans* isomers which are thermodynamically more stable than *cis*-isomers. Each molecule also has two chiral centers, C-3 and C-3'. Thus, considering astaxanthin, it may consist of three configurational isomers, two enantiomers (3S, 3'S and 3R,3'R) and a meso form (3R, 3'S). From these three isomer configurations, the most abundant one in nature is the 3S,3'S. Naturally produced astaxanthin can also be found in association with other compounds such as fatty acids and proteins, depending on the source of the pigment. For example, astaxanthin produced by algae is always bound to lipids, while synthetic astaxanthin, is not associated with any compound (Higuera-Ciapara *et al*., 2006).

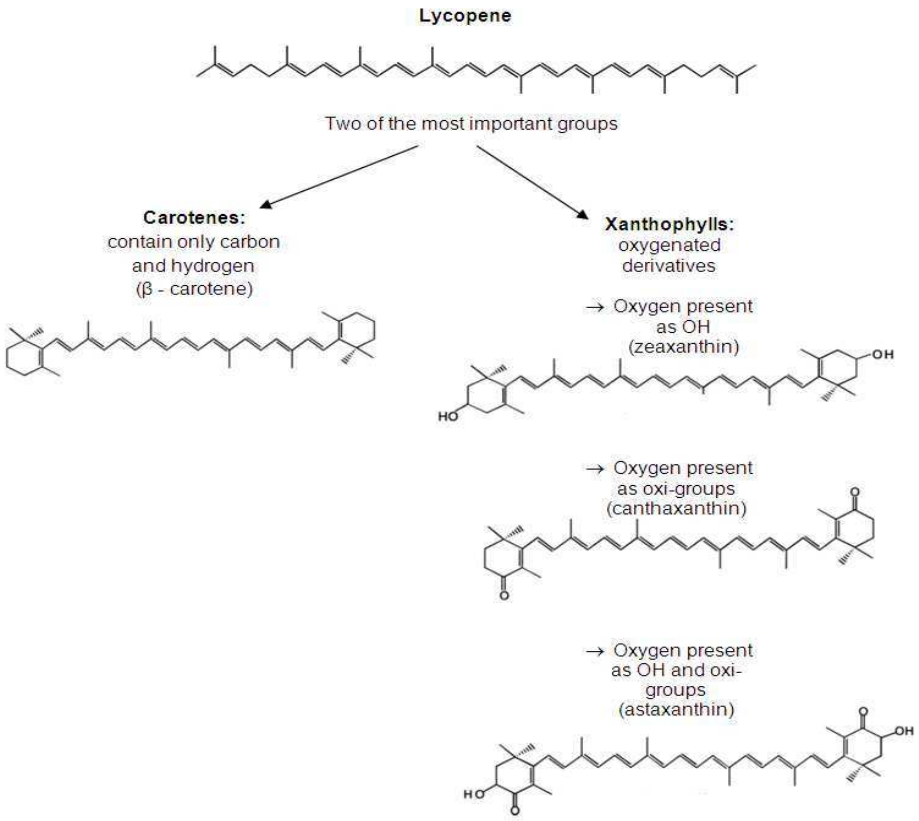


Figure 6 – Flow diagram representing two of the most important groups of carotenoids, carotenes and xanthophylls (Modified from Higuera-Ciapara *et al*., 2006; Martin *et al*., 2008; Tapiero *et al*., 2004).

iii. Enhancement of host immune response against pathogens

The role of probiotics in enhancing the immune response has extensively been studied in humans and animals, including fish. Certain bacterial compounds are known to act as

immunostimulants (Verschuere *et al*., 2000). These compounds elicit different host responses namely phagocytic activity and lysozyme production. Phagocytic activity plays an important role in antibacterial defences. Before antibodies are produced, phagocytic activity serves as an early activator of the inflammatory response. Probiotics used in aquaculture that are able to stimulate phagocytic cells in the host, include the lactic acid bacteria *Lactobacillus acidophilus*, *L. rhamnosus* and *L. lactis* (Nayak, 2010). Pirarat *et al*. (2006), showed that *L. rhamnosus* offer protection against a common bacterial *Edwardsiella tarda* infection in tilapia. Fish mortality was significantly lower in the experimental group that were fed probiotics compared to the control group. Subsequent studies indicated an enhanced phagocytic ability of host immunological cells (Pirarat *et al*., 2006). Furthermore, studies done on rainbow trout (*Oncorhynchus mykiss*) also showed enhanced immune phagocytic cell activity when the fish were fed other strains of probiotics (Villamil *et al*., 2002).

Lysozyme is an essential bactericidal enzyme of innate immunity and plays a vital role in defence against diseases in fishes (Nayak, 2010). The enzyme hydrolyses *N*-acetylmuramic acid and *N*-acetylglucosamine, constituents of peptidoglycan in the cell walls of bacteria (Balcázar *et al*., 2007). It was found that probiotic bacteria such as *Carnobacterium maltaromaticum*, *C. divergens* and *L. rhamnosus* are able to increase the lysozyme activity in rainbow trout (*O. mykiss*) (Kim and Austin, 2006). In brown trout (*Salmo trutta*), probiotic bacteria *Lactococcus lactis* and *Leuconostoc mesenteroides* also showed an increased lysozyme activity (Balcázar *et al*., 2007). This increased lysozyme levels then acts against infectious agents by attacking their cell walls causing lysis of the cells (Grinde *et al*., 1988).

iv. Antiviral effects

Aquaculture species are not only vulnerable to fungal and bacterial diseases, but also a number of viral diseases. However, viruses can be inactivated by biological substances such as extracellular agents of probiotic bacteria, although the exact mechanism of inactivation is not clear (Wang *et al*., 2008). Bacteria (*Vibrio* sp., *Pseudomonas* sp. and *Aeromonas* sp.) isolated from salmonid hatcheries showed antiviral activities against infectious hematopoietic necrosis virus (IHNV). Also, *Moraxella* showed antiviral capacity that is specific for poliovirus (Balcazar *et al*., 2006). Two dominant aquaculture bacterial strains found in shrimp hatcheries, *Vibrio* NICA 1030 and NICA 1031, were also tested for antiviral activity. *Vibrio* cells were exposed to IHNV and *Oncorhynchus masou* virus

(OMV). Researchers found that both these strains produce antiviral substances against these viruses (Direkbusarakom *et al*., 1998). Not much research has been done on the use of bacteria as antiviral agents and this field holds potential for further research in the future.

1.6 Development of probiotics

The selection and development of a safe and effective probiotic is a multistep process (Fig. 7) (Verschuere *et al*., 2000; Vine *et al*., 2006). Research for probiotics is usually prompted by the need for alternative treatments for diseases. After an in-depth literature review, the first step is to isolate potential probiotic bacterial strains from suitable environments. These strains should preferably be isolated from the environment where they will be applied. However, there have been reports where probiotics isolated from terrestrial animals were successfully used on aquatic animals (Planas *et al*., 2004).

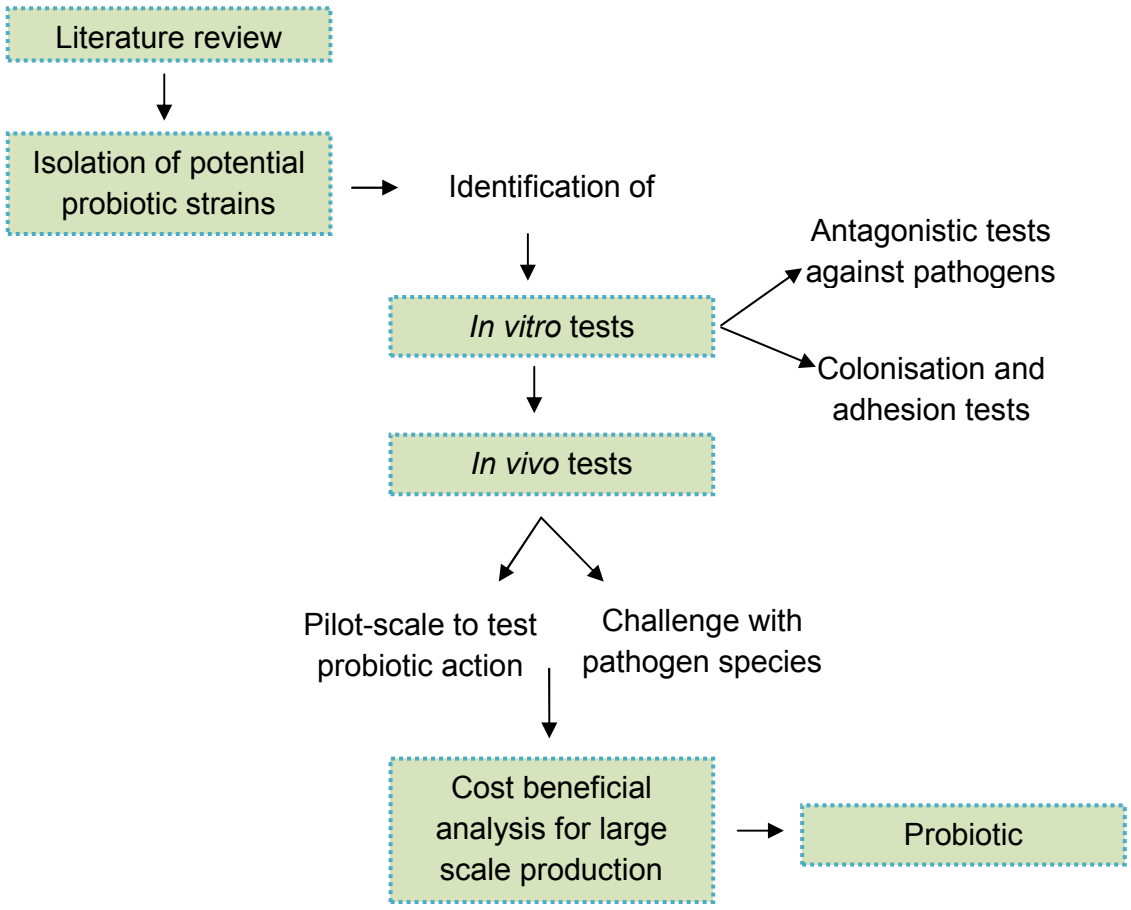


Figure 7 - Multistep process for the development of an aquaculture probiotic (adapted from Verschuere *et al*., 2000; Vine *et al*., 2006)

After the identification of isolates, *in vitro* tests must be performed in order to screen and preselect potential probiotic bacteria that will be examined further. A common *in vitro* test is the antagonism test that determines the capability of a strain to compete with pathogens. It is assumed that strains that are able to compete with pathogens *in vitro* can be considered as potential probiotics. However, it is not known if strains which are unable to compete with pathogens *in vitro* have the potential to act as probiotics. Therefore, antagonism tests should be interpreted with caution (Verschuere *et al*., 2000).

The type of growth medium plays an important role in the production of inhibitory metabolites by bacteria (Olsson *et al*., 1992). Strains unable to compete with pathogens *in vitro*, may be able to compete with pathogens *in vivo*. Thus, in *in vivo* studies, the selected aquaculture species can be challenged with a pathogen. If the survival rate is higher in probiotic-treated hosts than in untreated hosts, the potential probiotic may be antagonistic against pathogens. Adhesion tests are also performed *in vitro* and these tests determine the ability of a strain to adhere to intestinal cells and to inhibit pathogens. This test is commonly used for selecting probiotics for humans, however, this test is seldom used in aquaculture. After selecting potential probiotic strains, a small pilot-scale trial should be performed to determine the probiotic action of the strain *in vivo* (Verschuere *et al*., 2000).

Various methods are used to apply probiotics to aquaculture fish species. Probiotics can be added to the artificial diet or the water, through bathing or via live food (Verschuere *et al*., 1999). A perfect probiotic is considered to be non-pathogenic to the host, adhere and survives in the GIT of the host, stimulate the immunity of the host, produces beneficial compounds and remain viable under storage (Farzanfar, 2006). Finally, if the strain was found to be a successful probiotic, cost beneficial analysis for large scale production of the probiotic should be done. It is important to keep probiotic production costs low in order to be commercially competitive (Verschuere *et al*., 2000).

1.7 Probiotics considered for use in aquaculture

By applying probiotics in aquaculture the use of antibiotics in this industry may be reduced. Therefore, the search for effective probiotics is important for sustainable aquaculture practises. To date a wide range of probiotics have been evaluated for use in aquaculture. These organisms include both Gram positive and Gram negative bacteria (Table 5), as well as yeasts.

Table 5 - Bacterial species used as probiotics				
	Isolate	**Source**	**Probiotic action**	**Reference**
Gram positive				
Lactic acid bacteria	*Carnobacterium* sp.	Rainbow trout (*Oncorhynchus mykiss*)	Controlling furunculosis	Irianto and Austin, 2002
	Carnobacterium sp.	GIT of Atlantic salmon (*Salmo salar* L.)	Inhibits the growth of two common fish pathogens *Aeromonas salmonicida* and *Vibrio anguillarum*	Jöborn *et al*., 1997
	Streptococcus	Salmonids	Not tested as a probiotic	Trust and Sparrow, 1974
	Streptococcus faecium M74	GIT of carp (*Cyprimus carpio*)	Inhancement of growth and feed conversion ratio	Bogut *et al.,* 1998
	Pediococcus sp. Ab1	GIT of the abalone *H. gigantean*	Increase alginate lyase and VSCFAs (volatile short chain fatty acids)	Iehata *et al*., 2010
	Lactococcus lactis AR21	Rotifer mass culture	Inhibitory effect against *Vibrio anguillarum* and enhancment of growth	Harzevili *et al*., 1998
Other	*Bacillus* S11	Black tiger shrimp (*Penaeus monodon*)	Compete with pathogens	Rengpipat *et al*., 1998
	Bacillus subtilis	Shrimp culture ponds	Antagonistic effect against *Vibrio* species	Vaseeharan and Ramasamy, 2003
Gram negative				
	Pseudomonas fluorescens	Unknown	antagonistic effect against the common fish pathogen *Vibrio anguillarum*	Gram *et al*, 1999
	Vibrio midae	GIT of South African abalone (*Haliotis midae*)	Enhancment of growth rate and disease resistance	Macey and Coyne, 2005
	Vibrio alginolyticus	Unknown	Reducing fish diseases caused by *Aeromonas salmonicida,Vibrio ordalii* and *Vibrio anguillarum*	Austin *et al*., 1995
	Aeromonas media	Pacific oyster (*Crassostrea gigas*)	Inhibitory effect against *Vibrio tubiashii*	Gibsona *et al*., 1998

i. Gram positive bacteria

This group of bacteria, especially lactic acid bacteria (LAB), are humans and terrestrial animals (Bomba *et al*., 2002; Heilig *et al.,* 2002; Ishibashi and Yamazaki, 2001; Schiffrin *et al*., 1997; Wang *et al*., 2004). Lactic acid bacteria are non-motile, non-sporulating, oxidase-and catalase-negative bacteria. Cell morphology can be coccobacilli, cocci or rods (Ringø and Gatesoupe, 1998). Lactic acid bacteria have a wide range of antimicrobial activity through the production of organic acids such as lactic and acetic acid. Some strains also produce ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin and reutericyclin. Bacteriocins and bacteriocin-like molecules are also produced by these bacteria (De Vuyst and Leroy, 2007).

The isolation of LAB from fish is hampered by the sub-dominance of these bacteria in fish (Balcazar *et al*., 2006, Titus, 2012). However, through a culture-independent method, 454 pyrosequencing, the presence of LAB in the GIT of trout and tilapia was confirmed. Members from the genera *Streptococcus*, *Aerococcus*, *Carnobacterium* and *Lactobacillus* were detected (Titus, 2012). Factors that play a role in isolating these bacteria from fish include the type of medium, incubation time and incubation temperature. Culture media usually used includes trypticase soy agar (TSA) and Muan, Rogosa and Sharpe (MRS). Studies found that the general optimum incubation temperature is between 20°C to 25°C. Incubation time for LAB is normally 2 to 3 days. However for isolation of LAB from coldwater fish, incubation for up to 4 weeks is recommended (Ringø and Gatesoupe, 1998).

Dominant LAB isolated from aquatic animals includes genera such as *Carnobacterium* (Irianto and Austin, 2002; Jöborn *et al*., 1997), *Streptococcus* (Bogut *et al.,* 1998; Trust and Sparrow, 1974), *Pediococcus* (Iehata *et al*., 2010) and *Lactobacillus* (Harzevili *et al*., 1998; Venkat *et al*., 2004). However, LAB is not the only Gram positive bacteria used as probiotics in aquaculture. *Bacillus* species have also been isolated from different aquatic environments and show promising results as probiotics (Rengpipat *et al*., 1998; Vaseeharan and Ramasamy, 2003).

ii. Gram negative bacteria

Gram negative genera that show potential as probiotics in aquaculture include *Pseudomonas* (Gram *et al*., 1999), *Vibrio* (Austin *et al*., 1995; Macey and Coyne,

2005; Macey and Coyne, 2006) and *Aeromonas* (Gibson *et al*., 1998). Although Gram negative bacteria are effective as probiotics, there are disadvantages in using this group. Most aquaculture pathogens are Gram negative. This makes transfer of antibiotic resistance genes between pathogenic and non-pathogenic bacteria more likely (Hall, 1997). Unlike most Gram positive bacteria, Gram negative bacteria are not able to produce endospores (Vine *et al*., 2006). These survival structures are differentiated cell types where the genome is surrounded by a cortex, a layer of modified peptidoglygan. The cortex is coated with a proteinaceous coat for the protection against lytic enzymes. Endospores are able to survive unfavourable physical conditions such as high temperatures, UV light and radiation. These structures are also able to withstand chemical substances including hydrogen peroxide and lysozyme (Enguita *et al*., 2003). Therefore, endospores makes culturing and storage of a probiotic bacterium such as LAB and other Gram positive bacteria easier. Compared to Gram positive bacteria, it is therefore more difficult to maintain viable cultures of Gram negative bacteria under storage conditions over a long period of time (Vine *et al*., 2006).

iii. Yeast

A less common group of organisms used as probiotics include yeast. Two yeast strains, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1, have been isolated from the GIT of *Haliotis midae* (Macey and Coyne, 2005). These two strains were added as a probiotic supplement to the feed of *H. midae*, and were shown to improve the survival and growth rate of the host. Also, these yeast isolates are able to survive and colonize the GIT of the host (Macey ad Coyne, 2006). Furthermore, *Debaryomyces hansenii* HF1 has also been isolated from the GIT of rainbow trout (Tovar *et al*, 2002). This strain has been incorporated as a dietary compound in sea bass larvae (*Dicentrarchus labrax*). *D. hansenii* HF1 was able to adhere to the GIT and increased the amylase secretion in the host (Tovar *et al*., 2002).

Phaffia rhodozyma has been studied intensively, especially for the production of the carotenoid astaxanthin (Andrewes *et al*., 1976; Schroeder and Johnson, 1993; Storebakken *et al*., 2004). Astaxanthin is an important pigment used in aquaculture for the pigmentation of some aquaculture species such as salmonids, red seabream, trout, lobster and shrimp (Guerin *et al.,* 2003; Higuera-Ciapara *et al*.,

2006). In Scholz *et al*. (1999) they evaluated the probiotic potential of *P. rhodozoma* to enhance vibriosis resistances in juvenile *Penaeus vannamei*. Yeast cells were included at 1% in the diet and were found to increase the weight of the shrimps and had a positive effect on their survival rate (Scholz *et al*., 1999).

1.8 Aim of this study

The aim of this study was to isolate potential probiotic bacteria from the gastrointestinal tract of the South African abalone (*Haliotis midae*). Furthermore, the aim was to evaluate the effect of these potential probiotic bacteria on rainbow trout (*Oncorhynchus mykiss*) and Mozambique tilapia (*Oreochromis mossambicus*).

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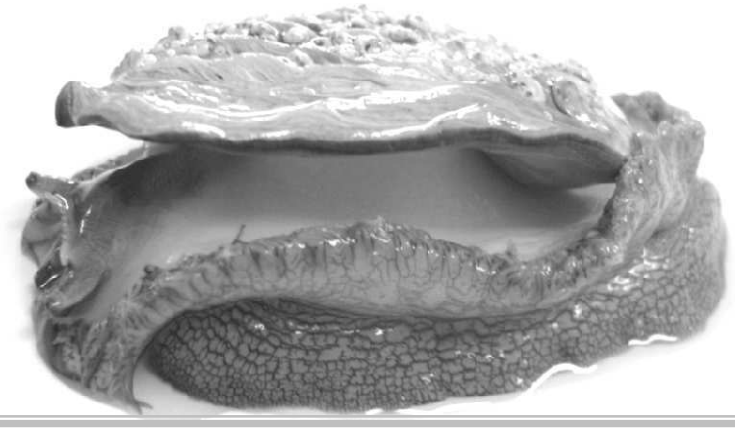
Chapter 2

Isolation of potential probiotic bacteria from the gastrointestinal tract of the South African abalone (*Haliotis midae*)



Abstract

The abalone industry in South Africa is becoming very important, providing jobs to many people and foreign income for the country. However, this industry faces two main problems, namely economic losses caused by diseases and the slow growth rate of abalone. One possible solution to these problems is the use of probiotics. The aim of this study, therefore, was to isolate potential probiotic bacteria from the gastrointestinal tract of the South African abalone, *Haliotis midae*. Nine different bacterial species were isolated and identified as *Corynebacterium variabilei*, *Staphylococcus carnosus, S. equorum, S. cohniii, Vibrio aestuarianus*, *V. nigripulchritudo, V. cyclitrophicus, Photobacterium leiognathi*, and *Paracoccus marcusii,* respectively. From these isolates, *P. marcusii* showed promising probiotic properties due to the production of astaxanthin. This bacterium was also found to grow optimally under conditions expected to be found in the gut of *H. midae. P. marcusii* was resistant to Trimethoprim, Streptomycin and Penicillin G and susceptible to Polymyxin B, Rifampicin, Norfloxacin, Chloramphenicol and Vancomycin. Based on the results, *P. marcusii* was chosen as a potential probiotic and possible pigment source in aquaculture and will be used in further studies.

Introduction

Abalone industry in South Africa

Globally the abalone industry is growing because harvesting of wild abalone is unsustainable. In South Africa the abalone industry is becoming very important, providing jobs to many local people and foreign income for the country (Macey and Coyne, 2005; Ten Doeschate and Coyne, 2008). Two of the main drivers for the development of this industry are over-exploitation of wild abalone stocks by poaching (Hauck and Sweijd, 1999) and high market prices. Favorable coastal waters, existing infrastructure and cheap labor also contribute to the rapid growth of the abalone industry in South Africa. Abalone farms are mainly located on the coast of the Western Cape Province, although there are some located on the coast of the Eastern and Northern Cape Province (Troell *et al*., 2006).

Problems associated with abalone farming and possible solutions

Economic losses caused by diseases are a problem in the abalone industry. The largest group of disease causing bacteria in abalone is from the family Vibrionaceae. The genus *Vibrio* includes more than 30 species and many are pathogenic (Pruzzo *et al*., 2005). Major abalone pathogens from this genus include *V. splendidus* and *V. harveyi* (Handlinger *et al.,* 2005; Nicolas *et al*., 2002). Disease outbreaks usually occur during the summer abalone spawning period, where there is a sharp increase in water temperature. During this period the abalone's energy reserves are limited and their immune systems are relatively suppressed (Travers *et al*., 2008). Other stress-related factors such as handling or poor water quality may also contribute to disease outbreaks (Handlinger *et al.,* 2005).

A number of fungi are also responsible for diseases in abalone. A Peronosporomycete, *Halioticida noduliformans,* was recently isolated from white nodules on the mantle of three abalone species. Peronosporomycetes infections of marine invertebrates cause many fatal diseases. In marine crustaceans such as shrimps and crabs, this fungus can lead to infertility. Other fungal species that are known to cause diseases in abalone are *Haliphthoros milfordensis*, *Halocrusticida awabi* and *Atkinsiella dubia* (Muraosa *et al.,* 2009).

In addition to diseases, the slow growth rate of abalone is another problem that the industry faces. Abalone have a complex life cycle consisting of free living trochophore larvae, free swimming veliger larvae, settling post larvae known as the spat, juvenile abalone and finally adult abalone (Fig. 1). The adult abalone spawn and the fertilized eggs again develop into free-living trochophore larvae (Fallu, 1991) in order to repeat the cycle. Under aquaculture conditions abalone grow faster than in their natural environments, but still needs four to five years to reach market size (*ca.* 80 g) (Macey and Coyne, 2005; Ten Doeschate and Coyne, 2008).

Attempts to reduce the loss of abalone due to disease include the use of antibiotics as treatment and in some cases as growth promoters. However, the use of antibiotics in aquaculture is questioned due to the spread of antibiotic resistance (Cabello, 2006; Farzanfar, 2006; Heuer *et al*., 2009; Hirsch *et al*., 1999). Therefore, the use of probiotics is considered to be a possible solution to these problems. Probiotics are live microbial supplements, which have a beneficial effect on the host by altering their microflora and/or the immediate environment (Verschuere *et al*., 2000) (Chapter 1). In aquatic animals the gastrointestinal tract is an open system that is always in contact with the surrounding water (Wang *et al*., 2008). The water environment contains a wide range of pathogenic and non-pathogenic bacterial strains (Schulze *et al*., 2006). Therefore, the main purpose of probiotics is to re-establish and maintain a beneficial symbiotic relationship between pathogenic and non-pathogenic microorganisms in the GIT of the abalone (Wang *et al*., 2008). Probiotic organisms are able to compete with pathogens through the production of antagonistic compounds (Balcázar *et al*., 2006), and better growth characteristics (Vine *et al*., 2006). They can also compete for adhesion sites in the GIT (Verschuere *et al*., 2000) or nutrient sources (Vine *et al*., 2006).

Gastrointestinal tract of abalone

The simple digestive system of an adult abalone consists of an oesophagus that extends to a large crop organ. In the crop, food is stored before it enters the stomach. The stomach forms a 180° loop at the posterior end of the abalone to extend interiorly adjacent to the crop. The crop and stomach are overlaid by a voluminous digestive gland. This gland is connected with the other digestive organs by ducts and tubules and occupies most of the visceral mass. The stomach

continues into a style sac, which is connected to a long intestine with five regions. Food enters region I and exits through region V which terminates in the anus (Campbell, 1965; Johnston *et al*., 2005).

Bacteria in the GIT of abalone play an important role in the digestion of nutrients through the production of different enzymes in the lumen (Iehata *et al.,* 2010). Several bacterial strains able to hydrolyse different polysaccharides have been isolated from the GIT of *H. midae*. Extracellular enzymes produced by these bacteria were able to degrade laminarin, carboxymethylcellulose (CMC), agar, carageenan and alginic acid. Furthermore, it was also found that isolates from different parts of the GIT, had different enzymatic activities (Erasmus *et al*, 1997). Enhancement of polysaccharide digestion through bacterial polysaccharolytic enzymes helps to improve absorption of nutrients and enhance the growth rate of abalone (Iehata *et al.,* 2010).

A successful probiotic bacterium should be able to attach and colonize the gastrointestinal tract (GIT) of the host (Balcázar *et al*., 2008). The structure together with the cell lining of the GIT plays an important role in the attachment of bacteria. Chemical and physical conditions such as pH, salinity, oxygen levels, available nutrients and redox potential inside the GIT vary among the different segments. Also, different cells types line each section of the tract and play a role in the secretion of compounds in the GIT, which affects the colonization ability of the bacteria (Campbell, 1965; Harris, 1993).

Abalone probiotics

Current abalone probiotics include yeasts and Gram-negative bacteria isolated from the GIT of the abalone (Iehata *et al*., 2010; Macey and Coyne *et al*., 2006). A Gram-negative bacterium, *Vibrio midae* and two yeasts, *Cryptococcus* sp. and *Debaryomyces hansenii* were isolated from the GIT of *H. midae* ((Macey and Coyne *et al*., 2006). It was found that these species are able to colonize the digestive tract and enhance the growth rate of the abalone. Lactic acid bacteria (LAB) isolated from terrestrial environments are also used as probiotics. However, very few studies have been done using LAB isolated from the gut of the abalone for probiotic use (Macey and Coyne *et al*., 2006). Recently, *Pediococcus* sp. Ab1 was isolated from the

gastrointestinal tract of the abalone *H. gigantean*. It was found that the addition of Ab1 to commercial abalone feed increased alginate lyase and volatile short chain fatty acids(VSCFAs) in the GIT by altering the gut microflora (Iehata *et al*., 2010). To date no lactic acid bacteria has been isolated from the gastrointestinal tract of the South African abalone, *H. midae*.

The aim of this study was, thus, to isolate potential probiotic bacteria from the gastrointestinal tract of the South African abalone (*Haliotis midae*), with the main focus on lactic acid bacteria.

Materials and methods

Sampling of abalone guts

Two batches of 10 abalone each were collected on 9 March 2010 and 4 May 2010 from Abagold Limited farm (Hermanus, South Africa). Abalone that were fed on different feeds, namely Abfeed, AquaNutro and Ulva, were collected. Under aseptic conditions abalone were shucked and the gut removed. After removal, the gut contents were expelled and each gut was transferred into sterile, saline solution (0.9% NaCl) containing acid-washed glass beads (Sigma, South Africa).

Culturing of enteric bacteria

Acid-washed glass beads (Sigma, South Africa) were used to homogenize each gut sample by vortexing on a Vortex-2 Genie for 5-7 min. Dilution series of 10^{-4} –10^{-7} was prepared from each homogenized gut sample. These dilutions as well as undiluted samples were plated out onto three different types of microbiological media prepared with natural sea water. Media used were Tryptone Soya agar (casein enzymic hydrolysate 17 g/l, papaic digest of soyabean meal 3 g/l, D-glucose 2.5 g/l, bile salts mixture 1.5 g/l, dipotassium hydrogen phosphate 4 g/l, sodium chloride 5 g/l and agar 12 g/l), De Man, Rogosa and Sharpe agar (Universal peptone 10 g/l, meat extract 5 g/l, yeast extract 5g/l, D(+)-Glucose 20 g/l, dipotassium hydrogen phosphate 2 g/l, diammonium hydrogen citrate 2 g/l, sodium acetate 5 g/l, magnesium sulfate 0.1 g/l, manganous sulfate 0.05 g/l and agar 12 g/l] and Zobell agar (polypeptone 5 g/l, yeast extract 1 g/l and agar 12 g/l). Plates were incubated at 20 °C for 3-7 days. Random colonies were picked from the growth on different plates and streaked out in order to obtain pure cultures. Pure cultures were stored at -80 °C in 100 mM $CaCl_2$ – 15 % glycerol.

Identification of isolated bacteria

Gram stains, catalase and oxidase tests were performed on pure cultures. The positive control for the oxidase test was *Pseudomonas aeruginosa* and for the catalase test *Staphylococcus aureus*. Based on these results, representative strains were selected for DNA extractions. Total genomic DNA extractions were done using

the ZR Fungal/Bacterial DNA kit™ (Zymo Research Corp. USA). The presence of DNA was confirmed on a 1% agarose gel stained with ethidium bromide (EtBr) and visualized under UV light. After successful DNA extractions, the 16S ribosomal RNA region was amplified by PCR using KAPA readymix in a 20 µl reaction. Bacterial specific primers (FORB primer 5'-AGAGTTTGATCCTGGCTCAG-'3 and REVB primer 5'-GGTTACCTTGTTACGACTT-'3) were used (Inqaba biosystems). The PCR conditions consisted of initial denaturing step at 94° C for 10 min, followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The reaction was completed with a final extension for 7 min at 72 °C and then cooled and held at 4 °C.

After confirming the PCR products on a 1 % agarose gel stained with EtBr, the products were digested with *Rsa*I (Fermentas Life Science, division of Thermo Fisher Scientific, Massachusetts, USA), and incubated at 37 °C for 8 hours. The reaction mixture contained 10µl of the PCR reaction product, 18µl nuclease-free water, 2 µl 10x Buffer Tango™ (Fermentas Life Science, division of Thermo Fisher Scientific, Massachusetts, USA) and 1µl *Rsa*I. The digested DNA was visualized with UV light on a 1.5 % agarose gel stained with etidium bromide (EtBr). Cultures chosen for sequencing were based on the restriction enzyme maps that were obtained following digestion with *Rsa*I. Cultures were sequenced on an ABI3130xl genetic analyser in the forward and reverse direction using the BigDye® terminator kit (Applied Biosystems, California, USA). Reaction mixtures consisted of 1.5 µl BigDye, 1.5 µl ½ BigDye, 5 µl nuclease free water, 1 µl PCR product and 1 µl FORB/REVB primer. Before sequencing, the PCR products were cleaned using the ZR DNA Clean and Concentrator™-5 Kit (Zymo Research Corp., USA).

SeqManII (DNAstar) software was used to create consensus sequences of each forward and reverse sequence of a strain. These consensus 16S rRNA gene sequences of the isolated strains were compared with other related taxa collected from NCBI (National Center for Biotechnology Information) database. Automatic alignments were done using CLUSTAL_X (Thompson *et al.,* 1997) and these were manually adjusted in Se-Al (Rambaut, 2007). Phylogenetic relationships were determined through neighbor-joining analysis using PAUP* v4.0b10 (Swofford, 2002). Bootstrap values were expressed as percentages of 1000 replications.

Identification of Paracoccus strain

Based on phiogenetic relationships, all the strains except strain 6.15, could be identified as a specific species. However, strain 6.15 grouped together with three closely related *Paracoccus* species. Therefore, in order to identify strain 6.15, the motility and starch utilization were determined. For the motility test, a wet preparation was made. One drop of sterile saline solution (0.9 % NaCl) was placed on a microscope slide. Cells grown overnight were carefully mixed with the drop of 9 % saline solution and covered with a cover slip. Motility of cells was examined under the 100x magnification of a microscope (Nikon eclipse E800).

Starch utilization tests were done on starch agar plates (agar 15 g/l, peptone 5 g/l, yeast extract 5 g/l and soluble starch 3 g/l). Strain 6.15 was streaked out in triplicate onto starch plates and incubated for 2 days at 26 °C. After growth occurred, the plates were covered with iodine. A clear/yellow zone around the colony in contrast to the dark blue color of the rest of the plate indicated starch hydrolysis (Kaur *et al*., 2012).

Optimum growth temperature of Paracoccus strain.6.15

Twelve test tubes containing 9.9 ml of Luria Bertani broth (tryptone 10 g/l, sodium chloride 10 g/l and yeast extract 5 g/l) were prepared. Each test tube was inoculated with 0.1 ml of *the Paracoccus* starter culture that had an absorbance of 1,662. After inoculation the absorbance of each test tube was measured. Triplicate test tubes were then incubated on a wheel at 20 °C, 26 °C, 30 °C and 37 °C. Absorbance values were measured every 24 hours for 120 hours.

Optimum growth pH of Paracoccus strain.6.15

Triplicate test tubes containing 9.9 ml Luria Bertani broth with a pH of 4, 5, 6, 7, 8, 9 and 10, were respectively prepared. The pH was adjusted with 10 M NaOH or 1 M HCl. Each test tube was inoculated with 0.1 ml of *Paracoccus* starter culture that had an absorbance of 1,806. The absorbance of each inoculated test tubes were measured and tubes were incubated on a wheel at 26 °C. Absorbance values were measured every 24 hours for 50 hours.

Optimum growth NaCl concentrations Paracoccus strain 6.15

Test tubes containing 10 g/l peptone and 5 g/l yeast extracted were prepared. Sodium chloride was added at concentrations of 0 %, 1.5 %, 3 %, 4.5 %, 6 %, 7.5 % and 9%. Each test tube was inoculated with 0.1 ml of *Paracoccus* starter culture that had an absorbance of 1,806. After the absorbance of each inoculated test tube was measured, test tubes were incubated on a wheel at 26 °C. Absorbance values were measured every 24 hours for 120 hours.

Antibiotic resistance of Paracoccus strain 6.15

Eight antimicrobial susceptibility tests disks from Oxoid were selected in order to test resistance of *Paracoccus* strain 6.15 against commonly used antibiotics. Selected antibiotics included Penicillin G and Vancomycin that both inhibit cell wall synthesis, Streptomycin and Chloramphenicol that inhibit protein synthesis, Norfloxacin and Rifampicin that inhibit nucleic acid synthesis, Polymyxin B that disrupts the cell membrane and Trimethoprim that blocks folic acid synthesis. Triplicate spread plates of *E.coli* (control) and *Paracoccus* strain.6.15 were made on Tryptone Soya agar. Antibiotic disks were placed on the spread plates and incubated for 3 days at 26 °C. After incubation, inhibition zones of each antibiotic disk were measured.

Results

Identification of isolated bacteria

One hundred and forty pure cultures were obtained from the guts of sampled abalone. After performing Gram stains, catalase and oxidase tests, 29 cultures were chosen for DNA extraction. The 16 S ribosomal RNA genes were successfully amplified through PCR (Fig. 2). The amplified DNA was digested with *Rsa*I and different digestion profiles were obtained. Based on the restriction fragment length polymorphism (RFLP) maps, shown in Figures 3 and 4, ten different strains were selected as representatives for sequencing (Table 1).

Phylogenetic analysis of the sequencing showed that the strains belonged to five different genera. Because of the difficulty in aligning diverse genera, five individual analyses were performed. Bootstrap values are shown at branching points and are expressed as percentages of 1000 replications. In Figures 5 - 9 different phylogenetic trees were drawn based on the five different genera that were identified, namely *Vibrio*, *Photobacterium*, *Corynebacterium*, *Staphylococcus* and *Paracoccus*. Properties of identified isolates are shown in Table 1.

Of the five different bacterial strains that were identified, *Paracoccus* sp was identified as the most promising candidate to be further evaluated as a potential probiotic in aquaculture (Kurnia *et al*., 2010). In addition, *Paracoccus* spp. is known to produce astaxanthin (Harker *et al*., 1998; Kurnia *et al*., 2010) and will, therefore, also be evaluated as a potential source of pigment to be used for trout.

Identification of Paracoccus strain 6.15

Paracoccus strain 6.15 grouped together with three astaxanthin producing species, *Paracoccus marcusii*, *P. carotinifaciens* and *P. haeundaensis* (Fig. 9). In order to identify this strain, known differences among these three species were listed (Table 3) and further tests were done. Strain 6.15 showed no motility and was not able to utilize starch. Based on phylogenetic, morphological and biochemical data, *Paracoccus* strain 6.15 was identified as *P. marcusii.*

Optimum growth conditions of Paracoccus marcusii

The **optimum growth temperature** of *P. marcusii* was found to be 26 °C (Fig. 10). Cells were also able to grow at 20 °C, 30 °C, and 37 °C. Growth of cells was slower at 20 °C, although at 90 hours the number of cells was the same as the culture incubated at 26 °C. Between 0 hours and 30 hours the absorbance values of the culture incubated at 30 °C were the same as that of 26 °C, however, after 30 hours cell number began to decrease. The least amount of growth was observed at incubation temperature of 37 °C.

The **optimum growth pH** of *P. marcusii* is between 7 and 8 (Fig. 11). At pH 6, 9 and 10 growth of the cells were slower, however, the number of cells after 48 hours were the almost the same as pH 7 and 8. Very poor growth was detected at pH 4 and 5.

The **optimum NaCl** concentrations were between 1.5 % and 3 % (Fig. 12). Relatively good growth was also detected at a NaCl concentration of 4.5 %. Low absorbance values were obtained at concentration of 6 % and almost no growth was detected at concentrations of 0 %, 7.5 % and 9 %.

Antibiotic resistance of Paracoccus strain 6.15

Paracoccus marcusii showed resistance to Trimethoprim, Streptomycin and Penicillin G (Fig. 13). It was however, sensitive to Polymyxin B, with an inhibition zone of 25.33 mm, Rifampicin with 33.67 mm, Norfloxacin with 13.67 mm, Chloramphenicol with 50.33 mm and Vancomycin with 20.33 mm (Table 4).

Discussion

In the search for potential probiotic bacteria, four Gram positive isolates (Fig. 5 and 6) and five Gram negative isolates were identified (Fig. 7 - 9) from the GIT of abalone. No lactic acid bacteria were, however, isolated. Although it is known that lactic acid bacteria are present in the gut of abalone (Iehata *et al*., 2010), the sub-dominance of this group of bacteria in fish complicates their isolation (Balcazar *et al*., 2006; Titus, 2012).

The Gram positive isolates were classified to belong to the genera *Corynebacterium* and *Staphylococcus*. Isolated species from both these genera were shown to be catalase positive and oxidase negative (Table 1). *Corynebacterium variabile* cells are non-motile, rod-shape and cells usually occur single, in pairs (typical in a V form) or form clumps (Collins, 1987). A variety of different *Corynebacterium* species have been isolated from the surface of smear-ripened cheese and have also been found in soil environments, plant material and other dairy products (Gelsomino *et al*., 2005). This species is not known to have any probiotic effects and was therefore, not identified as a potential probiotic to be used in aquaculture. Three different *Staphylococcus* species were also isolated and identified as *S. carnosus, S. equorum* and *S. cohnii*. Not much research has been done on *Staphylococcus* in aquaculture environments. However, the role of *Staphylococcus* in food spoilage is well known. This organism causes gastroenteritis when eating staphylococcal enterotoxin-contaminated food. It has also recently been found that *S. equorum* is capable of causing life-threatening respiratory tract infections in patients with cystic fibrosis (Kajikazawa *et al.,* 2007). Because of the pathogenic effect of species in this genus, these isolates were disregarded as potential probiotic species.

Vibrio is a dominant genus of bacteria found in marine animals and aquatic environments. Three different *Vibrio* species have been identified in this study namely *V. aestuarianus*, *V. nigripulchritudo* and *V. cyclitrophicus*. These small rod-shaped cells are Gram negative and catalase and oxidase positive (Table 1). *Vibrio* species normally have a polar flagellum and are motile (Tison and Seidler, 1983). They are considered to be opportunistic pathogens (Labreuch *et al.,* 2006) and diseases caused by *Vibrio* species known as vibriosis results in major economic losses in aquaculture (Goarant *et al*., 2007). Two of the *Vibrio* species, isolated in

this study, *V. aestuarianus* and *V. nigripulchritudo*, are well known for causing diseases in oysters and shrimps (Garnier *et al.,* 2008; Goarant *et al*., 2007; Labreuche *et al.* 2006; Labreuche *et al*., 2010; Tison and Seidler 1983). These two species have not yet been found to cause any disease in abalone. However, gene transfer between *Vibrio* species well is known and virulence genes can easily be transferred between species (Lorenz and Wackernagel, 1994). Therefore, these *Vibrio* species were also not considered as potential probiotics.

Photobacterium leiognathi, a bioluminescent species closely related to *Vibrio* was isolated. This Gram negative, rod shaped bacterium is catalase and oxidase positive (Table 1). Bioluminescent bacteria are well known to form symbiotic relationships with some species of marine fish. In addition to bioluminescence that helps lure prey, these bacteria aid in supplying oxygen and nutrients for growth of the fish. Luminescence in bacteria is the product of luciferase, which is a mixed-function of oxidase. To produce blue-green light (luminescence), the oxidase uses oxygen, reduced flacin mononucleotide and long-chain fatty aldehydes (Ast *et al.,* 2007). Excess cells in the light organ are released into the surrounding water through the gut of the fish (Dunlap *et al.,* 2004). Some bioluminescent bacteria are also known to be free living, as in the case of *P. leiognathi* (Zarubin *et al*., 2012). The oligotrophic environment in which this free-living bacterial species live does normally not have enough nutrients for optimum growth. Therefore, one of the functions of these bioluminescent cells is to attract predators such as zooplankton. After engulfment of the bacteria, zooplankton starts to emit light, attracting bigger fish that prey on them. Through this, the free-living bioluminescent bacteria are able to reach the GIT of the fish, which have more nutrients than the environment. In the GIT they are able to proliferate and then be excreted into the environment together with the faeces (Zarubin *et al*., 2012). *Photobacterium leiognathi* is not considered to be a potential probiotic. This isolate is free living and for a bacterium to be a potential probiotic, colonization of the GIT is very important. However, the cells can possibly release enzymes inside the host that may have some positive effects such as enhancing digestion.

Based on phylogenetic relationships, strain 6.15 was grouped together with three closely related astaxanthin producing bacteria namely *Paracoccus marcusii, P. carotinifaciens* and *P. haeundaensis* (Fig. 9). These three species are Gram

negative, non-sporulating, oxidase and catalase positive orange colonies (Table 2). Strain 6.15 was identified as *P. marcusii,* since it was found that cells were non-motile and unable to utilize starch.

Paracoccus marcusii showed promise to be used in aquaculture due to the production of astaxanthin. This carotenoid is commonly used as a pigment that is included in the feed of a variety of different aquaculture species such as trout, red see bream, salmon and shrimp (Bjerkeng *et al*., 1999; Choubert *et al*., 2009; Gomes *et al.,* 2002). Aquatic animals are not able to synthesize these pigments and under aquaculture conditions fish do not come in contact with natural sources of this pigment. This results in dark gray meat, which is unappealing for consumers. Therefore, it is important to include pigments in the diet for meat to look fresher and more appealing for consumers. Also, astaxanthin plays a role in many other essential biological functions of fish species including enhanced sexual maturity, embryo development, and egg survival. This pigment can also increase the defence potential against oxidative stress and thus improves liver function (Higuera-Ciapara *et al*., 2006). In a study performed on shrimp, it was shown that astaxanthin improves the immune response and tolerance to stress (Gabaudan, 1996; Higuera-Ciapara *et al*., 2006).

In order to evaluate the probiotic and pigmentation effect of *P. marcusii*, *in vivo* studies are performed, which requires large numbers of this bacterium were needed. Therefore, the optimum growth conditions were determined for large scale production of *P. marcusii*. The optimum growth temperature of 26°C, together with the ability to grow relatively well at 20°C, correlated with the water temperature from which the bacterium was isolated, which ranged from 18°C to 22°C. It was also observed that optimum growth occurred at a pH and NaCl range similar to that of sea water namely 7-8 and 1.5-3%, respectively.

It was also important to know if *P. marcusii* was resistant to commonly used antibiotics. Although the use of antibiotics in aquaculture is questioned (Farzanfar, 2006; Heuer *et al*., 2009; Hirsch *et al*., 1999), cultured fish still obtain large quantities of prophylactic antibiotic to control diseases. Another concern is the spreading of antibiotic resistance between aquatic bacteria, but also between aquatic and terrestrial bacteria (Farzanfar, 2006; Heuer *et al.,* 2009). In this study, it was found

that *P. marcusii* is resistant to Trimethoprim, Streptomycin and Penicillin G to and susceptible to Polymyxin B, Rifampicin, Norfloxacin, Chloramphenicol and Vancomycin. It is suspected that if *P. marcusii* were supplemented to fish exposed to antibiotics to which this bacterium is susceptible, no probiotic or pigmentation effects will be visible. However, if fish were exposed to antibiotics to which *P. marcusii* is resistant, possible probiotic and/or pigmentation effects would be expected.

Conclusion

In this study, it was aimed to isolate potential probiotic bacteria from the GIT of abalone, particular LAB. However, no lactic acid bacteria were isolated. Possible reasons may be because of the low concentrations of these bacteria in the gut of the abalone. However, *Paracoccus marcusii* shows possible probiotic properties and industrial applications based on its production of pigments. Further *in vivo* studies need to be conducted in order to determine if *P. marcusii* can be used as an effective aquaculture feed additive.

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Figures

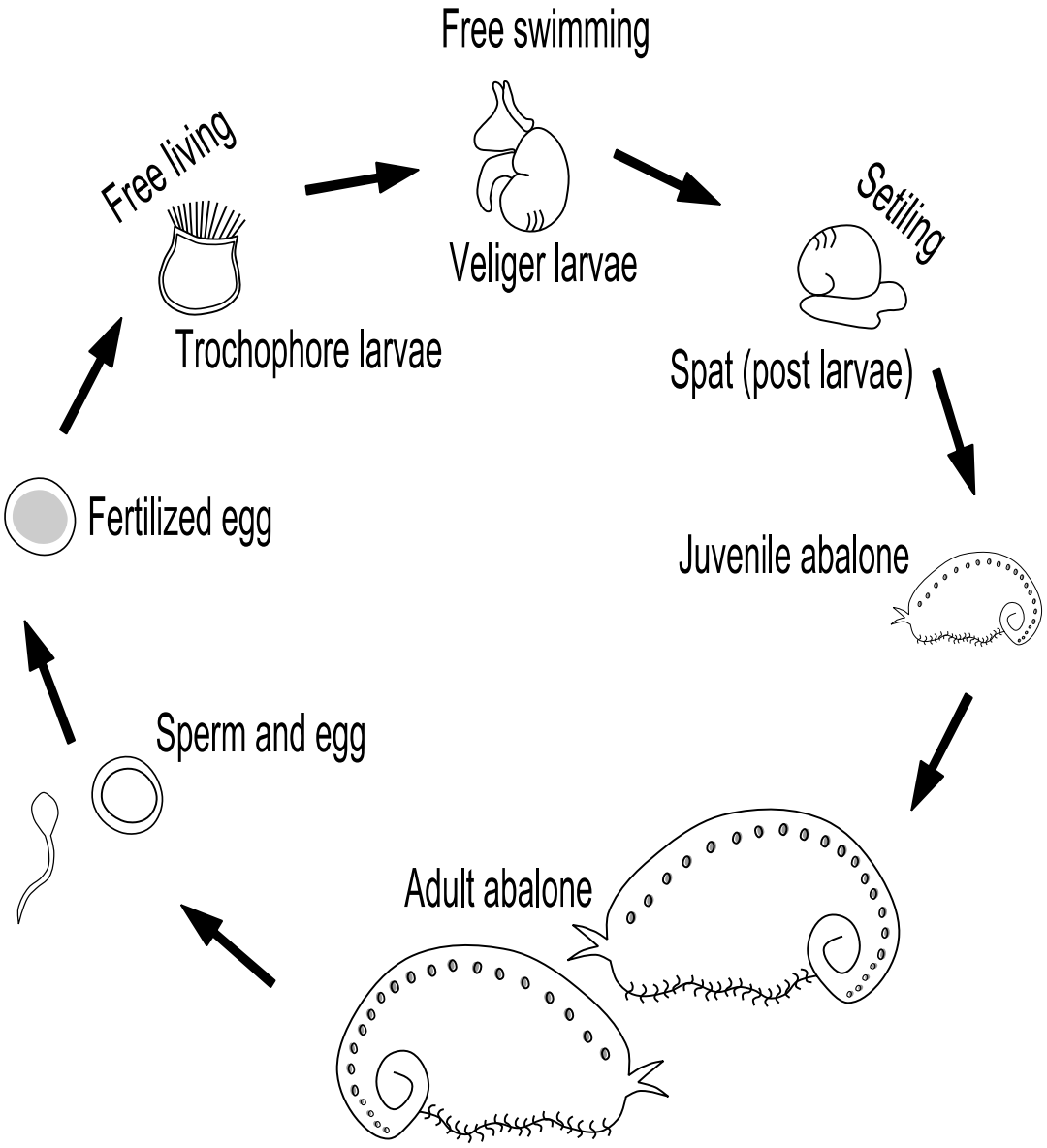


Figure 1 - Life cycle of abalone. Sperm fertilize the egg to form a fertilized egg that develops into trochophore larvae, veliger larvae and then spat. The spat develops into juvenile abalone that grows to adult abalone (modified from Fallu, 1991).

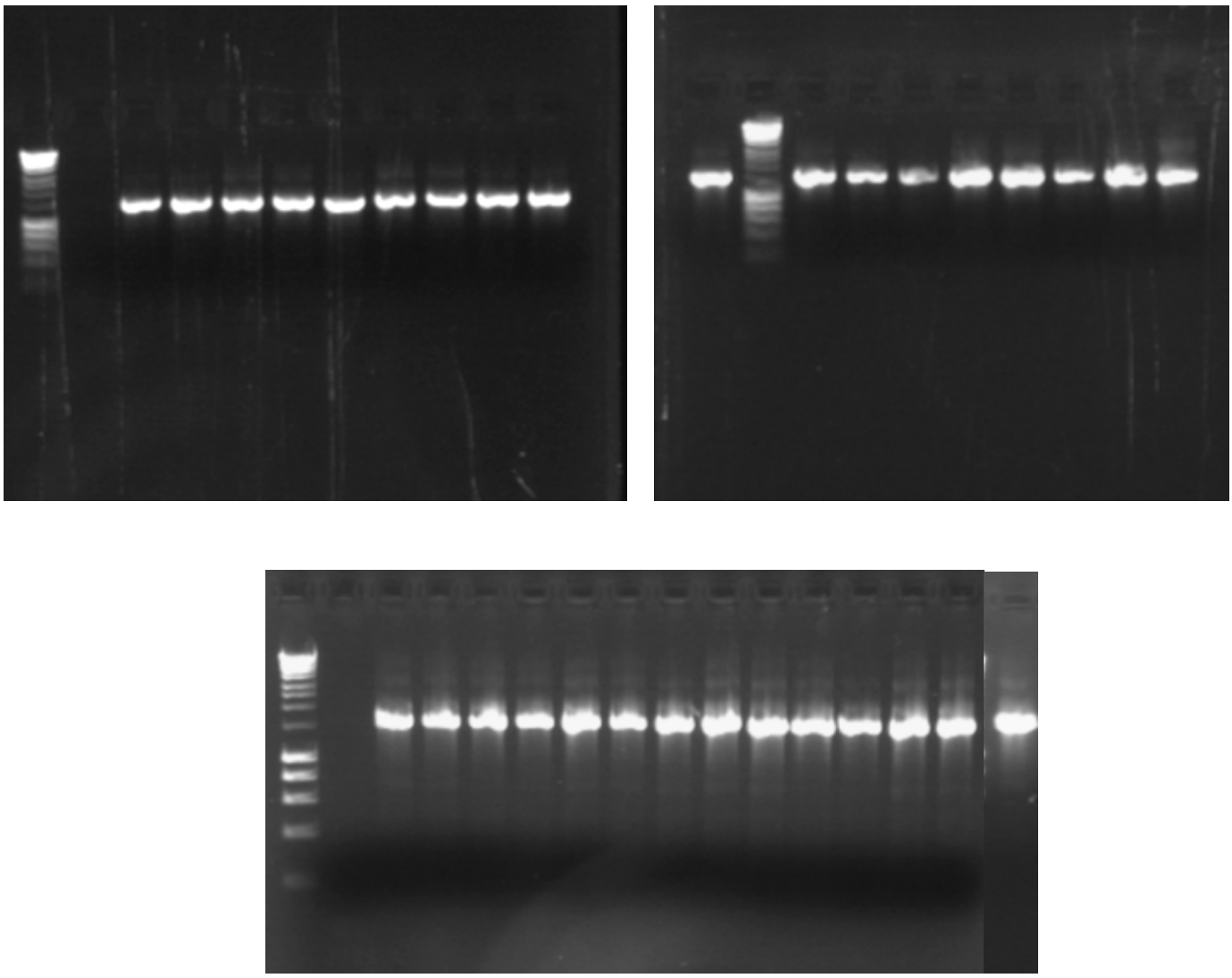
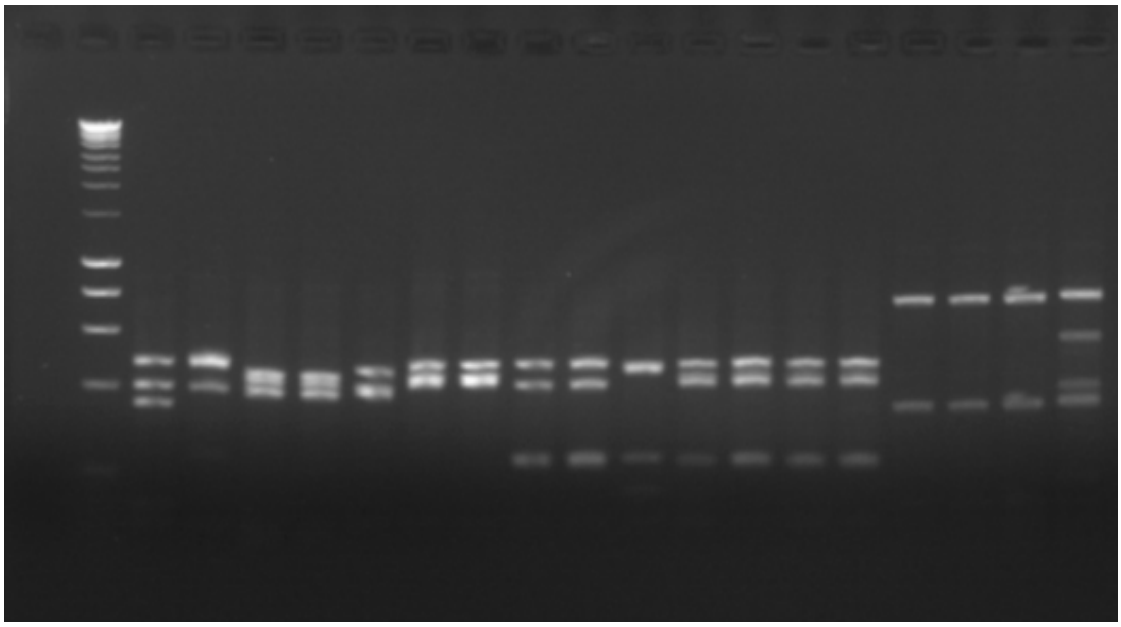


Figure 2 – Total genomic DNA extractions of bacterial isolates selected based on Gram staining, catalase and oxidase testing. The 16S ribosomal RNA gene was amplified to be digested with *RsaI*.

a.



b.

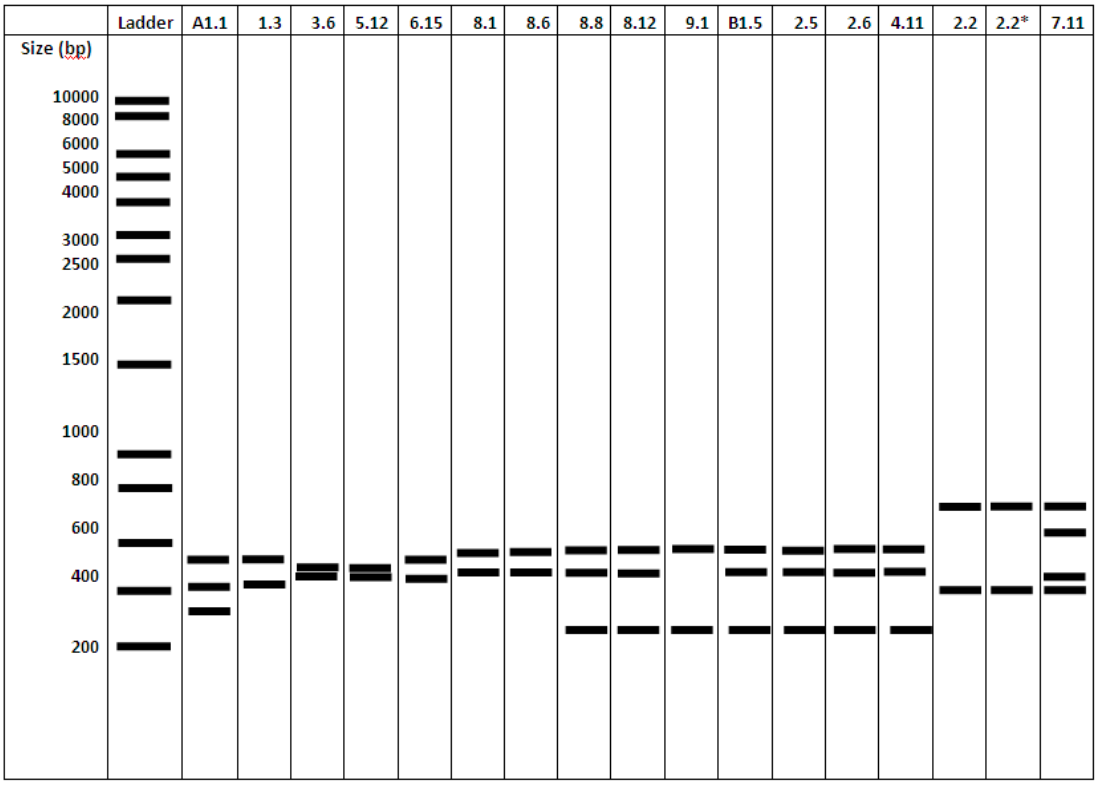
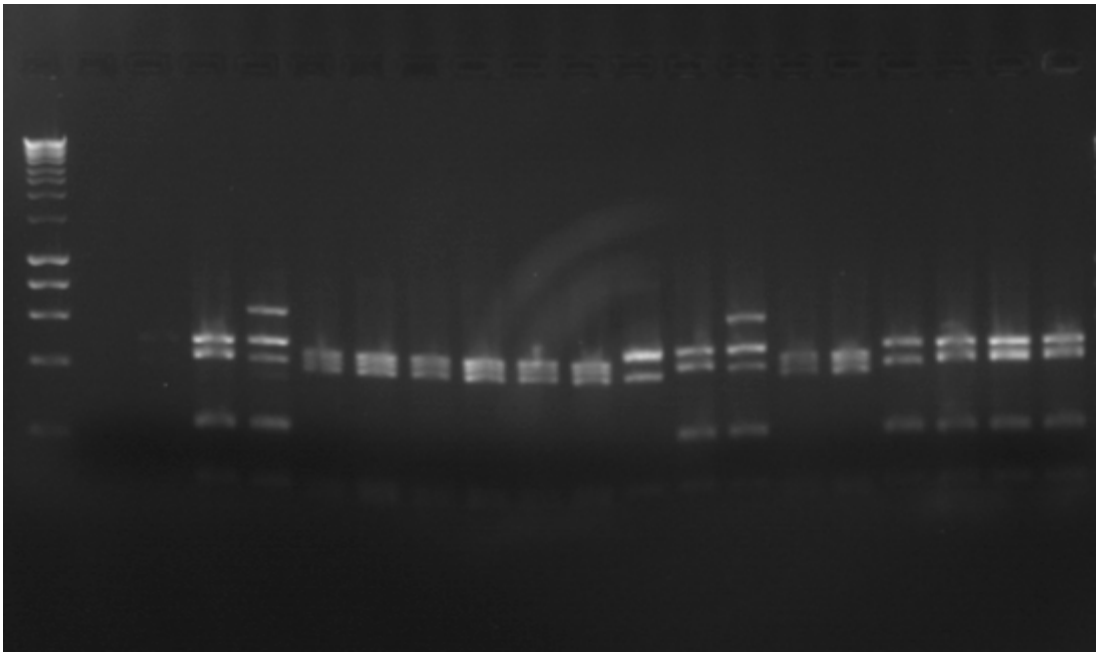


Figure 3 – Isolates from abalone sampled on 9 March 2010. a. Agarose gel of amplified 16S ribosomal RNA region DNA, digested with *Rsa*I. b. RFLP maps drawn from agarose gel picture in Figure 3a. Different patterns indicate possible different species.

a.



b.

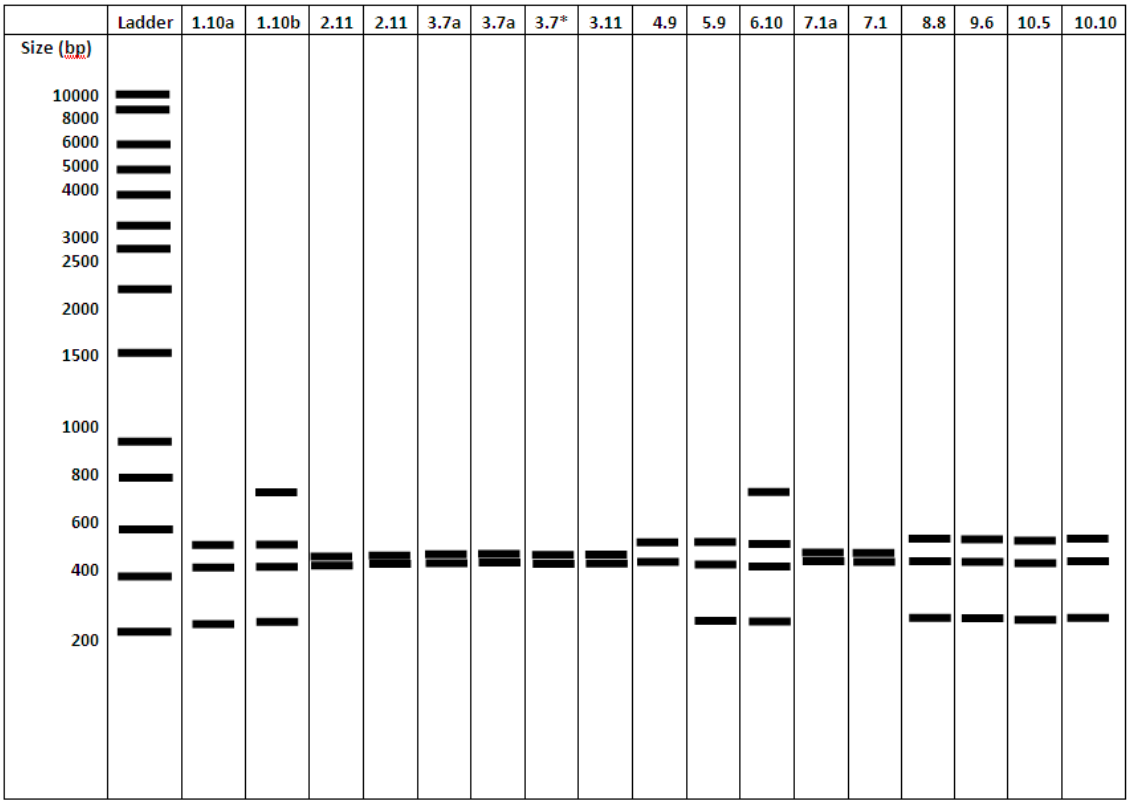


Figure 4 – Isolates from abalone sampled on 4 May 2010. a. Agarose gel of amplified 16S ribosomal RNA region DNA, digested with *Rsa*I. b. RFLP maps drawn from agarase gel in Figure 4a. Different patterns indicate possible different species.

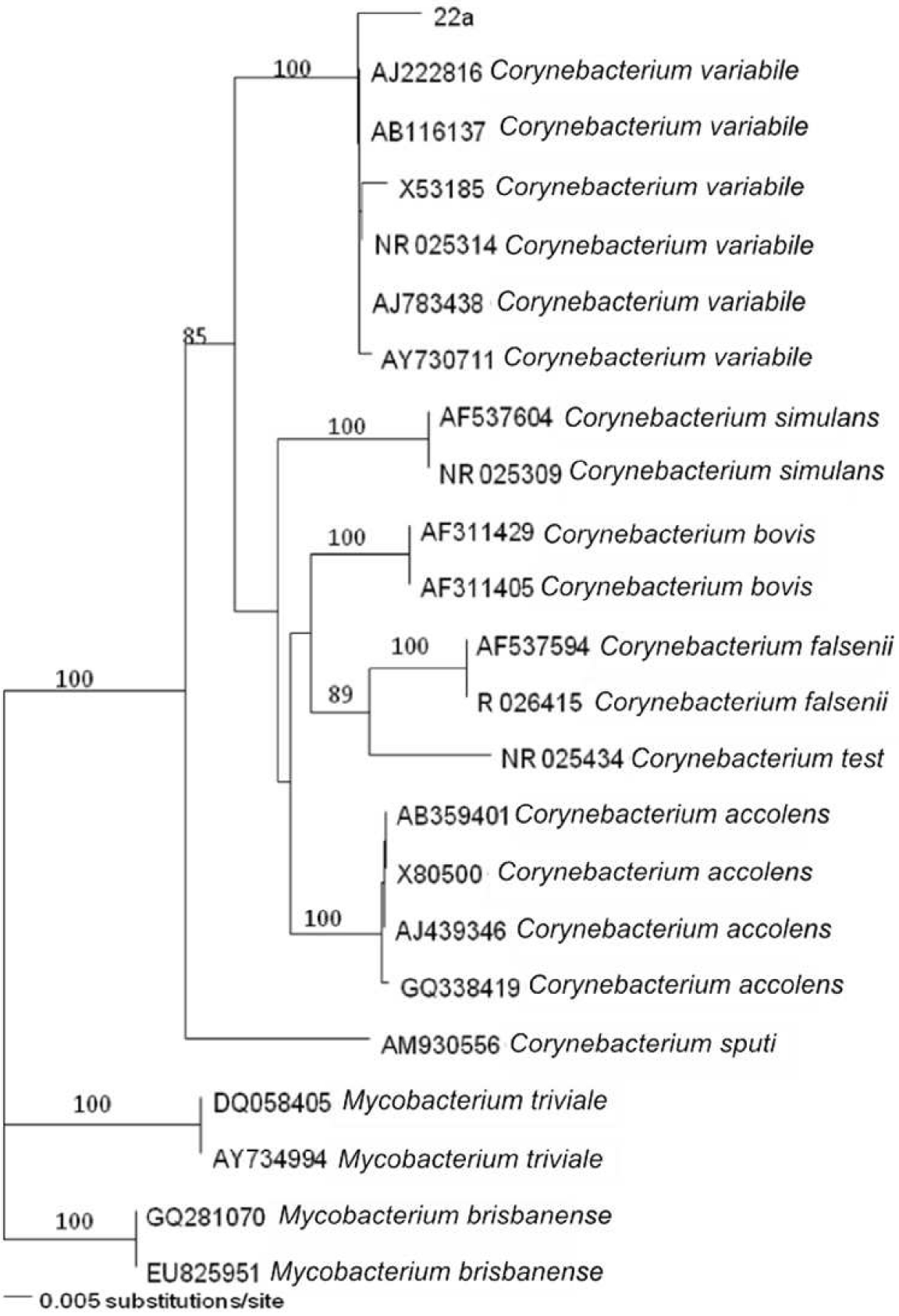


Figure 5 – Neigbour-joining tree based on the phylogenetic analysis of the 16S rRNA sequences of different *Corynebacterium* from Genbank species and isolate 2.2. Tree rooted with *Mycobacterium* from Genbank.

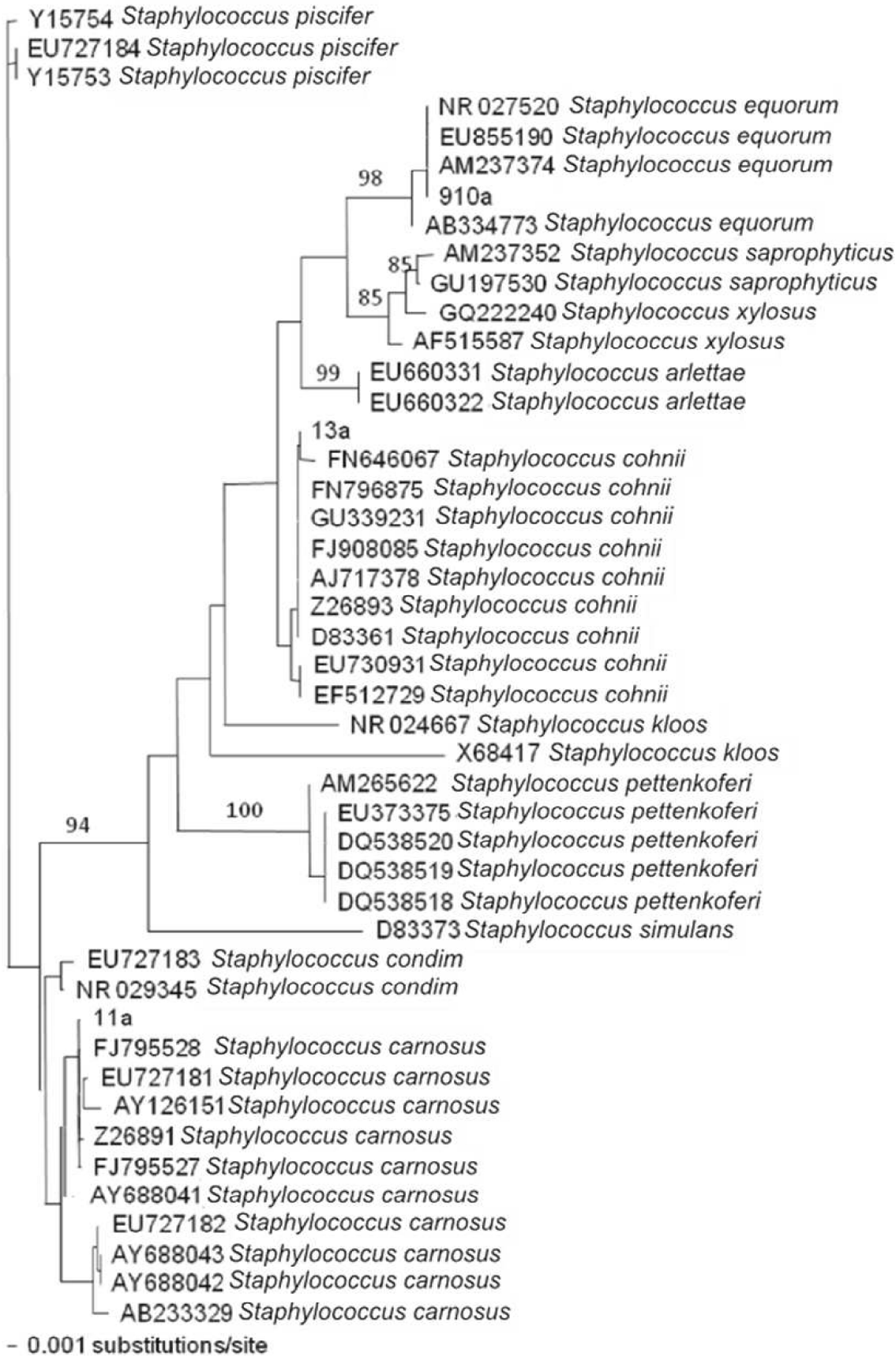


Figure 6 – Neigbour-joining tree based on the phylogenetic analysis of the 16S rRNA sequences between different *Staphylococcus* species from Genbank and isolates 1.1, 1.3 and 9.1. Trees were rooted to midpoint.

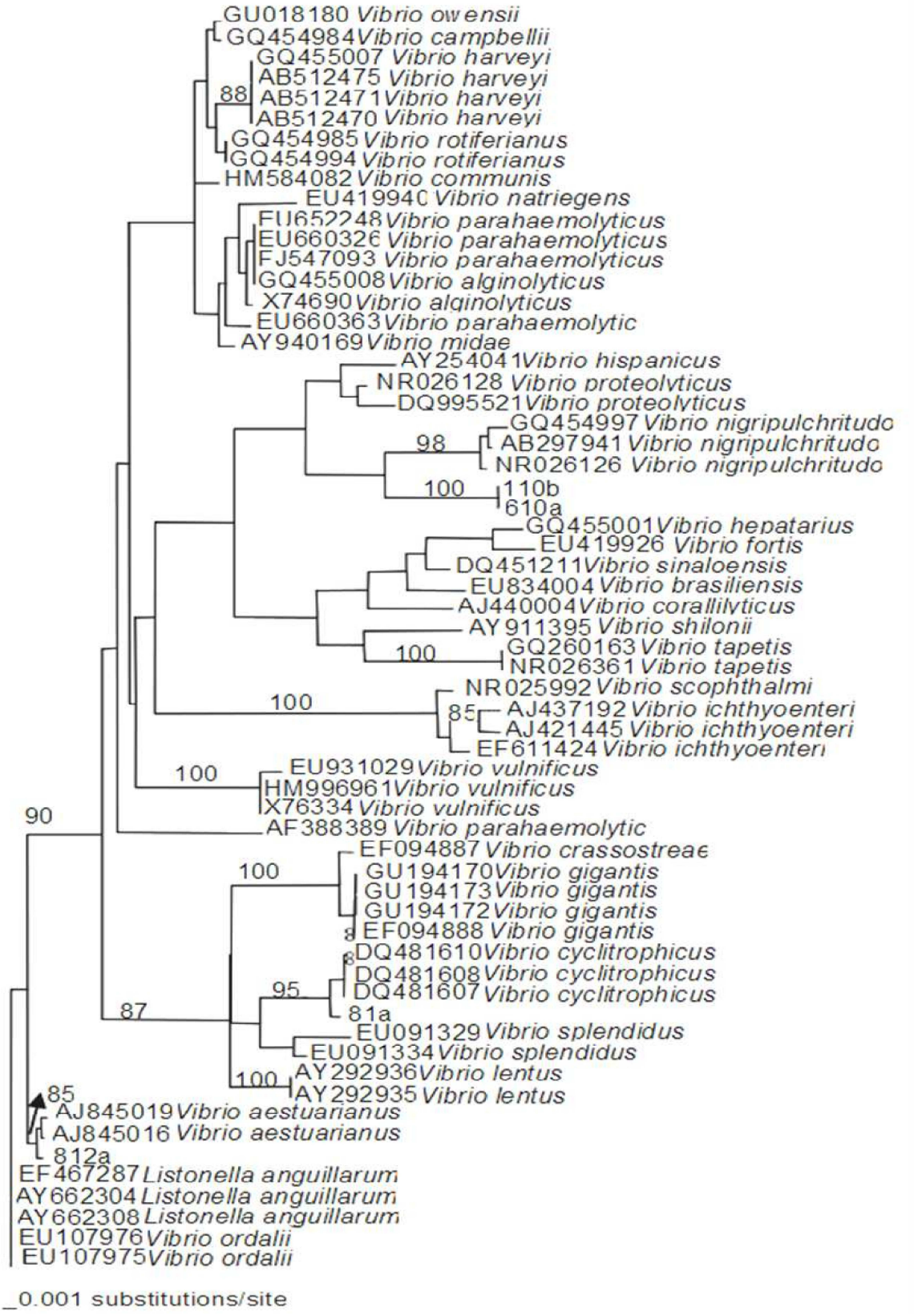


Figure 7 – Neigbour-joining tree based on the phylogenetic analysis of the 16S rRNA sequences of different *Vibrio* species from Genbank and isolates 8.1, 1.1 and 6.10. *Listonella anguillarum* was chosen as outgroup.

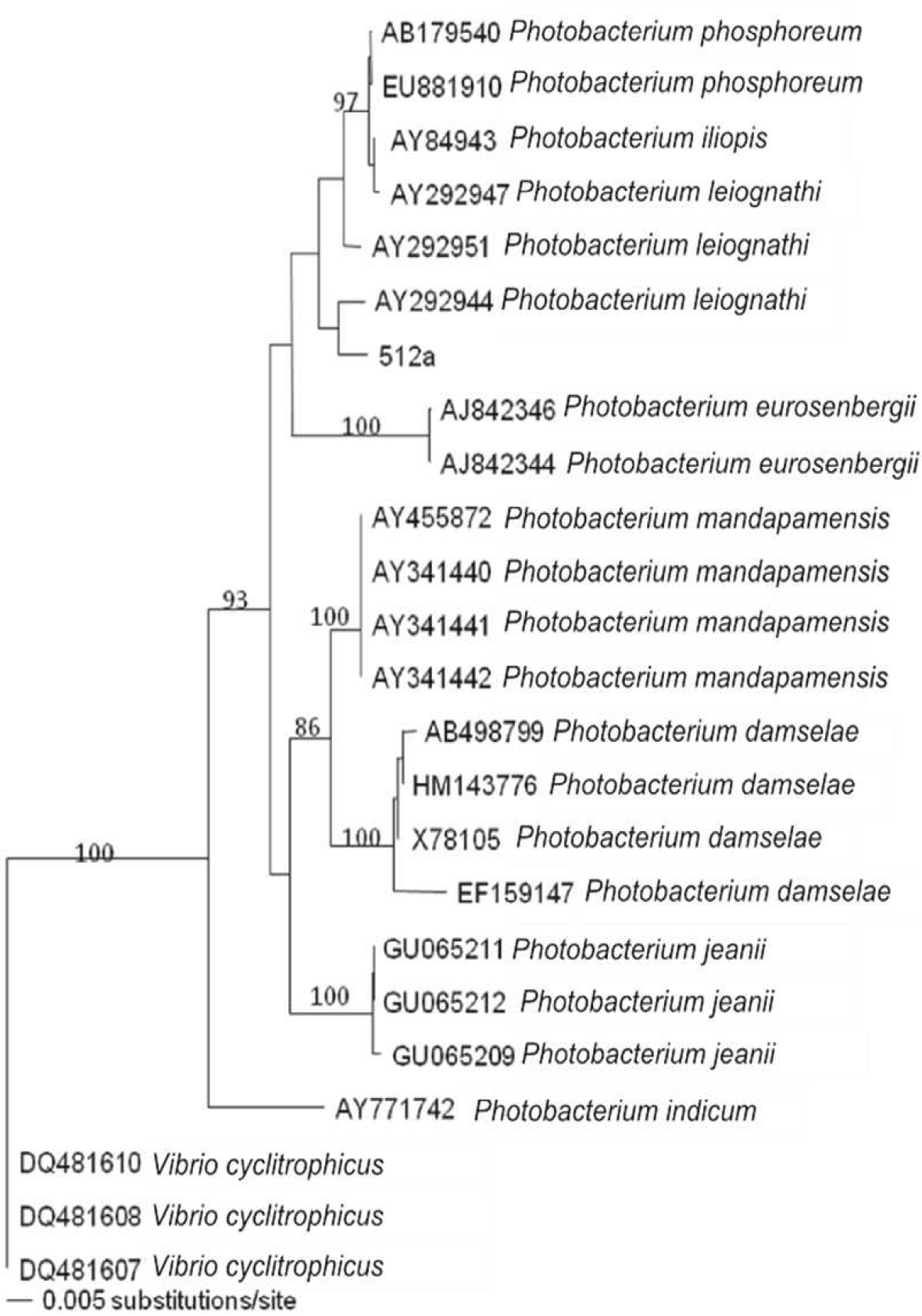


Figure 8 – Neigbour-joining tree based on the phylogenetic analysis of the 16S rRNA sequences of different *Photobacterium* species from Genbank and isolate 5.12. *Vibrio cyclitrophicus* from Genbank was chosen as the outgroup.

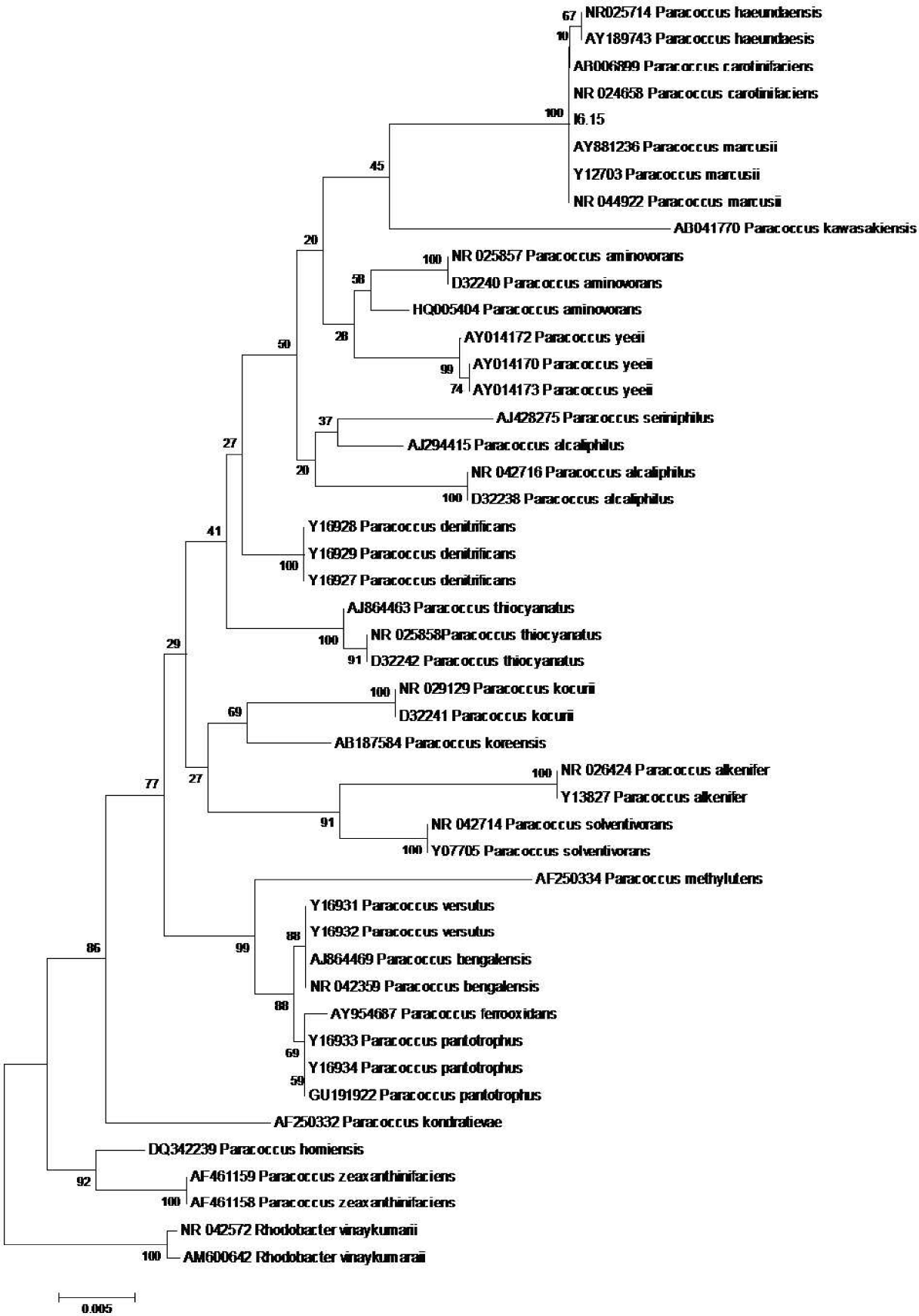


Figure 9 – Neigbour-joining tree based on the phylogenetic analysis of the 16S rRNA sequences of different *Paracoccus* species from Genbank and isolate 6.15. *Rhodobacter vinaykumarii* from Genbank was used as outgroup.

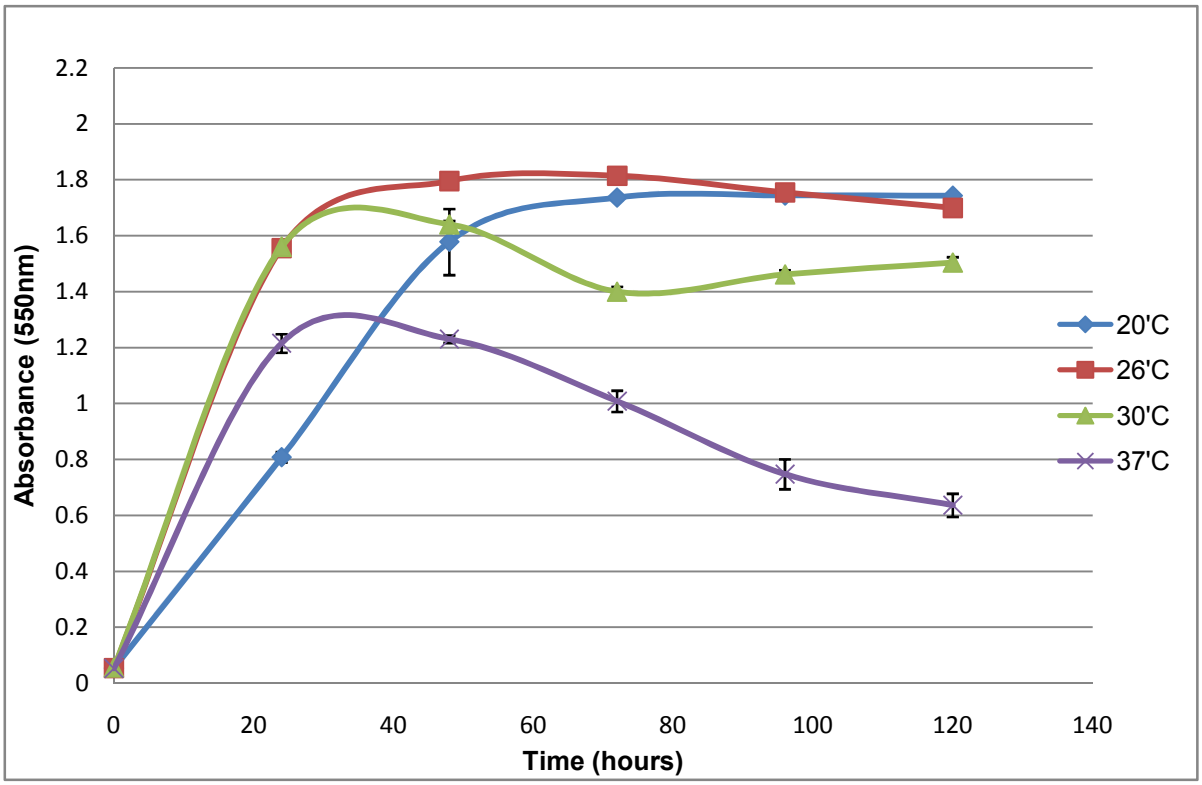


Figure 10 - Growth curve of the optimum temperature of *Paracoccus marcusii* isolate 6.15. Triplicate testubes containing Luria Bertini broth were inoculated with isolate 6.15 and incubated at 20 °C, 26 °C, 30 °C and 37 °C. Absorbance values were measured every 24 hours for 120.

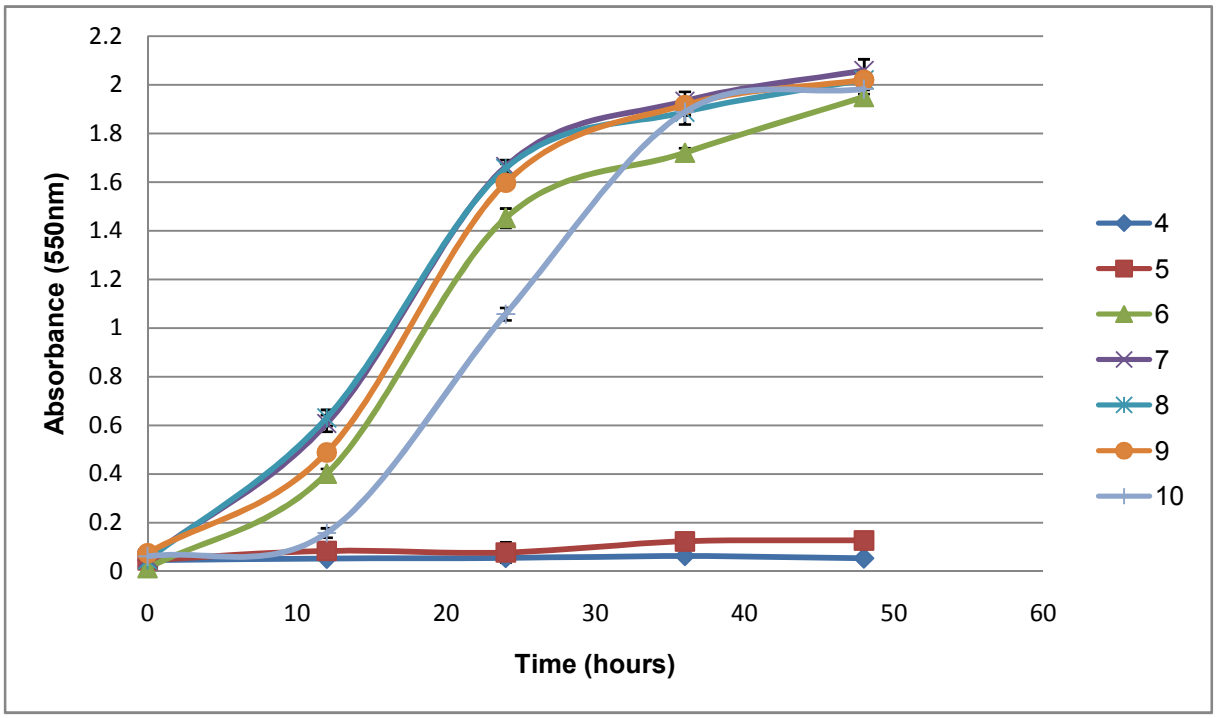


Figure 11- Growth curve of the optimum pH of *Paracoccus marcusii* isolate 6.15. Triplicate testubes containing Luria Bertini broth with different pH values of 4, 5, 6, 7, 8, 9 and 10 were inoculated with the isolate. Absorbance values were measured every 24 hours for 50

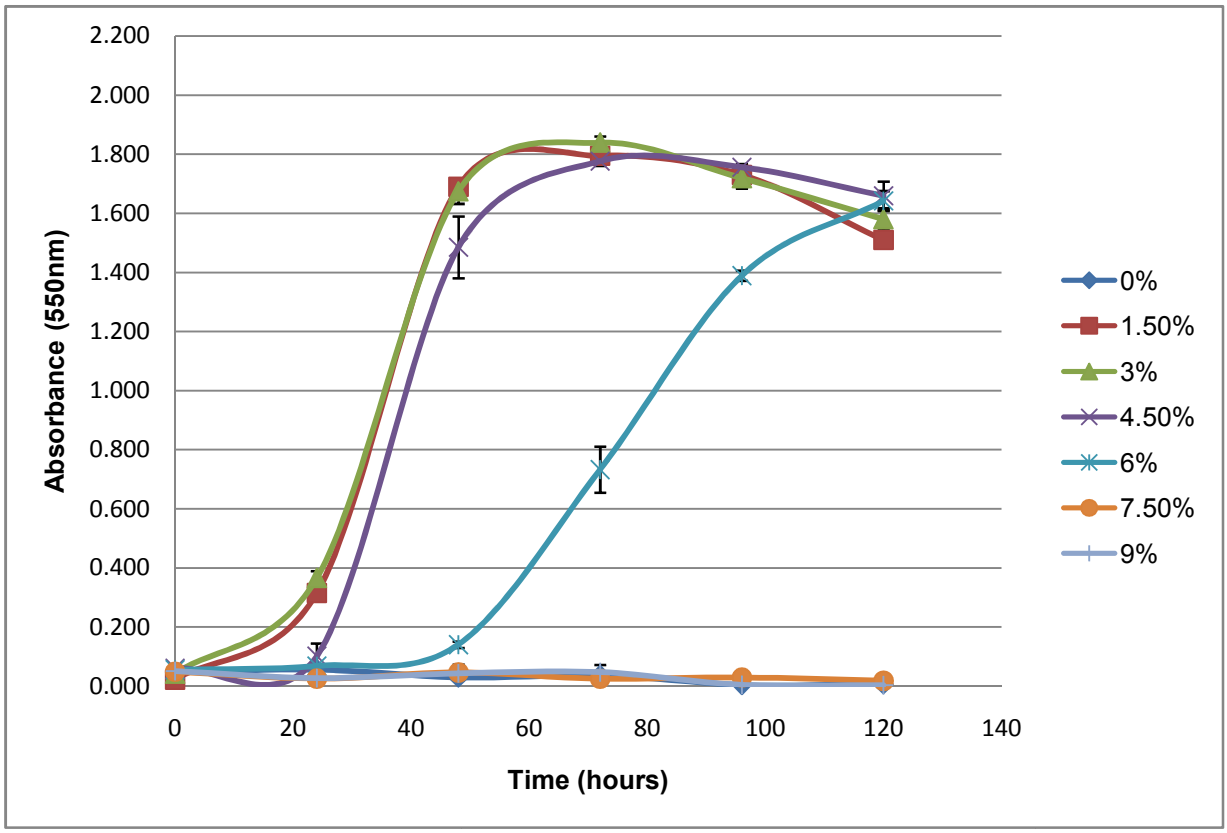


Figure 12 - Growth curve of the optimum NaCl concentrations of *Paracoccus marcusii* isolate 6.15. Test tubes containing 10 g/l peptone and 5 g/l yeast extract were prepared. Sodium chloride was added at concentrations of 0 %, 1.5 %, 3 %, 4.5 %, 6 %, 7.5 % and 9 % and test tubes were inoculated with the isolate. Absorbance values were measured every 24 hours for 120.

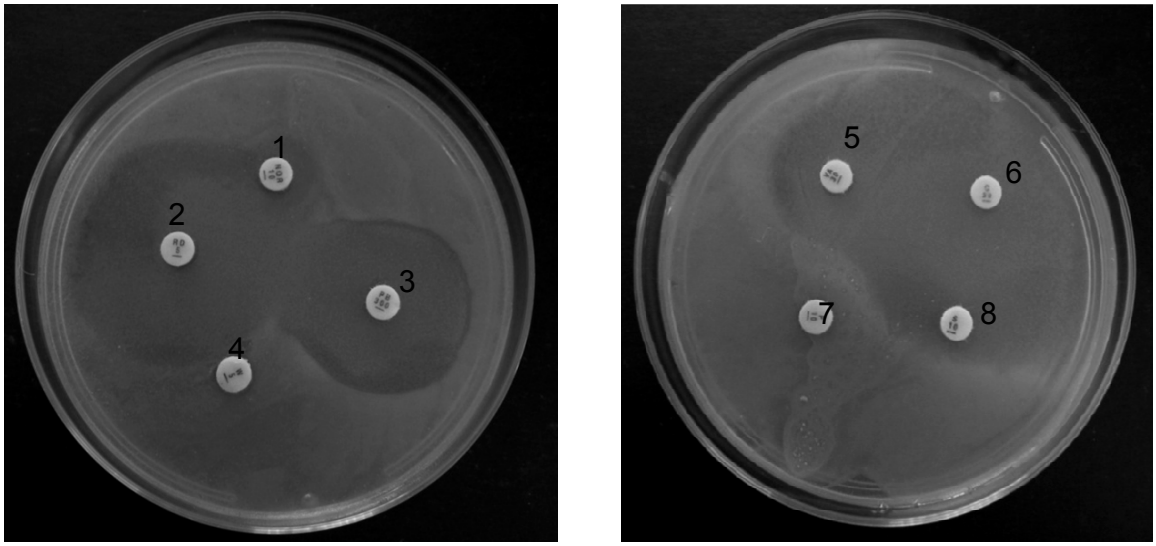


Figure 13 - Zones of inhibition of *Paracoccus marcusii* with different antibiotics. 1 – NOR (Norfloxacin), 2 – RD (Rifampicin), 3 – PB (Polymyxin B), 4 – W (Trimethoprim), 5 – VA (Vancomycin), 6 – C (Chloramphenicol), 7 – P (Penicillin G) and 8 – S (Streptomycin).

Tables

Table 1 - Properties of identified cultures isolated from the gut of abalone (*Haliotis midae*)

Diet of abalone	Media	Isolate number	Gram staining and cell shape	Catalase test	Oxidase test	Identification
Abfeed	MRS	1.1	Gram positive cocci	+	-	*Staphylococcus carnosus*
Abfeed	MRS	1.3	Gram positive cocci	+	-	*Staphylococcus cohnii*
Abfeed	Zobell	1.1	Gram negative rods	+	+	*Vibrio nigripulchritudo*
Abfeed	MRS	2.2	Gram positive rods	+	-	*Corynebacterium variabile*
Abfeed	Zobell	5.12	Gram negative rods	+	+	*Photobacterium leiognathi*
AquaNutro	Zobell	6.1	Gram negative rods	+	+	*Vibrio nigripulchritudo*
Abfeed	Zobell	6.15	Gram negative cocci	+	+	*Paracoccus* sp.
AquaNutro	MRS	8.1	Gram negative rods	+	+	*Vibrio cydirophicus*
AquaNutro	Zobell	8.12	Gram negative rods	+	+	*Vibrio aestuarianus*
Abfeed	TSA	9.1	Gram positive cocci	+	-	*Staphylococcus equorum*

Table 2 - Similarities among astaxanthin producing *Paracoccus* species (Harker *et al.*, 1998; Lee *et al.*, 2004; Tsubokura *et al.*, 1999)
Gram negative
Non-sporulating
Orange colonies (produce astaxanthin)
Oxidase and Catalase positive
Aerobic respiration

Table 3 - Differences among astaxanthin producing *Paracoccus* species

	P. marcusii (Harker *et al.*, 1998)	***P. carotinifaciens*** (Tsubokura *et al.*, 1999)	***P. haeundaensis*** (Lee *et al.*, 2004)
***Motility**	-	+	-
Flagella	N/A	peritrichous flagella	N/A
Nitrate reduction	-	-	+
Cell shape	Cocci to short rods	Rods	Rods
Arrangement	Pairs or short chains	No chains	No chains
Cell size	1-2 µm by 1.0-1.5 µm	0.3-1 µm by 1.0-5.0 µm	0.3-0.7 µm by 0.8-2.5 µm
Optimum pH	?	7	8
DNA G+C content	66 mol%	67 mol%	66.9 mol %
Utilization of:			
Mannitol	+	+	-
Maltose	+	+	-
Mannose	+	+	-
***Starch**	-	-	+
Arabinose	+	-	+
Glucose	+	+	-

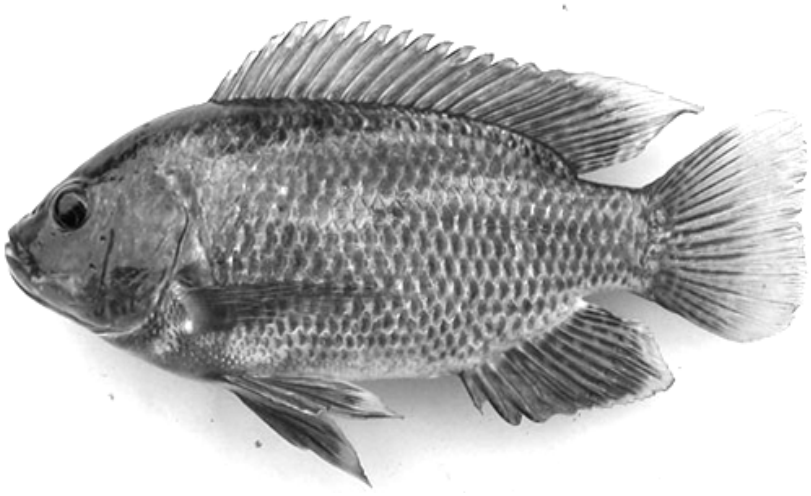
* The *Paracoccus spp*. isolated in this study showed no motility and was unable to degrade starch

Table 4 - Antibiotic resistance of *Paracoccus marcusii* strain 6.15

Antibiotic	**Zone diameters in mm**							
	***E. coli* (control)**				***Paracoccus marcusii***			
	Plate A	**Plate B**	**Plate C**	**Average**	**Plate A**	**Plate B**	**Plate C**	**Average**
Polymyxin B (PB)	12	15	14	13.67	26	25	25	25.33
Trimethoprim (W)	23	16	18	19.00	-	-	-	-
Rifampicin (RD)	9	8	8	8.33	33	34	34	33.67
Norfloxacin (NOR)	35	25	30	30.00	14	13	14	13.67
Chloramphenicol (C)	25	23	23	23.67	51	50	50	50.33
Streptomycin (S)	21	22	20	21.00	-	-	-	-
Vancomycin (VA)	-	-	-	-	20	18	23	20.33
Penicillin G (P)	-	-	-	-	-	-	-	-

Chapter 3

Application of *Paracoccus marcusii* as probiotic for Mozambique tilapia (*Oreochromis mossambicus*)



Abstract

Antibiotics are commonly used to treat diseases and enhance the growth rate of fish under aquaculture conditions. However, due to negative impacts of these agents, the search for effective probiotics in aquaculture continues, particularly for the establishment of the commercial production of local aquaculture species such as Mozambique Tilapia (*Oreochromis mossambicus)*. This chapter aims to evaluate the probiotic effect of *Paracoccus marcusii* (Chapter 2) on the growth rate and immunity of Mozambique tilapia. *Paracoccus marcusii* was administered orally at 10^7CFU/kg feed. Throughout the study it was found that the addition of the probiotic bacterium did not have any negative effect on the number of gastrointestinal tract (GIT) microorganisms of the host. At the end of the trial, significant differences in growth and immune parameters were observed between fish feeding on the negative control and the probiotic-coated feed. Fish supplemented with the probiotic feed showed a 185.79% increase in body weight, which was higher than the negative control of 166.67%. The feed conversion ratio (FCR) of probiotic-supplemented fish was lower than that of the negative control which was 198.92 and 237.06, respectively although *P. marcusii* did not have any effect on the immunoglobulin concentrations of the fish, the bacterium did enhance the lysozyme activity in the blood serum. In this study it was therefore observed that *P. marcusii* may be an effective probiotic for Mozambique tilapia, through the improvement of the growth rate and lysozyme activity of the fish.

Introduction

The name 'tilapia' descends from the African Bushman word meaning 'fish' (El-Sayed, 2006). This group of freshwater fish are endemic to African countries, Jordan and Israel. Tilapia is considered as an ideal candidate for aquaculture due to its fast growth rate and the ability to tolerate a wide range of environmental conditions. It is also relatively resistant to a variety of stress factors and diseases and is able to reproduce in captivity. Tilapia is also a low-priced protein source that can be exported as a high-value product (El-Sayed, 2006; Lim and Webster, 2006). Over 70 species have been identified, however, only a few of these species are used in aquaculture (Lim and Webster, 2006). The three main species used in aquaculture include *Oreochromis niloticus* (Nile tilapia), *O. aureus* (blue tilapia) and *O. mossambicus* (Mozambique tilapia) (Lim and Webster, 2006). From 1990 to 2002, tilapia aquaculture has grown significantly with the main production in Africa, Asia, North America and South America (Fig. 1) (El-Sayed, 2006).

In South Africa, *O. mossambicus* (Fig. 2) is naturally found in the river systems of Limpopo and the Western province, and is the species most often used for aquaculture in South Africa (Hey, 1944). *O. mossambicus* can be distinguished from *O. niloticus* and *O. aureus* by yellow pigmentation in the gular region, tails without vertical banding, an upturned, protruding snout and the black colour of older males (Lim and Webster, 2006). *O. mossambicus* is only distributed in the southern parts of Africa, and is becoming threatened by Nile tilapia that occurs in the Limpopo river systems (Van der Waal, 2001).

Tilapia is described as herbivorous, although this fish species has the ability to adapt to different food sources and will sometimes eat small insects and fish (Nagase, 1964). The length of the gastrointestinal tract of herbivorous fish is normally longer than that of carnivorous fish (Nagase 1964, Sklan *et al*., 2004, Smith *et al*., 2000). However, the length of the gastrointestinal tract of tilapia is shorter than that of a typical herbivorous fish, although longer than carnivorous species (Nagase, 1964). In order to accommodate such a long tract inside the body of the fish, intestinal coils are formed. These complex coils are species-specific and also play an important role in the absorption of nutrients. The gastrointestinal tract of tilapia consists of five main regions namely the hepatic loop, proximal major coil, gastric loop, distal major coil

and the terminal segment. The gastrointestinal tract of tilapia lacks intestinal ceca. This structure tends to develop better in carnivorous fish and plays a role in absorption of nutrients, fermentation and storage of food. In tilapia the length of the tract accommodates for these functions by the complex coiling (Smith *et al*., 2000).

One of the atribute that makes this an ideal aquaculture species is its ablity to survive in harsh conditions. This species can tolerate temperatures as low as 6-10°C or as high as 35-42°C for short periods of time. Optimum growth is usually between 20°C and 35°C (Ndong *et al*., 2007). Tilapia is also able to tolerate, grow and even reproduce in brackish water (Al-Harbi and Uddin, 2005). As with all aquaculture species, under stressful conditions tilapia are more susceptible to diseases (Marzouk *et al*., 2008; Salinas *et al*., 2006). Some common disease-causing organisms in tilapia include the *Lymphocystis* virus, bacteria such as *Flexibacter* (Ndong *et al*., 2007), *Streptococcus iniae* (Perera *et al*., 1997) and *Vibrio parahaemolyticus* (Balfry *et al*., 1997) and protozoan diseases like *Trichodina* and *Ichthyophthirius* (Ndong *et al*., 2007).

Antibiotics are commonly used in aquaculture to treat diseases and as prophylactics to enhance growth of the fish. Nowadays, due to the negative impact of antibiotics on the environment, the use of probiotics is increasing. A variety of different studies have been conducted in order to find effective probiotics for the use in tilapia aquaculture. The effect of *Enterococcus faecium* on the growth performance and immune response of tilapia (*Oreochromis niloticus*) were evaluated (Wang *et al*., 2008). After 40 days, tilapia supplemented with the probiotic showed a better increase in final weight and daily weight gained than fish fed only a basal diet (Wang *et al*., 2008). *Enterococcus faecium* fed to Nile tilapa had a positive effect on the weigth of fish. However, the immune response of the tilapia supplemented with the probiotic did not show any remarkable difference, although the respiratory burst activity of the phagocytes in the blood was higher (Wang *et al*., 2008). A number of different *Bacillus* species have also been evaluated as possible tilapia probiotics. *Bacillus pumilus* (Wang *et al*., 2008), *Bacillus subtillis* (Marzouk *et al*., 2008) and *Bacillus amyloliquefaciens* (Ridha and Azad, 2011) all show promising results to enhance the growth rate and immunity of the fish. Other bacterial species that show possibilities include *Lactobacillus* species (Ridha and Azad, 2011), particularly, *L. acidophilus* (Lara-Flores *et al*., 2003) as well as *Streptococcus faecium* (Lara-Flores

et al., 2003). A few studies have also evaluated yeasts as potential probiotic supplements for different fish species (Lara-Flores *et al.,* 2003; Marzouk *et al*., 2008). In most of the studies it was found that bacterial supplements were more effective than yeast (Marzouk *et al*., 2008; Ridha and Azad, 2011). However, one study found that the yeast *Saccharomyces cerevisiae* was more effective than bacterial supplements (Lara-Flores *et al.,* 2003). It was also shown that *S. cerevisiae* was able to adhere and colonize the gut of tilapia in order to prevent colonization by pathogens (Marzouk *et al*., 2008).

During the course of this study (Chapter 2), *Paracoccus marcusii* was isolated from the gastrointestinal tract of South African abalone (*Haliotis midae*), and identified as a potential bacterial species to be used in aquaculture. Due to the production of astaxanthin in the cells, *P. marcusii* is also considered as a possible source of pigmentation that can be used in aquaculture. Studies have showed that these pigments are not only deposited in the muscle, but can enhance the growth rate of fish and improve their immunity (Gabaudan, 1996, Higuera-Ciapara *et al*., 2006). The aim of this study was, therefore, to evaluate the probiotic effect of *Paracoccus marcusii* on the growth and immunity of Mozambique tilapia (*O. mossambicus*) in aquaculture systems.

Materials and methods

Experimental layout and fish

The trial took place at Welgevallen Experimental Farm, University of Stellenbosch and extended over a period of 8 weeks as part of a larger trial on the effect of feed additives in Tilapia. The facility consisted of a recirculating aquaculture system, which has 88 x 80 litre tanks. These tanks were continuously supplied with water sourced from a borehole in the Eersteriver, Stellenbosch. Temperature and quality of the water was monitored throughout the extent of the trial.

A total of 80 mixed-sex Mozambique tilapia (*O. mossambicus*) with a mean weight of 40 g was obtained from the Aquaculture division, University of Stellenbosch. Eight fish were randomly allocated into each of the tanks. Fish were fed a basal diet for a week before starting with experimental diets.

Experimental treatments

Two different diets were prepared and each diet represented a treatment (Table 1). All treatments were repeated 5 times and were randomly assigned to each of the 10 tanks. Treatment 1 served as the negative control which consisted of a basal diet of pellets coated with fish oil. Pellets from treatment 2 were coated with fish oil containing the potential probiotic bacterium, *Paracoccus marcusii* (isolate 6.15), isolated from the gastrointestinal tract of abalone (*Haliotis midae*).

In preparation of treatment 2 large amounts of lyophilized *P. marcusii* (isolate 6.15) cells were required (Fig. 3). Thus, for large scale production, cells were cultured in 3 litre Erlenmeyer flasks containing 1 litre specialized medium each (5 g/l yeast extract, 10 g/l peptone, 3 % NaCl, pH 7-8). Flasks were incubated at 26 °C for 7-14 days. These optimum growth conditions were determined in Chapter 2. After cultivation, cells were harvested through centrifugation in 400 ml centrifuge bottles at 10000rpm for 15 minutes. The supernatant were discarded and pellets were lyophilized until dried and stored at 4°C in a dark container. One gram of the lyophilized cells was mixed with 0.9 litres of fish oil. The oil was then mixed with 1 kg of pellets until evenly coated.

In order to determine the number of viable *P. marcusii* isolate 6.15 cells in 1 gram of lyophilized cells, dilution series of 10^{-1} – 10^{-6} were prepared. The dilutions were plated out in triplicate onto nutrient agar (1 g/l meat extract, 2 g/l yeast extract, 5 g/l peptone, 8 g/l sodium chloride) and incubated at 26 °C for 5 days. Total colony forming (CFU) units were counted and the average number of viable *P. marcusii* cells in 1 g of lyophilized cells was determined.

Viability and optimum storage temperature of Paracoccus marcusii (isolate) *cells on feed*

Samples from treatment 2 were collected directly after mixture. Within 24 hours, 1g of pellets was dissolved in 9ml of sterile saline solution (0.9 % NaCl). From this mixture, 1 ml was plated out in triplicate onto nutrient agar (meat extract 1 g/l, yeast extract 2 g/l, peptone 5 g/l, sodium chloride 8 g/l and agar 15 g/l). Plates were incubated at 26 °C for 5 to 7 days. Total CFU of *P. marcusii* were determined. Samples collected after mixture was then divided into 3 different containers and stored at temperatures 20 °C, 4 °C and -20 °C. Viability of *P. marcusii* cells mixed with the pellets was determined once every month for 4 months as describe above.

Viable cell counts of GIT microbes

Experimental samples were taken at the start (sampling 1), after 22 days (sampling 2) and at the end of the trial (sampling 3). At each sampling, 1 fish from every tank was placed in a small container of water containing a high concentration of the commercial anaesthetic (AQUI-S 40 mg/l, Bowker, 2004). This was done in order to kill anesthetize the fish without causing any extra stress. After measuring the length and weight of the fish, each GIT was aseptically removed and placed in a 10 ml sterile saline solution (0.9 % NaCl) containing acid-washed glass beads (Sigma, South Africa) and stored at 4°C until used.

Within 24 hours after the GIT of the fish were removed, each GIT was homogenized by vortexing on a Vortex-2 Genie for 10 minutes. Dilution series of 10^{-2} – 10^{-5} were prepared from each homogenized GIT sample. Dilutions were plated out in triplicate onto Tryptone Soya agar (TSA) (Casein enzymic hydrolysate 17 g/l, papaic digest of soyabean meal 3g/l, D-Glucose 2.5 g/l, bile salts mixture 1.5 g/l, dipotassium

hydrogen phosphate 4 g/l, sodium chloride 5 g/l and agar 12 g/l). Plates were incubated at 26 °C for 2 days. Total colony forming units (CFU) for each plate was counted and the average CFU for each treatment was determined.

Growth, feed conversion ratio and mortalities of fish

The initial mean weight together with the final mean weight of fish from the 2 treatments was measured. The total weight of dry feed given of each treatment was also determined together with the total weight gained by the fish. In order to monitor the effect of the different feeds on the growth rate of the fish, the percentage increase in body weight (1) and feed conversion ratio (FCR) (2) were determined. The mortalities of each tank were also noted throughout the trial.

% increase in body weight = Final mean weight / initial mean weight (1)

FCR = Total weight of dry feed given / Total weight gain (2)

Blood analysis

At the last sampling, one fish from each tank was randomly collected and anesthetized using the commercial anaesthetic AQUI-S 40 mg/l (Bowker, 2004). Blood (1.5 ml) was drawn from the caudal vein of each fish using 0.8 x 25 mm needles and 5 ml syringes. The syringes were rinsed with an anticoagulant heparin. Blood was placed in a 2 ml eppendorf tube and stored on ice. Within 4 hours after collecting blood, samples were centrifuged at 3000 rpm for 10 minutes. The supernatant (blood serum) was transferred to a new 1.5 ml tube and stored at -20 °C.

Lysozyme activity

Lysozyme activity in the blood serum of each sample was determined using a turbidity assay (Parry *et al*., 1965). The working solution was prepared, containing 37.5 mg of *Micrococcus lysodeikticus* (Sigma M3770) added to 50 ml of 0.05 M phosphate buffered saline (pH 6.2). Standard lysozyme (Sigma L7651) solutions

were also prepared with concentrations of 30 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1 µg/ml, 0.5 µg/ml and 0 µg/ml. Fifty microliter (50 µl) of each sample or standard and 250 µl of the working solution were added in triplicate to the wells of a microtiter plate. After mixing the plate, the absorbance was determined at 530 nm at time 0 and after 20 min. A standard curve was prepared and the lysozyme concentrations of the samples were determined.

Protein concentration

Total protein concentration in the blood serum was determined using the linearized Bradford method (Zor and Zvi, 1996). Standard protein solutions, bovine serum albumin (Biorad) were prepared at concentrations of 710 µg/ml, 473 µg/ml, 284 µg/ml, 189 µg/ml, 142 µg/ml, 114 µg/ml, 95 µg/ml and 0 µg/ml. Fifty microliter (50 µl) of each sample or standard and 200 µl of the Biuret reagent (Sigma) were added in triplicate to the wells of a microtiter plate. The absorbance was determined at 450 nm and 590 nm.

The number of total serum immunoglobulin content was determined using the method of Siwicki and Anderson (1993). After determining total protein concentration, the immunoglobulin was precipitated using 12 % polyethylene glycol (Sigma). The difference between the protein serum concentration before and after precipitation gives the total immunoglobulin present in the blood serum (Siwicki and Anderson, 1993).

Results

Cell concentration on experimental diets

Treatment 1 did not contain any *P. marcusii* (isolate 6.15) cells. Total CFU of *P. marcusii* in 1 gram lyophilized cells was 2.47 x 10^7. Thus, treatment 2 contained 2.47 x 10^7 CFU per 1 kg of feed.

Viability of Paracoccus marcusii cells isolate 6.15 on feed

The viability of *Paracoccus marcusii* (isolate 6.15) on the pellets after mixture was determined over a period of 4 months under different storage temperatures (Fig. 4). Samples were taken once every month. An average of 91.8 CFU/ml were obtained with the first sampling in October 2011. After the feed were stored at different temperatures for a month (November 2011), colony forming units for samples stored at 20 °C was 67.33, for 4 °C it was 16.33 and for -20 °C it was 18. Data obtained from the last two months (December 2011 and January 2012) were not included in Fig. 1, as no *P. marcusii* colonies could be detected.

Viable cell counts of GIT microbes

Viable cell counts (CFU/ml) were determined from the GIT of all the sampled fish (Fig. 5) over the extent of the trial. An average of 100.44 x 10^3 CFU/ml was obtained for the base line sampling (17 October 2011), where all the fish were fed a basal diet. After feeding on different treatments, the second sampling on 10 November 2011 revealed significant differences between the CFU/ml of the first sampling. Total CFU/ml increased significantly for both the treatments. Fish from treatment 1 had a lower CFU/ml number of 145.11 x 10^3 than fish sampled from treatment 2 which had 175.22 x 10^3 CFU/ml. At the third sampling (12 December 2011),the total CFU of both treatments decreased significantly. Treatment 1 had 100.22 x 10^3 CFU/ml and treatment 2 had 120.77 x 10^3 CFU/ml. From all the samples, no orange *P. marcusii* colonies were detected.

Growth, feed conversion ration and mortalities of fish

There was a significant difference in the percentage increase in body weight of fish at the end of the trial between the two treatments (Fig. 6). The increase in body weight of fish from treatment 1 was 166.67% and for treatment 2 it was 185.79 %. The feed conversion ratio (FCR) given in Fig. 7 also revealed a significant difference between the two treatments. Treatment 2 showed the lowest FCR of 198.92, in comparison with treatment 1 which has a better FCR of 237.06. Treatment 1 had the highest number of mortalities, with a total of 14. Treatment 2 had only 3 mortalities in total (Table 2).

Blood analysis

Fish fed on treatment 2 showed a significant higher lysozyme concentration of 23.3 µg/ml, in comparison with fish from treatment 1 with a concentration of 11.3 µg/ml (Fig. 8). Total protein concentration in the blood serum of fish (Fig. 9) from treatment 2 (22.7 µg/ml) were significantly higher than of treatment 1 (18.1 µg/ml). However, no significant difference in the immunoglobulin concentration in the serum was detected between the two treatments (Fig. 10). The immunoglobulin concentration for treatment 1 was 2.6 µg/ml and for treatment 2 it was 3.1 µg/ml.

Discussion

Increasing demand for the use of probiotic bacteria in aquaculture to improve the growth and health of fish has prompted the search for potential probiotic bacteria (Balcazar *et al*., 2007; Kim and Austin, 2006; Pirarat *et al*., 2006; Ridha and Azad, 2011; Wang *et al*., 2008). In this study we evaluated the probiotic effect of *P. marcusii* (isolate 6.15) on Mozambique tilapia was evaluated through monitoring the growth and immune response of fish cultured in an aquaculture setup.

In preparation of the probiotic treatment, large numbers of *P. marcusii* (isolate 6.15) cells were cultured at the optimum growth conditions as described in Chapter 2. In the present study 1 kg of feed was amended with 1 gram freeze-dried cells to obtain a concentration of 2.47 x 10^7 CFU/ g feed. Similar studies have included a much higher concentration of probiotic to the feed, with concentrations reaching up to 10^{10} CFU / g feed (Balcazar *et al*., 2007; Pirarat *et al*., 2006). However, this study continued with the low inclusion levels of the probiotic. This was due to culturing limitations and also because the profit margin in this species is small and additive should be inexpensive.

Before starting the trial it was important to determine the viability of the probiotic after mixing with the feed, and the optimum storage temperature to prolong shelf life. It was important that the number of probiotic cells supplemented to the fish remain constant throughout the duration of the trial. In this study, it was found that *P. marcusii* cells coated on the feed were only viable for 1 month at an optimum storage temperature of 20 °C (Fig. 4). Storage of the probiotic feed at 4 °C and -20 °C significantly reduced the viability of the bacterial cells. Therefore, feed was mixed with the probiotic every 2 weeks and stored at 20 °C, for the duration of the trial, to ensure the viability of *P. marcusii* cells on coated feed.

Healthy microbial GIT communities play a vital role in the well being of fish (De Silva and Anderson, 1995). These microorganisms may provide a nutritional benefit through the production of different enzymes, compete with pathogens and enhance the immune response of the host (Anand *et al*., 2009; Erasmus *et al*., 1997; Harris *et al*., 1991; Izvekova, 2005; Izevekova and Komova, 2005). It was, therefore, important to determine if the addition of *P. marcusii* (isolate 6.15) has any negative

effect on the number of intestinal microflora which could affect the health of the fish. Bacterial cell counts of two samples collected on two separate sampling occasions showed a slight increase in total CFU/ml when compared to the negative control. This increase was not as a result of the addition of *P. marcusii* (isolate 6.15) because no orange colonies were detected on the plates, but could be as a result of the increased size of the fish. Therefore, *P. marcusii* did not have any negative effect on GIT bacterial numbers of tilapia.

The results further indicate a significant difference in the percentage increase in body weight, between probiotic-fed fish and the negative control (Fig. 6). Fish supplemented with the probiotic showed a higher percentage increase in body weight. The feed conversion ratio is an indication of the ability of fish to convert feed mass into body mass. The FCR of probiotic-fed fish was significantly lower than that of the negative control (Fig. 7). A lower FCR value indicates that less feed is necessary to produce one kilogram of fish body mass (Winfree and Stickney, 1980).

Only 3 mortalities occurred in the tanks where fish were fed the probiotic supplemented feed and this was significantly lower than the 14 mortalities in the negative control tanks. This indicate that, although the number of probiotic cells added to the feed was significant lower than that of previous studies (Balcazar *et al*., 2007; Pirarat *et al*., 2006), *P. marcusii* showed a definite enhancement in the growth performance of the fish.

In an ideal system it would be expected that *P. marcusii* (isolate 6.15) would have a positive effect on the immune response of the fish. Fish have many specific and non-specific immune response mechanisms to protect them against diseases (Ellis, 1999). In order to evaluate the effect of *P. marcusii* on the immune system of fish, the lysozyme activity and immunoglobulin concentrations in the blood serum were determined. Lysozyme is bacteriocidal enzymes and aid in defence against different bacterial infections (Ridha and Azad, 2011). The enzyme hydrolyses N-acetylmuramic acid and N-acetyglucosamine which form part of the peptidoglycan layer of bacterial cell walls. Various studies (Balcazar *et al*.,2007; Kim and Austin, 2006; Pirarat *et al*., 2006, Wang *et al*., 2008), have shown that probiotic bacteria are able to increase the lysozyme activity in fish and in this way improve the immune response of fish. In this study, we also found that the potential probiotic bacterium,

P. marcusii (isolate 6.15), increased the lysozyme activity in the blood serum of the fish (Fig. 8). Immunoglobulin proteins also play an important role in the immune system of fish. These proteins are present in the blood and body fluids and act as immune effector molecules to protect fish against diseases (Tang, *et al*., 2008). In this study there was, however, no significant difference between the immunoglobulin concentrations of the two treatments (Fig. 10).

The exact association between *P. marcusii* (isolate 6.15) and the GIT of the host was not investigated in this study. However, it was hypothesized that this bacterium does not colonize the GIT of the fish, but rather moves through the GIT as a transient bacterium or is lysed in the GIT. This was evident from the fact that no *P. marcusii* colonies were observed when the viable cell counts of the GIT were detected. Although it is speculated that the bacterium do not colonize but rather moved through the GIT or were lysed, the bacterium still displayed a probiotic effect. Tilapia is a herbivorous fish species and has a relative long mid- and hind-gut section (Buddington *et al*., 1987). This may help to prevent the bacteria being wash out of the GIT too quickly, preventing them to have any probiotic effects. Longer GITs provide enough time for the bacterium to act as a probiotic before leaving the host. If the bacteria were lysed, enzymes and carotenoids were possibly released in the GIT, which may have resulted in better growth performance and enhanced immunity.

Conclusion

The findings in this study demonstrate the potential use of *P. marcusii* as a probiotic bacterium for Mozambique tilapia (*Oreochrois mossambicus*) in aquaculture systems. *Paracoccus marcusii* was able to improve the growth rate, FCR and survival rate of the fish. Although *P. marcusii* (6.15) did not have any effect on the immunoglobulin concentrations in the blood, increased lysozyme activity in the blood was detected. This bacterium could, therefore, be effective as a probiotic by improving the growth rate and possibly strengthening the immune system of the fish. However, further studies need to be done in order to optimize dosage concentration of the probiotic supplement. It is also important for future studies to determine the type of association between *P. marcusii* and the host, together with the specific probiotic action of the bacterium in the host.

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Figures

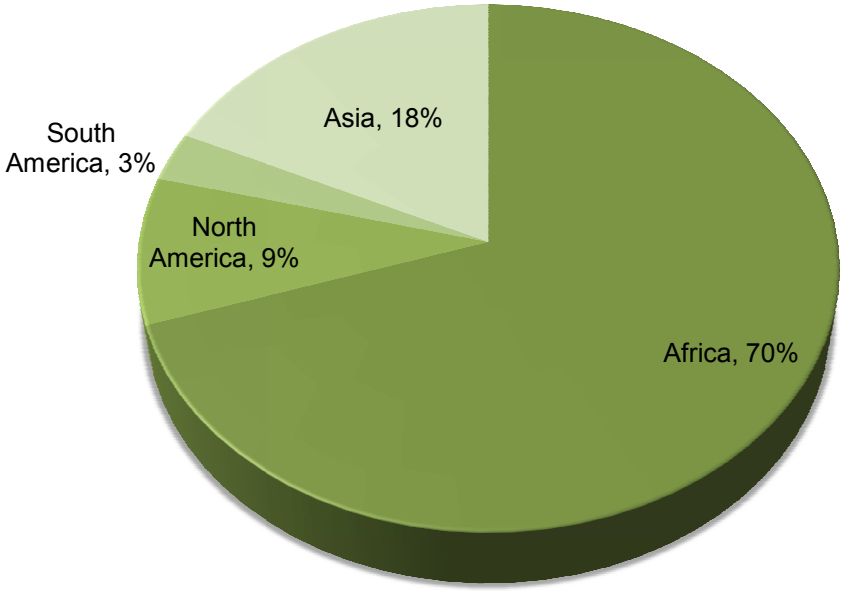

Figure 1 – Main regions for tilapia production under aquaculture conditions (adapted from El-Sayed, 2006).

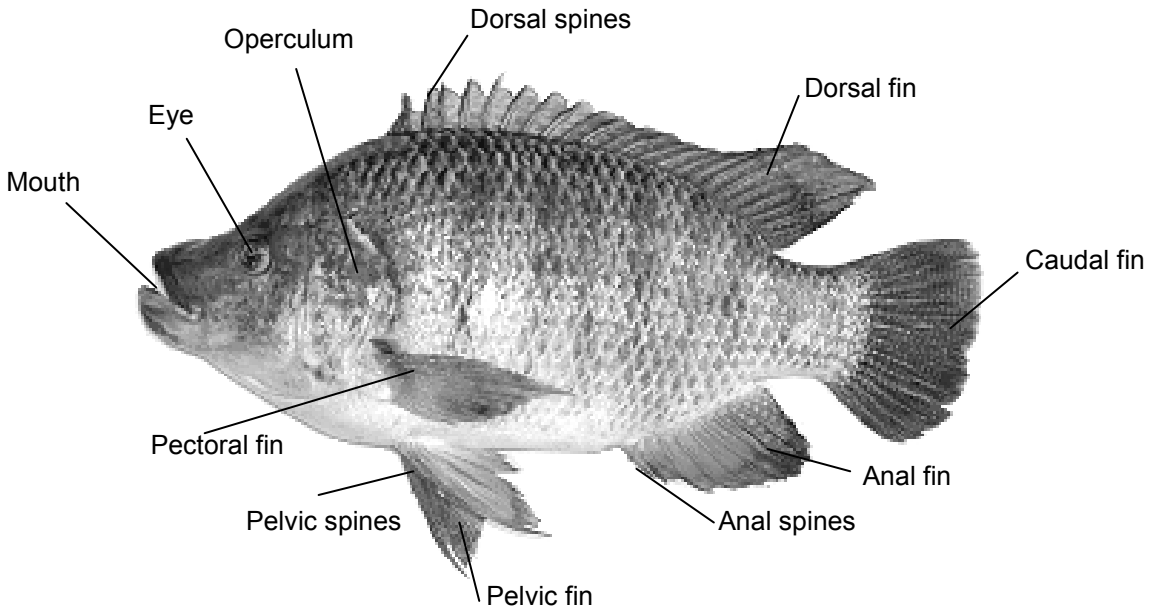

Figure 2 – Common anatomical characteristics of *Oreochromis mossambicus* (adapted from Lim and Webster, 2006).

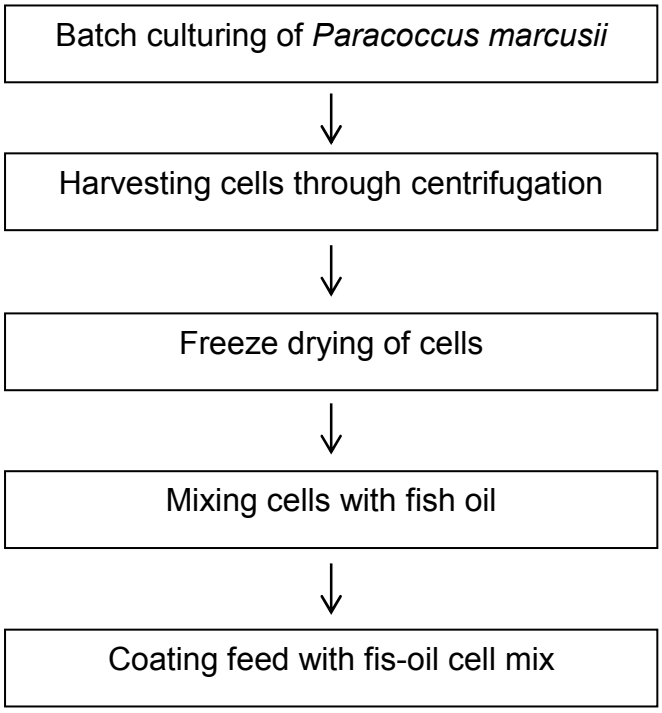

Figure 3 – Diagram of the preparation of treatment 3. *Paracoccus marcusii* (isolate 6.15) were batch cultured. Cells were harvested through centrifugation and freeze-dried. The freeze-dried cells were used to mix with the fish oil to coat the feed.

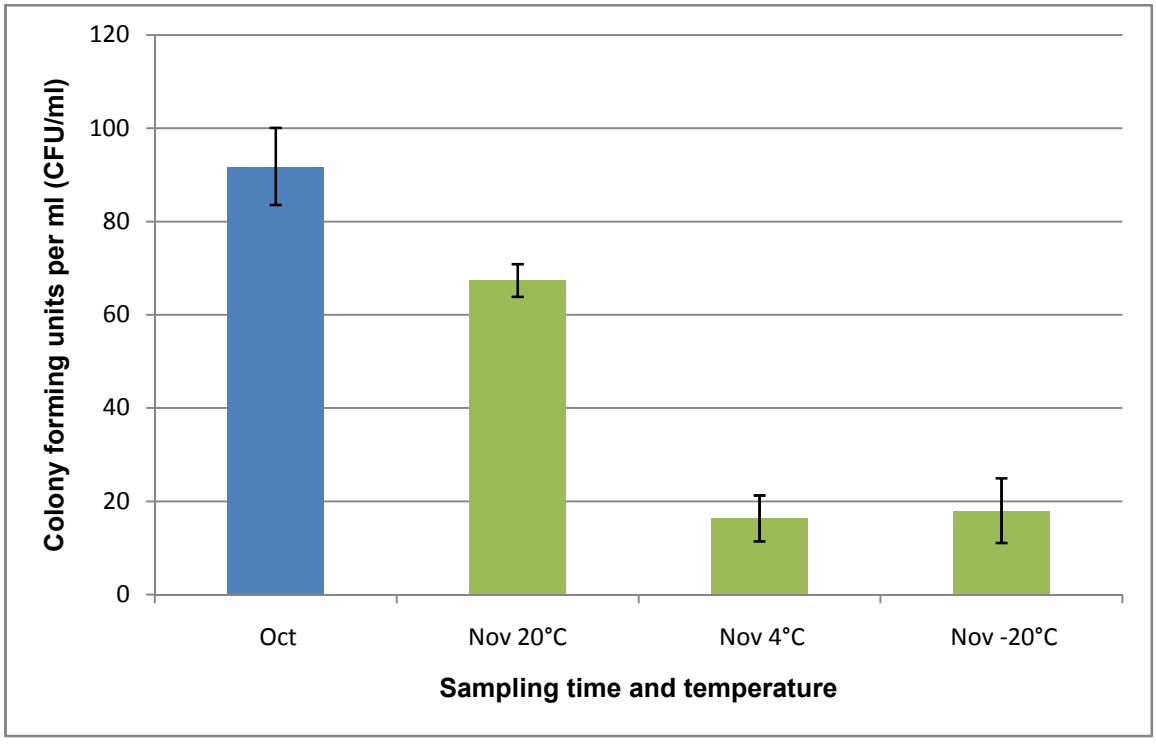

Figure 4 – Viability of *Paracoccus marcusii* (isolate 6.15) on coated feed. Feed was mixed in October 2011 and then stored at 3 different temperatures namely 20°C, 4°C and -20°C. Colony forming units were determined from October 2011 to January 2012, however, no growth was observed in December and January.

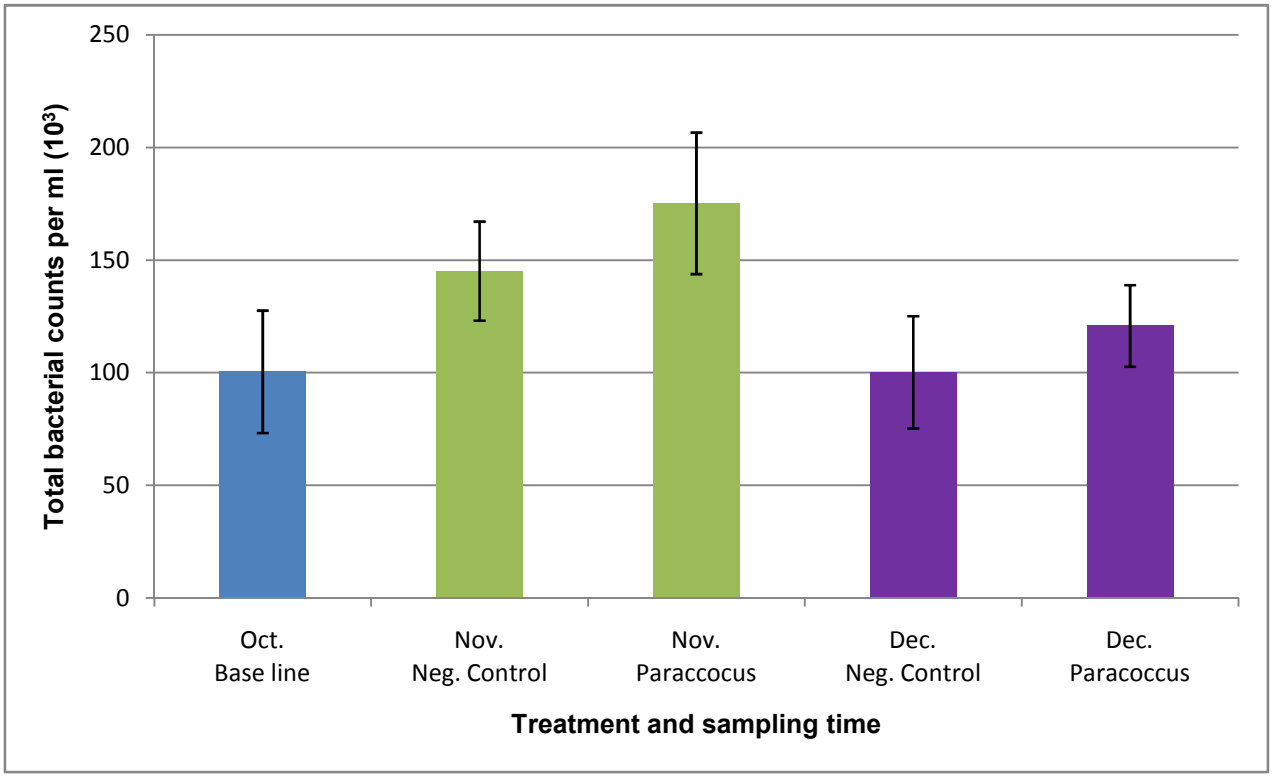

Figure 5 – Viable cell counts from the gastrointestinal tract of Mozambique tilapia fed on two different diets over two months. For the base line all the fish fed on the same diet. One fish from each tank was sampled every sampling time in order to remove the GIT.

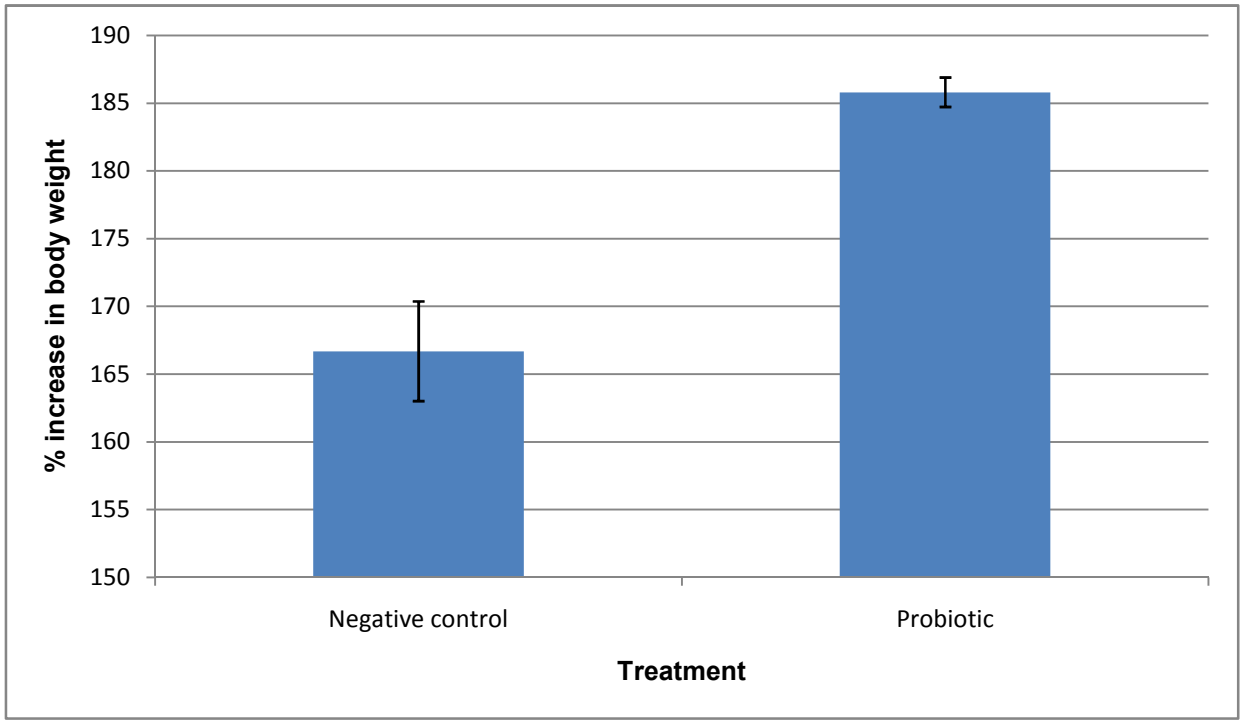

Figure 6 - Percentage increase in body weight of Mozambique tilapia following the course of the trial. The initial mean weight together with the final mean weight of tilapia from the two different treatments was measured in order to obtain the % increase in body weight.

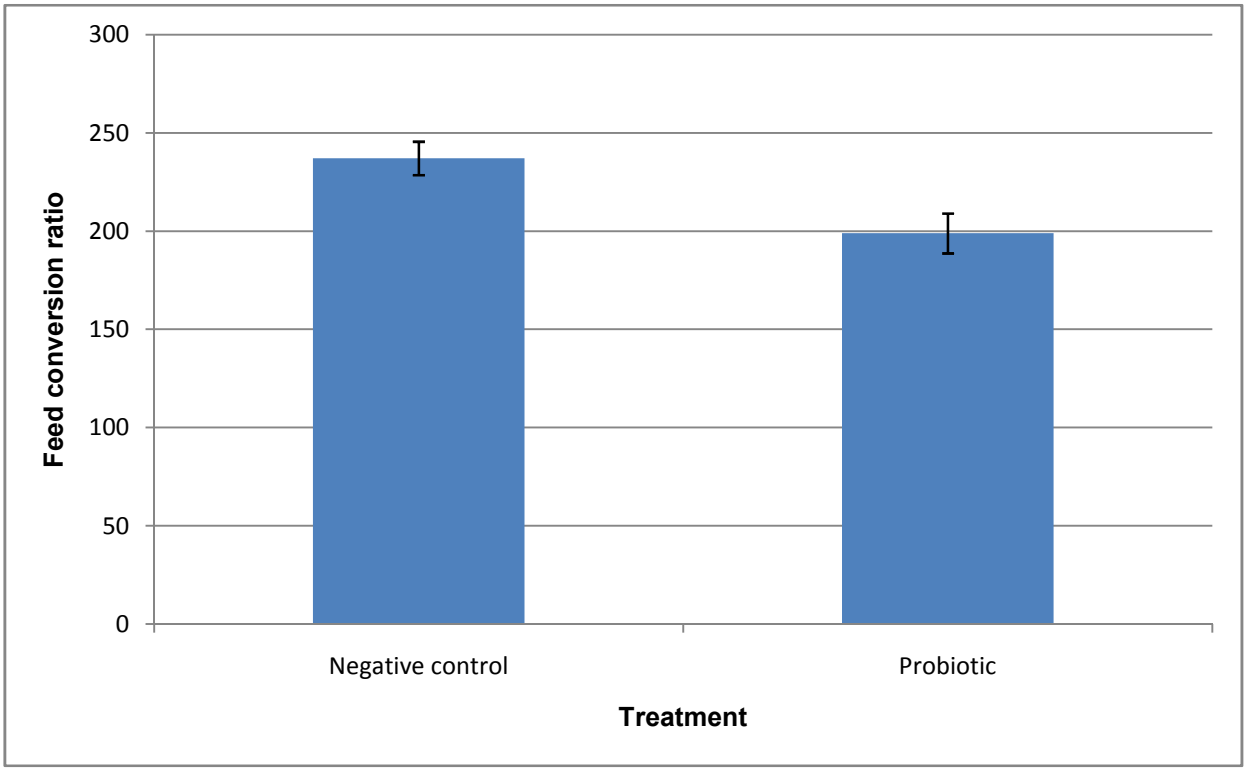

Figure 7 – Feed conversion ratio of Mozambique tilapia fed different diets over the course of the trial. Total dry feed given of each treatment was determined together with total weight gained by the tilapia from each treatment.

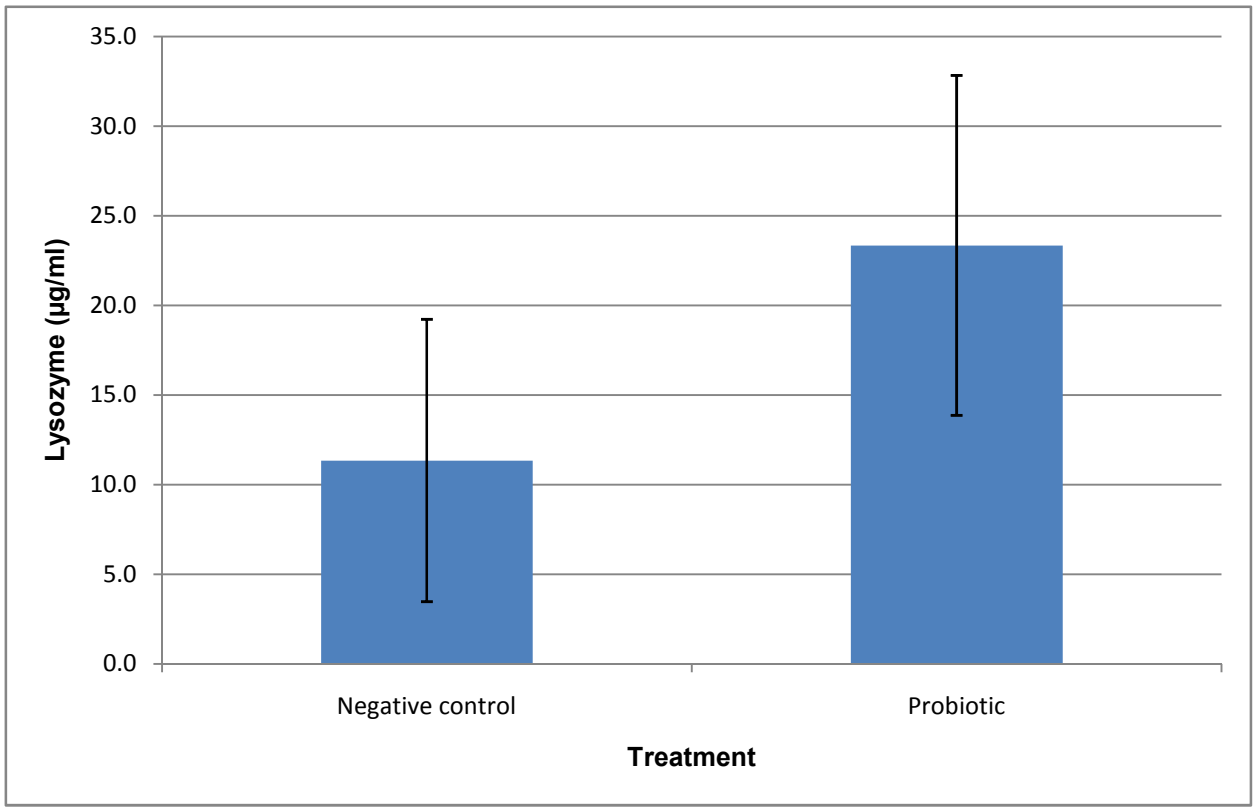

Figure 8 – Lysozyme activity from blood serum of Mozambique tilapia. Blood samples were collected at the end of the trial. Lysozymy activity was determined using a turbidity assay.

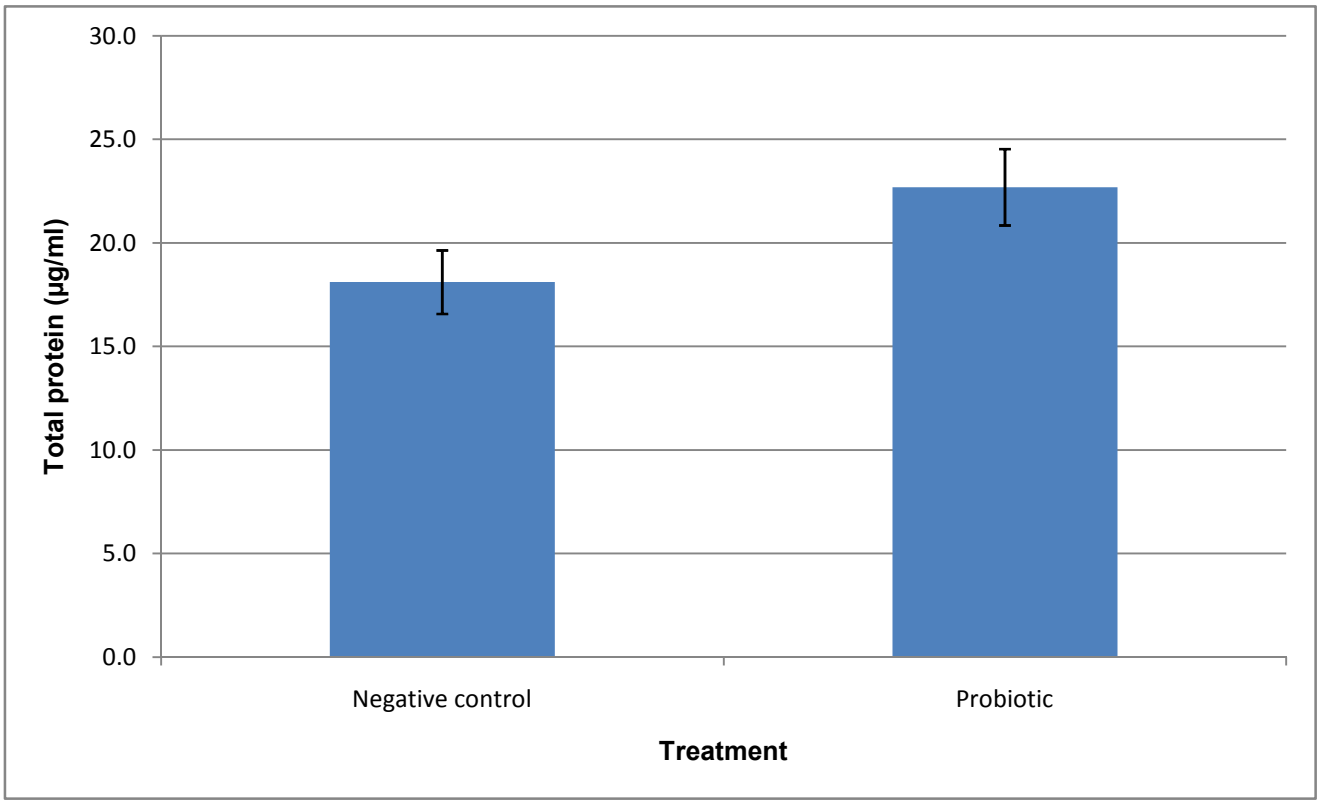

Figure 9 – Total protein concentration in blood serum of Mozambique tilapia. Blood samples were collected at the end of the trial. Total protein concentrations were determined uding the linearized Bradford method.

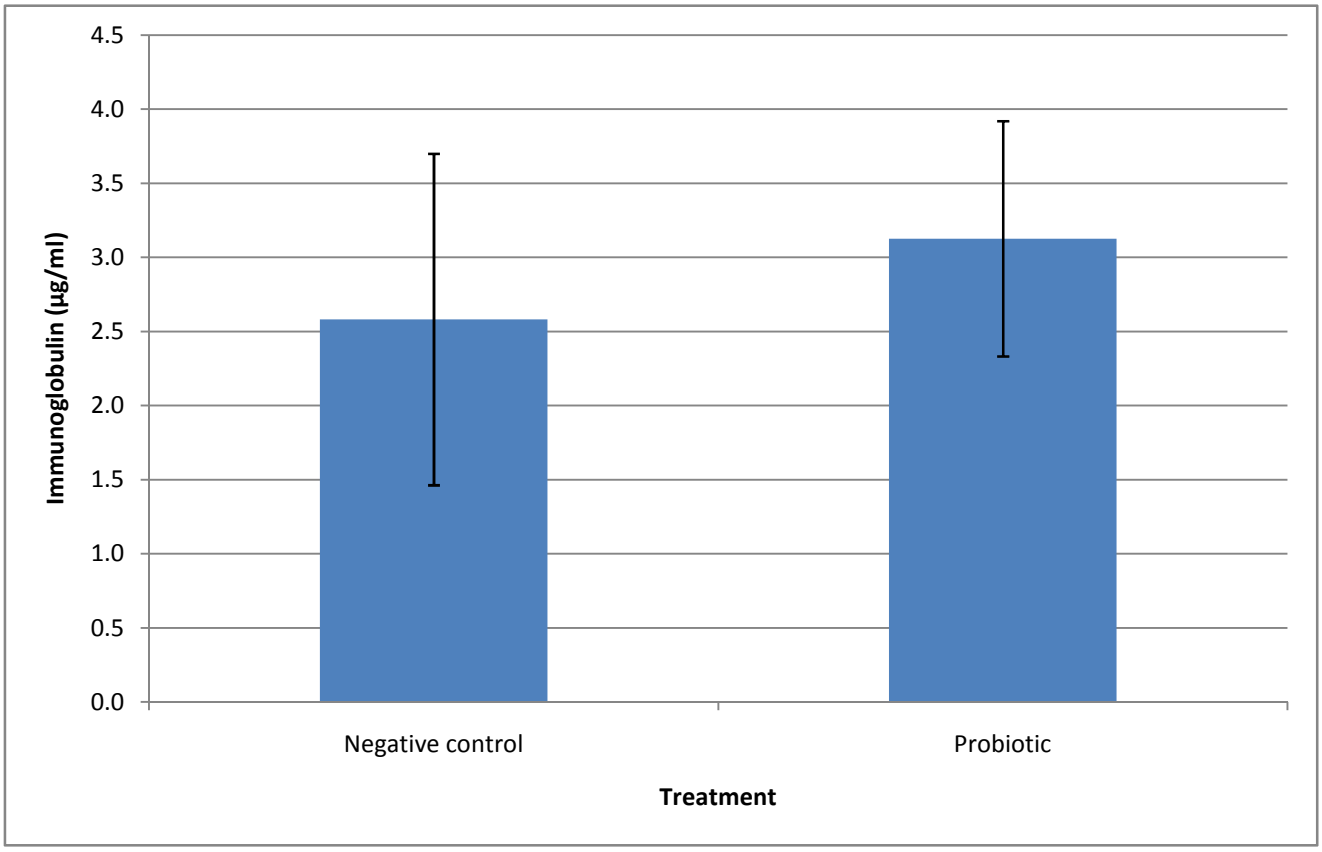

Figure 10 – Total immunoglobulin concentration in the blood serum at the end of the trial of Mozambique tilapia. Total immunoglobulin content was determined using the method of Siwicki and Anderson, 1993.

Tables

Table 1 : Experimental treatments		
Treatment	**Diet**	**Contents**
1	Negative control	Basal diet
2	Potential probiotic	Pellets coated with *Paracoccus marcusii*, 1 g dried cells/kg feed mixed with fish oil

Table 2 - Total mortalities during the duration of the trial			
Treatment	**Tank no.**	**Total fish per tank**	**Total mortalities**
1. Negative control	29	6	0
	36	6	2
	43	6	5
	50	6	6
	57	6	1
2. Probiotic	31	6	1
	38	6	0
	45	6	0
	52	6	2
	59	6	0

Chapter 4

Probiotic and pigmentation effect of *Paracoccus marcusii* on rainbow trout (*Oncorhynchus mykiss*)

Abstract

Astaxanthin is an important carotenoid used in aquaculture for the pigmentation of species such as trout. *Paracoccus marcusii* (isolate 6.15), isolated from the GIT of the South African abalone (*Haliotis midae*), was investigated in this chapter as a possible alternative source for pigmentation of aquaculture species. This isolate also showed potential as a probiotic bacterium. The aim of this study was to evaluate the potential of *P. marcusii* (isolate 6.15) as a pigmentation source and possible probiotic for rainbow trout (*Oncorhynchus mykiss*) under aquaculture conditions. Fish were fed three different diets, namely a positive control (Carophyll® pink - DSM), *P. marcusii* and a negative control (no pigment added). After a period of 10 weeks, fish fed on the positive control showed pigmentation of the meat. No pigmentation was visible for fish feeding on *P. marcusii* and negative control treatments. There was also no significant increase in the growth rate or the immune response of the fish feeding on *P. marcusii*. Microbial GIT communities of fish feeding on the three different treatments showed no distinctive similarities or differences in diversity. However, similarities and differences in community diversity were detected from the samples of the same sampling times.

Introduction

Trout species are naturally found in river systems, lakes, dams and mountain streams. They have a world-wide distribution, including Alaska, British Columbia, Europe, North Africa, the Middle East and western Asia. These species are able to endure temperatures ranging from 0°C to 27°C, with the optimum between 14°C to 18°C (Sedgewick, 1995). Trout is widely used in aquaculture and some of the most commonly used species in aquaculture include rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) (FAO, 2005-2011). Of these three species, rainbow trout (Fig. 1) is by far the most abundant because of its fast growth, good feed conversion and easy reproduction (Sedgewick, 1995).

Diseases and the use of probiotics in trout farming

It is well known that fish under aquaculture conditions are more susceptible to diseases (Marzouk *et al*., 2008, Salinas *et al*., 2006; Sedgewick, 1995). Therefore, diseases in rainbow trout are a major problem (Sedgewick, 1995) and are caused by a variety of organisms including viruses, bacteria, fungi and parasites (Henryon *et al*., 2005; Noble and Summerfelt, 1996). Commonly encountered viral diseases include infectious pancreatic necrosis, viral hemorrhagic septicaemia and infectious hematopoietic necrosis (Henryon *et al*., 2005, Noble and Summerfelt, 1996). Bacteria are one of the largest groups of disease causing organisms in trout. Major bacterial diseases include furunculosis caused by *Aeromonas salmonicida* (Irianto and Austin, 2003), streptococcosis caused by *Streptococcus iniae*, lactococcosis caused by *Lactococcus garvieae* (Brunt and Austin, 2005; Eldar and Ghittino, 1999) and *Flavobacterium branchiophilum* that cause bacterial gill disease. Some parasitic diseases are caused by *Gyrodactylus*, *Chilodonella, Trichodina* and *Trichophrya* (Noble and Summerfelt, 1996).

Treatment of trout diseases usually includes the use of antibiotics (Heuer *et al*., 2009; Hirsch *et al*., 1999). However, because the use of antimicrobial agents is questioned (Cabello, 2006; Hirsch *et al*., 1999; Wang *et al*., 2008), the search for effective probiotics increases. A number of studies have evaluated the potential probiotic effect of different bacterial isolates (Brunt and Austin, 2005; Brunt *et al*., 2007; Kim and Austin, 2006; Nikoskelainen *et al*., 2001; Robertson *et al*., 2000). Two bacterial

strains, *Carnobacterium maltaromaticum* B26 and *Carnobacterium divergens* B33, were isolated from the GIT of healthy rainbow trout and showed enhancement of the cellular and humoral immune responses. *Carnobacterium maltaromaticum* also showed an increased phagocytic activity and *Carnobacterium divergens* showed an increase in respiratory burst and serum lysozyme activity (Kim and Austin, 2006). *Carnobacterium* sp. isolated from Atlantic salmon was also administered to rainbow trout and was able to reduce diseases caused by *A. salmonicida* and *Vibrio ordalii* (Robertson *et al*., 2000). In some instances, human probiotics such as *Lactobacillus rhamnosus*, were administered to trout. In this case it was found to reduce the mortality of fish challenged with the pathogen *A. salmonicida* (Nikoskelainen *et al*., 2001; Panigrahi *et al*., 2005).

Trout pigmentation

The distinctive red to pink colour of the meat makes trout species very attractive for consumer consumption (Kurnia *et al*., 2010). In nature, this carnivorous fish species consume sources of pigments such as algae, which give them their red to pink colour. However, under farming conditions, the fish do not come into contact with these natural sources of pigments, which results in dark grey meat. This colourless meat is not appealing for consumers and significantly decreases the value of the fish (Higuera-Ciapara *et al*., 2006). Therefore, under aquaculture conditions pigments are added to the feed of trout (Choubert *et al*., 2009). Two of the main pigments used in trout farming are the carotenoids astaxanthin and canthaxanthin. Of these two pigments, astaxanthin has a better ability to bind to the muscle actomyosin than canthaxanthin (Bjerkeng *et al*., 1992).

In fish, carotenoids are mainly absorbed in the mid and hind GIT (Storebakken and No, 1992). These pigments are lipid soluble and the same absorptive pathways are used as for lipids (Sheridan, 1988). Nonpolar carotenes such as β-carotene are located in the hydrophobic core of lipid emulsion, bile salt miscelles and chylomicron whereas polar xanthophylls such as astaxanthin are located on the surface of these components. The amount of carotenoid which is available for muscle pigmentation (bioavailability) is influenced by absorptive and post absorptive factors. One of the factors that determines bioavailability is the digestibility of carotenoids, which refers to the proportion absorbed in the gut (Torrissen *et al*., 1990). Digestion involves a

number of steps (Fig. 2), starting with the breakdown of the food matrix which can be mechanical and chemical. The composition of the food matrix has a big influence on the availability of the pigment (Storebakken and No, 1992). If a very stable matrix is used in order to protect the pigment during production or in storage, the pigment may not become soluble. The second step is solubilisation of astaxanthin into mixed bile salt micelles and then movement across the water layer next to the microvilli. The pigment is then taken up by the enterocyte and incorporated into chylomicrons, which are then transported to actomyosin (Bjerkeng *et al*., 2007; Furr and Clark, 1997).

Astaxanthin is naturally produced by a variety of different organisms, including plants (Ausich, 1997; Cunningham and Gantt, 2005), microalgae (Ausich, 1997; Lorenz and Cysewski, 2000), yeasts (Sanpietro and Kula, 1998) and bacteria (Guyomarc'h *et al*., 2000; Lee *et al*., 2004; Yokoyama *et al*., 1995). All these different species produce astaxanthin using the same biosynthetic pathway, with only small differences between them. The biosynthetic pathway for astaxanthin (Fig. 3) begins with the conversion of farnesyl pyrophosphate (FPP) to geranyl-geranyl pyrophosphate (GGPP). This reaction is catalyzed by GGPP synthase which is encoded by the *crtE* gene. Two GGPP molecules are then linked through phytoene synthase encoded by the *crtYB* gene to form phytoene. Phytoene dehydrogenase (encoded by *crtI* gene) introduces four double bonds in the phytoene to form lycopene. The ψ acyclic ends of the lycopene are converted to β rings through lycopene cyclase, also encoded by the *crtYB* gene, to form β-carotene and γ-carotene (Lee and Kim, 2006a; Lee and Kim, 2006b; Martin *et al*., 2008). β-carotene hydroxylase (*crtZ* gene) and ketolase (*crtW* gene) are two enzymes required to convert β-carotene into astaxanthin. Hydroxylase converts β-carotene to zeaxanthin through introducing two hydroxyl groups (-OH) at the 3 and C-3' end. Ketolase incorporates two keto groups (C=O) at C-4 and C-4' of β-carotene to form canthaxanthin. These two enzymes are then further responsible for converting canthaxanthin and zeaxanthin to astaxanthin (Lee and Kim, 2006b; Martin *et al*., 2008).

Commercial production of astaxanthin is becoming very important as the demand for this red pigment is growing. Large amounts of the pigment need to be produced for aquaculture use all over the world (Guerin *et al*., 2003; Higuera-Ciapara *et al*., 2006). Astaxanthin can be chemically or biologically synthesised. Some advantages of chemical synthesis of carotenoids are that it produces carotenoids of distinct purity

and consistency. Also, the total costs of the product are relatively low (Bjerkeng *et al*., 2007). A disadvantage in the chemical synthesis of carotenoids is the complexity of carotenoid synthesis. Although in some cases technology and knowledge developed for the synthesis of a certain carotenoid can be used to synthesize others, new chemical routes are often required to produce carotenoids. Another problem is that chemical synthesis produces mixtures of stereoisomers. Some of these stereoisomers are not found in nature and may not be as active as naturally occurring carotenoid isomers. Undesired side effects may also occur in these stereoisomers (Ausich, 1997; Higuera-Ciapara *et al*., 2006; Katsuyama *et al*., 1987).

One way to solve the problems associated with chemical synthesis is to synthesize carotenoids biologically. In this production process, only natural stereoisomers are produced and the knowledge of the biosynthesis of one carotenoid can be used to synthesize others. However, a negative aspect of biosynthesis is that the production is more expensive than that of a chemical synthesis process. Carotenoids are produced intracellularly (Ausich, 1997) and are often associated with other molecules such as lipids and proteins (Higuera-Ciapara *et al*., 2006). Therefore, purification of the carotenoids is needed and this process adds to the expense of the final pigment product (Bowen *et al*., 2002; Bjerkeng *et al*., 2007).

Two species which are primarily used for commercial biosynthesis of astaxanthin include the microalgae *Haematococcus pluvialis* (Bubrick, 1991; Lorenz and Cysewski, 2000; Olaizola, 2000) and the yeast *Phaffia rhodozyma* (Andrewes *et al*., 1976; Sanpietro and Kula, 1998). Supercritical carbon dioxide extraction is used to extract astaxanthin and other carotenoids from these species (Lim *et al*., 2002; Machmudah *et al*., 2006; Nobre *et al*., 2006). The extraction of carotenoids is very expensive and therefore, studies have been done on the effect of including intact *Haematococcus pluvialis* and *Phaffia rhodozyma* cells in the feed of fish (Sommer *et al*., 1991). It was demonstrated that astaxanthin extracted from *H. pluvialis* can effectively be utilized by rainbow trout in both an esterified and unesterified form. However, when intact cells were administered, the limiting factor for the utilization of carotenoids was the extent to which the cell walls or cysts were cracked or broken. Intact cell walls can completely inhibit the utilization of the pigments in the muscle of the fish (Bowen *et al*., 2002). Studies done on *P. rhodozyma* also showed that the degree of cell wall disruption affects the utilization of astaxanthin in the muscle of the

fish (Bjerkeng *et al*., 2007). Through these studies it was shown that cell wall disruption is crucial in order for the intracellular astaxanthin to be available for absorption (Bowen *et al*., 2002; Bjerkeng *et al*., 2007).

Another possible source of pigmentation for aquaculture is the astaxanthin producing Gram negative bacterium, *Paracoccus marcusii* (isolate 6.15), isolated from the gastrointestinal tract of South African abalone (*Haliotis midae*). Gram negative bacteria have weaker cell walls than algae and yeasts, it is therefore, hypothesized that the cell wall of *Paracoccus marcusii* will be disrupted in the GIT, releasing astaxanthin to bind to the muscle. The aim of this study was therefore to evaluate the potential probiotic effects of *Paracoccus marcusii* on the growth performance and health of rainbow trout as well as to determine the ability of intact *P. marcusii* (isolate 6.15) cells to serve as a pigment source to be used for rainbow trout farming.

Materials and methods

Pilot trial

Before setting up the experimental trial, a pilot trial was done to evaluate the potential of *P. marcusii* as a pigmentation source. Five different diets were prepared namely a negative control with no pigment added, positive control with a commercial pigment added and three different concentrations of *P. marcusii* added, 0.3 g/kg, 0.6 g/kg and 1 g/kg freeze-dried cells. The duration of the trial was 2 weeks. Fish were sampled at the end of the trial and the colour of the meat was evaluated. After a successful pilot trial, the experimental trial was started.

Experimental trial

Experimental layout and fish

The trial took place at Jonkershoek Experimental Farm, University of Stellenbsoch and extended over a period of 10 weeks. The facility consisted of 38 porta pools from which 15 pools were used in this study. Each porta pool was continuously supplied with water sourced from the upper Eersteriver. The temperature and quality of water was monitored throughout the course of the trial.

A total of 1200 mixed-sex Rainbow trout (*Oncorhynchus mykiss*), with an average weight of 63.69 g and length of 156.31 mm, were equally divided into 15 tanks. Fish were fed a basal diet for a week before starting with experimental diets on 14 June 2012.

Experimental diets

Three different diets were prepared and each diet represented a treatment (Table 1). All treatments were repeated 5 times and were randomly assigned to 15 of the porta pools. Treatment 1 served as the negative control (NK) and consisted of a basal diet of pellets coated with fish oil. Treatment 2 served as the positive control (PK). Pellets were coated with fish oil containing commercial pigments (Carophyll® pink - DSM). Pellets of treatment 3 were coated with fish oil containing the potential

probiotic bacterium (P), *Paracoccus marcusii* that was isolated from the GIT of abalone (*Haliotis midae*). Treatment 3 was prepared as previously describe in Chapter 3.

Sampling

Experimental samples were taken throughout the trial every 2 weeks (Table 2). During every sampling, 3 fish from each porta pool were caught and placed in water containing a high concentration of antistatic (AQUI-S, 40mg/l) in order to anesthetize the fish with as little stress possible. Fish were labelled and kept on ice until dissected in the lab. All samples were processed within 5 hours.

Before dissection, the length and weight of the fish were obtained. Thereafter the bench together with the equipment were treated with 70% (v/v) ethanol to minimize contamination. The gut of each fish was then aseptically removed and after discarding all gut contents, the gut was placed into 10 ml of sterile saline solution (0.9% NaCl). The weight of the gutted and gilled fish, together with the weight of the liver was obtained. Fish was filleted and the colour of the meat was measured using a colorimeter (color-guide 45°/0° from BYK Gardner, USA). At sampling 5, blood was also drawn from each fish.

Growth and feed conversion of fish

The initial mean weight together with the final mean weight of fish from the three treatments was measured. The total weight of dry feed given to each treatment was also determined together with the total weight gained by the fish. In order to monitor the effect of the different feeds on the growth rate of the fish the feed conversion ratio (FCR) (1) were determined.

$$\text{FCR} = \text{Total weight of dry feed given} / \text{Total weight gain} \qquad (1)$$

Colorimetric analysis

The colour of each fish fillet was determined using a colorimeter (color-guide 45°/0° from BYK Gardner, USA). The colorimeter was placed on the meat and triplicate measurements were made on three different places of the fillet in order to give a mean value (Nickell and Bromage, 1998). Three parameters were measured using the colorimeter, namely the lightness (L*), red/green chromaticity (a*) and yellow/blue chromaticity (b*) (Fig. 4). Lightness values range from 0 to 100, where 0 is absolute black and 100 is absolute white. Positive values of a* indicate to a red colour, whereas negative values indicate to a green colour and positive b* values indicate a yellow colour, whereas negative values indicate blue colour (Choubert, 2010).

Blood analysis

Blood samples were collected at sampling 5 (16 August 2012), using the method described in Chapter 3. Before the centrifugation of the blood samples in order to collect the blood serum, whole blood samples were used to determine the percentage of red blood cells in the blood. Lysozyme activity was determined as described in Chapter 3. The percentage red blood cells were determined through filling Hematocrit capillary tubes two-thirds with the whole blood of the different samples. These tubes were then placed in a Hematocrit centrifuge for 5 minutes. The percentage packed cell-volume was determined by the Hematocrit tube reader (Aly *et al*., 2008).

Bacterial community analysis

DNA extractions

In order to extract DNA from the GIT samples collected in 10 ml sterile saline solution (FSO), each GIT was homogenised. The 10 ml FSO bottle contained glass beads (Sigma, South Africa) which aided in homogenising each sample on a vortex (Vortex-2 Genie) for 5 minutes. Total genomic DNA was extracted from the homogenised GIT of each sample using the ZR Fungal/Bacterial DNA kit™ (Zymo Research Corp. USA). The presence of DNA was confirmed on a 1% agarose gel stained with

ethidium bromide (EtBr) and visualized under UV light. DNA were then used for ARISA-PCR reactions.

ARISA-PCR

PCR reactions were done using the forward primer ITSF-FAM-(5'-GTCGTAACAAGGTAGCCGTA-3') and the reverse primer ITSReub-(5'-GCCAAGGCATCCACC-3') (Inqaba Biosystems). The forward primer was fluorescently labelled with 6-carboxy-fluorescein. These primers amplify variable lengths of the ITS regions between the small 16S and large 23S rRNA genes in bacteria. The reaction mixture contained 0.5 µl genomic DNA, 0.2 µl of each forward and reverse primers, 5 µl KapaTaq ready-mix (KapaBiosystems, RSA) and 4.1 µl ddH_2O with a final volume of 10 µl. PCR conditions started with initial denaturing at 95 °C for 5 minutes, 38 cycles of denaturing at 95 °C for 45 seconds, annealing at 56 °C for 50 seconds and elongation at 72 °C for 70 seconds and finally elongation at 72 °C for 7 minutes. Samples were then held at 4 °C before reactions were amplified in a GeneAmp PCR System 2400 (Applied Biosystems, California, USA). The presence of DNA was confirmed on a 1% agarose gel stained with EtBr and visualized under UV light.

Analysis

Products obtained from PCR were send to the Central Analytical Facility at the University of Stellenbosch. Capillary analysis on an automated Genetic Analyser ABI 3010XI was done with the ROX 1.1 size standard for bacteria (Slabbert *et al*., 2010). Based on different fragment lengths and fluorescent intensities, electropherograms were generated. GeneMapper® Version 4.1 Software (Applied Biosystems) was used to analyse electropherogram profiles and sized using the applicable size marker. The peak height over peak size was preferred. This data was exported to Microsoft Excel 2007 for further analysis. The Whittaker index of similarity (S_W) was calculated to test similarities between samples.

$$S_W = \sum_{i=1}^{n} \left(\frac{|bi1 - bi2|}{2} \right)$$

Each sample was compared in a pairwise manner and a distance matrix was generated. Variables b1 and b2 represents the percentage contributions to the *i*th operational taxonomic units (OTU) of two samples. This proportional similarity index ranges from a scale of 0 to 1, where 0 is completely similar and 1 completely dissimilar (Hewson and Fuhrman, 2006). The similarities/dissimilarities between ARISA profiles were analysed using unweighted pair-group clustering algorithm with arithmetic averages (UPGMA) to calculate dendrograms and ordination methods using Statistic 10 Software (Statsoft).

The Shannon diversity index (*H'*) determines the richness and relative abundance of OTUs.

$$H' = -\sum pi \, log_e \, pi$$

This index is defined as the negative sum of each OTUs proportional abundance (*pi*) multiplied by the natural logarithm of its proportional abundance (Hill *et al*., 2003).

Results

Pilot trial

Fish sampled at the end of the 2 weeks revealed a significant difference in their meat colour. No pigmentation was visable from fish feeding on the negative control. However, fish feeding on the positive control together with all 3 different concentrations of *P. marcusii* (isolate 6.15) show pigmentations (Fig. 5 a-e).

Growth and feed conversion of fish

The increase in body weight of fish feeding on the 3 different diets was consistent during the course of the trial with no significant difference between the different diets (Fig. 6). For the first and second sampling times, the average weight of the fish from the different treatments were relatively similar. At sampling times 3 and 4, the weight of fish from the negative control was higher than that of the positive control and probiotic treatments. The weight of fish feeding on the probiotic was the lowest at sampling 3 and 4. However, at the end of the trial, the average weight of the fish feeding on the different diets were the same. No significant differences were detected between the feed conversion ratios (FCR) of fish feeding on the different diets (Fig. 7). The FCR of the negative control was 37.53, the positive control was 36.98 and for the probiotic treatment 39.899.

Colorimetric analysis

Meat pigmentation of fish sampled from the different diets was evaluated over time (Figs. 8 - 10). At the start of the trial, sampling time 1, no pigmentation was visible. The average a*-value was -1.977, b*-value was 8.47 and L-value was 51.29. Fish supplemented with the positive control showed a drastic increase in red colour (a*-value) after 2 weeks, with an average a*-value of 12.26. This high a*-value continued over the next month with a*-values of 13.88 and 15. There was a decrease in a*-value for the positive control at the end of the trial, with a value of 9.06, however, it was still significant higher than the negative control and probiotic treatments. No significant differences were visible over time between the probiotic supplementedfeed and the negative control with an average a*-value of 1.89 (Fig. 8).

After 2 weeks, fish supplemented with the positive control also showed a significant increase in the yellow colour (b*-value) of 18.61, which remained high throughout the trial. Fish from the negative control and probiotic treatment were significant lower and had similar b*-values throughout the trial, with an average of 11.74 (Fig. 9). However, the lightness (L-value) of fish supplemented with the positive control had an average of 43.12, which was significant lower than the negative control and probiotic treatment with an average of 48.05 (Fig. 10).

Blood analysis

No significant differences were detected in hematocrit levels in fish sampled from the three different treatments. The average hematocrit level of fish sampled from the positive control was 52.25%, the negative control 53.75% and the probiotic treatment 50.83% (Fig. 11). There were also no significant differences in the lysozyme activity of the different treatments. The positive control had a lysozyme concentration of 10.54 µg/ml, the negative control 10.59 µg/ml and the probiotic treatment 10.47 µg/ml (Fig. 12).

ARISA analysis

Similarities between bacterial community structures of fish showed distinct cluster patterns in samples taken at the same sampling time (Fig. 13). However, no distinct patterns were formed when considering the different treatments (Fig. 14). These results can be supported by the Shannon diversity index of samples taken from the different treatments over a specific time period (Fig. 15). There was no significant difference in the Shannon diversity index of the different treatment at each sampling time. However, a significant decrease in the diversity of the bacterial community structure over time was visible with a p-value of 0.42875. The Shannon diversity index for the three treatments at time 1 was 2.9. At time 3 the index ranged between 2.5 and 2.7 and at time 5 between 2.1 and 2.3.

Discussion

Paracoccus marcusii (isolate 6.15) showed promise to be used in aquaculture as a potential probiotic bacterium and pigmentation source. This species produces the carotenoid pigment astaxanthin, which is commonly used for pigmentation of aquaculture species such as trout, salmon, shrimp and red see bream (Higuera-Ciapara *et al*., 2006). In this study the ability of *P. marcusii* to act as a probiotic and pigmentation source for rainbow trout (*O. mykiss*) under aquaculture conditions was evaluated.

The pigmentation result of the experimental trial was unexpected since the pilot study showed positive results. Also Kurnia and co-workers showed that different *Paracoccus* species were able to enhance skin coloration and the growth rate of red sea bream (Kurnia *et al.,* 2010). Various factors could have played a role in these findings. One of the problems might have been that the concentration of *P. marcusii* cells in the feed which was too low resulting in low numbers of *P. marcusii* cells in the GIT of the fish and subsequently low astaxanthin concentrations. However, it is not suspected to be the problem because pigmentation was visible in the pilot trial. More likely, the pigment could have been destroyed before fish were able to absorb it. Astaxanthin is a highly unstable molecule which is easily degraded when exposed to light, heat or oxygen (Storebakken *et al*., 2004; Tachaprutinum *et al.,* 2009). Decomposition of astaxanthin might have occurred during the freeze-drying of cells or more probably during storage of the feed. Freeze-drying could possibly have damaged the cells walls, exposing the astaxanthin to the environment. Astaxanthin could then have been exposed to oxygen or light, during storage which led to the decomposition of the pigment.

In order to determine the probiotic effect of *P. marcusii,* growth enhancement together with immune parameters were evaluated. Fish feeding on the *P. marcusii* diet did not show any increase in their growth rate or FCR. Furthermore, the probiotic bacterium did not have any effect on the measured immune parameters of the fish. However, there was no negative effect of this bacterium on the growth and immune system of the fish.

This study further investigated the effect of the three different diets on the microbial GIT communities of the fish. It was found that there was no similarity in the community structure between different treatments (Fig. 13). Therefore, different feeds did not have any effect on similarities of microbial communities in the GIT. However, it was observed that the community structure of fish sampled at the same sampling time were highly similar (Fig. 12). It is suggested that the age of fish might have an effect on the GIT microbial community structure. Another reason might be the effect of changing water quality over time. Fish are closely in contact with their water environment, and a change in water quality might have a direct effect on the microbial GIT communities. Also, the diversity of communities between the different diets was the same, with a significant decrease in diversity of communities as the feeding trials continued, with a significant p-value (Fig. 14). It is therefore, reasonable to assume that the different treatments used in this study did not have any effect on the similarity or diversity of microbial GIT communities of the fish.

Conclusion

This study has shown than *P. marcusii* did not have any probiotic or pigmentation effect on rainbow trout under aquaculture conditions. Possible reasons for obtaining these results were too low bacterial numbers being added to the feed or decomposition of astaxanthin. Therefore, further studies are needed to investigate the optimum bacterial concentration to be added to the feed. It is also important to determine the concentration of astaxanthin produced by each cell and to evaluate the stability of the pigment over time.

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Figures

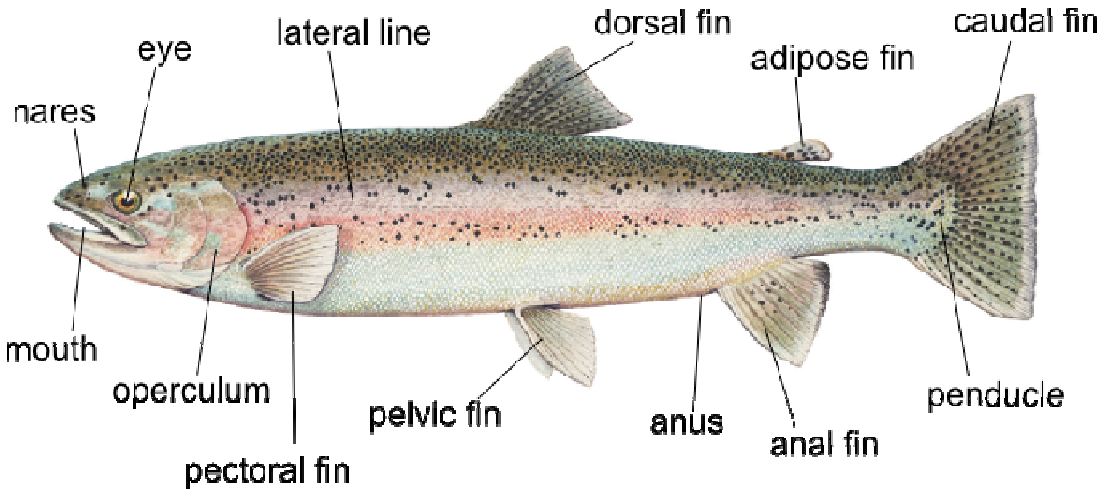

Figure 1 – Common anatomical characteristics of *O. mykiss* (adapted from Sedgwick, 1995).

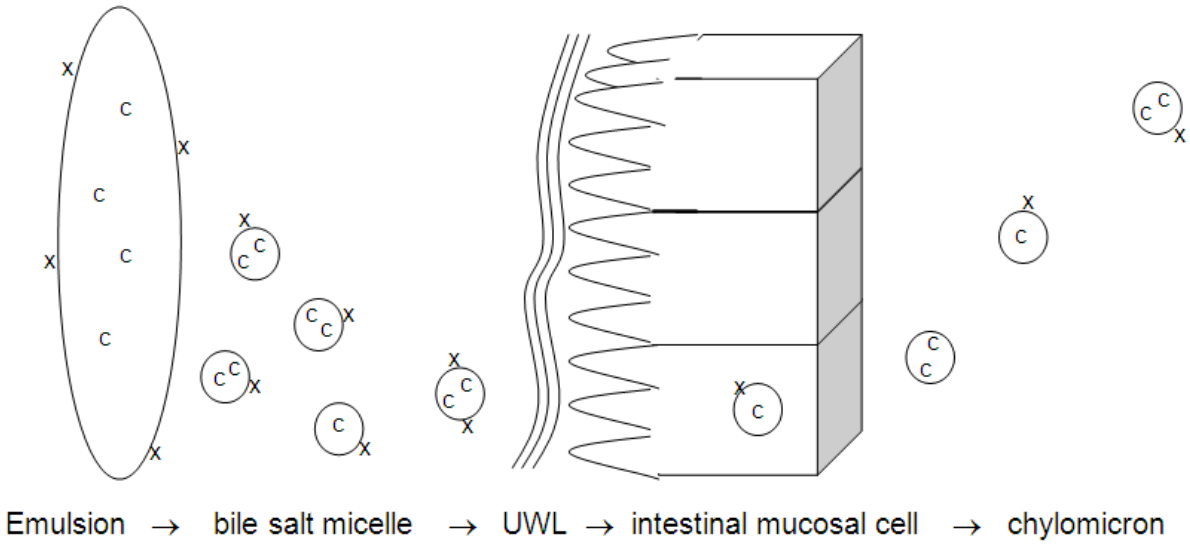

Figure 2 – Absorption of carotenoids in the gastrointestinal tract. Carotenoids transferred from lipid emulsion to salt micelles, moving through the unstirred water layer (UWL) and mucosal cell and then incorporation into lymphatic chylomicrons. C: nonpolar carotenes (such as β-carotene) located in the hydrophobic core of lipid emulsion, bile salt miscelles and chylomicron. X: polar xanthophylls (such as astaxanthin) located on the surface of the components (Modified from Furr and Clark, 1997).

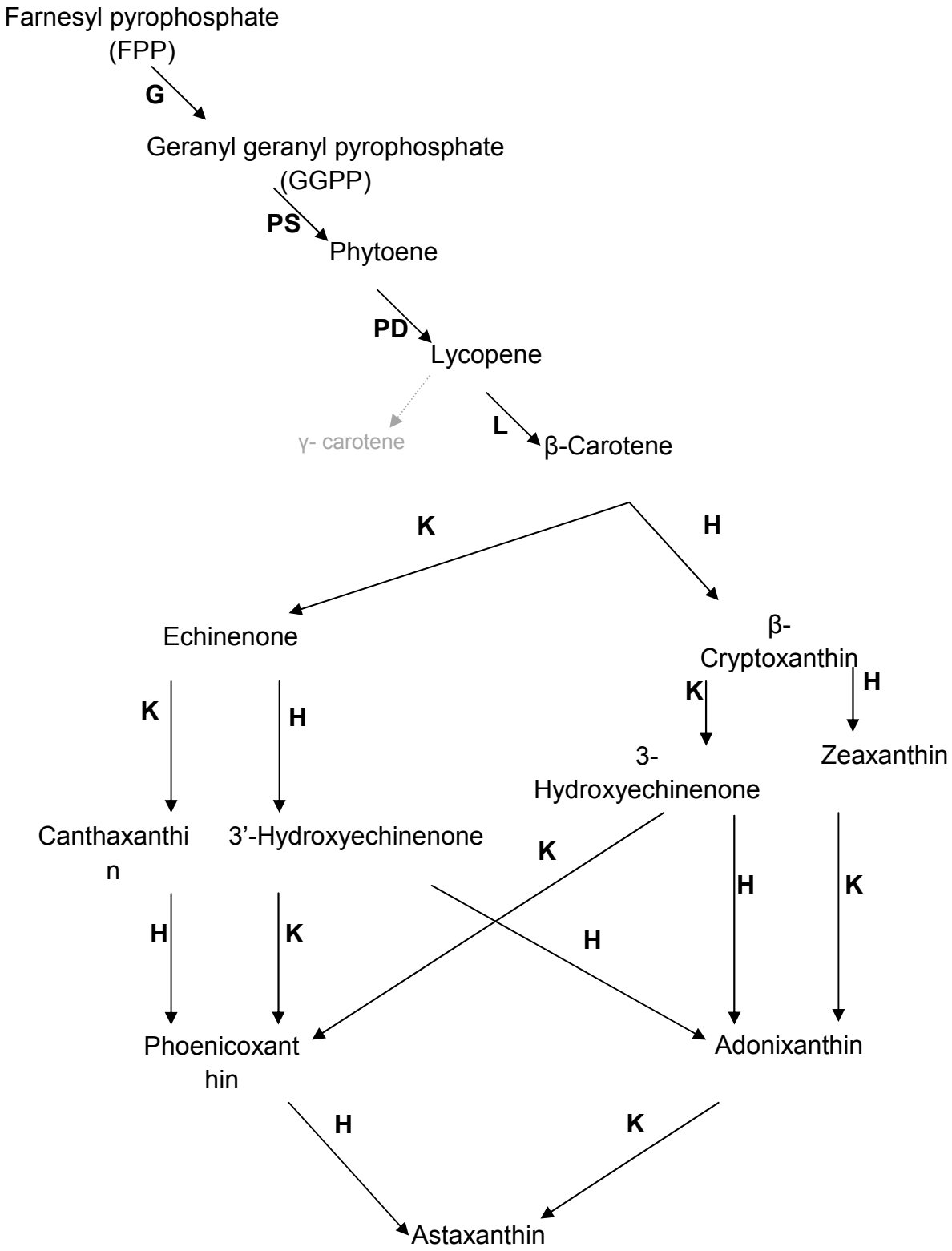

Figure 3 – Biosynthetic pathways for the conversion of farnesyl pyrophosphate (FPP) to astaxanthin. Enzymes responsible to catalyze reactions are: **G**-GGPP synthase, **PS**-phytoene synthase, **PD**-phytoene dehydrogenase, **L**-lycopene β-cyclase, **H**-β-carotene hydroxylase and **K**-β-carotene ketolase (Modified from Lee and Kim, 2006a; Lee and Kim, 2006b; Martin *et al*., 2008).

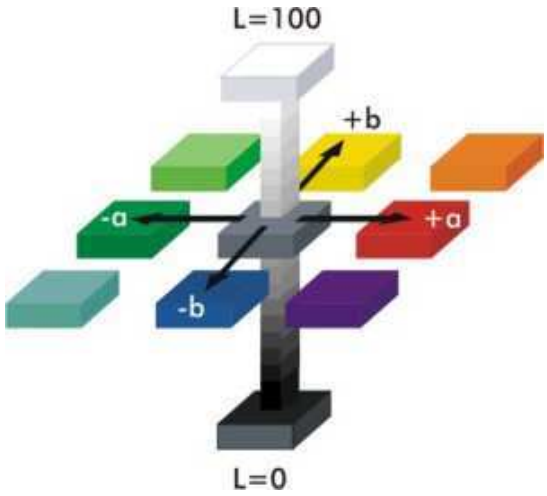

Figure 4 – CIE L, a*, b* system. L=100 is absolute with and L=0 is absolute black. Positive a* indicated a red colour and negative a* green colour. Positive b* indicate yellow and negative b* blue (BYK Gardner, USA).

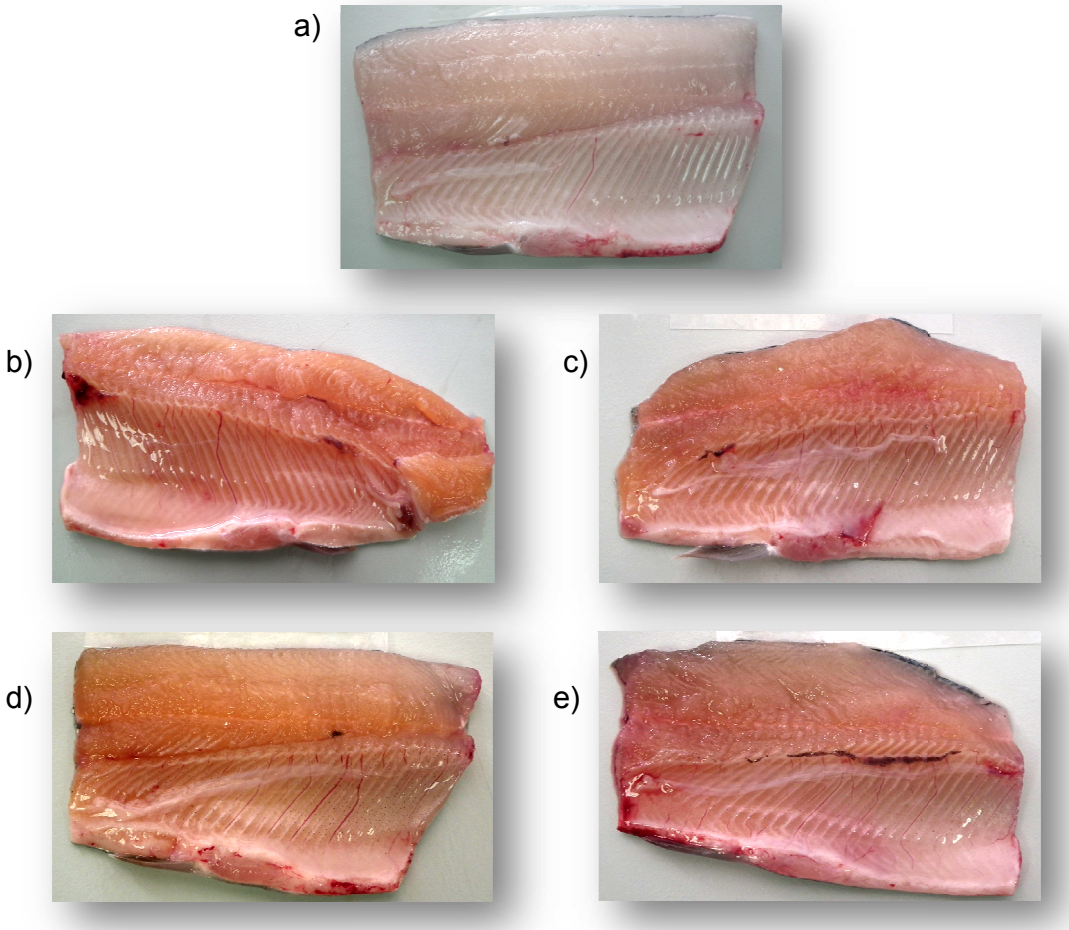

Figure 5 – Fillets taken from fish feeding on: a) Negative control, no pigmentation of the meat, b) Positive control, pigmentation of meat with a salmon pink colour, c) 0.3 g dried cells / kg feed, d) 0.6 g dried cells / kg feed, e) 1 g dried cells / kg feed. All three fillets taken from fish feeding on the bacterium showed a salmon pink colour, just as the positive control.

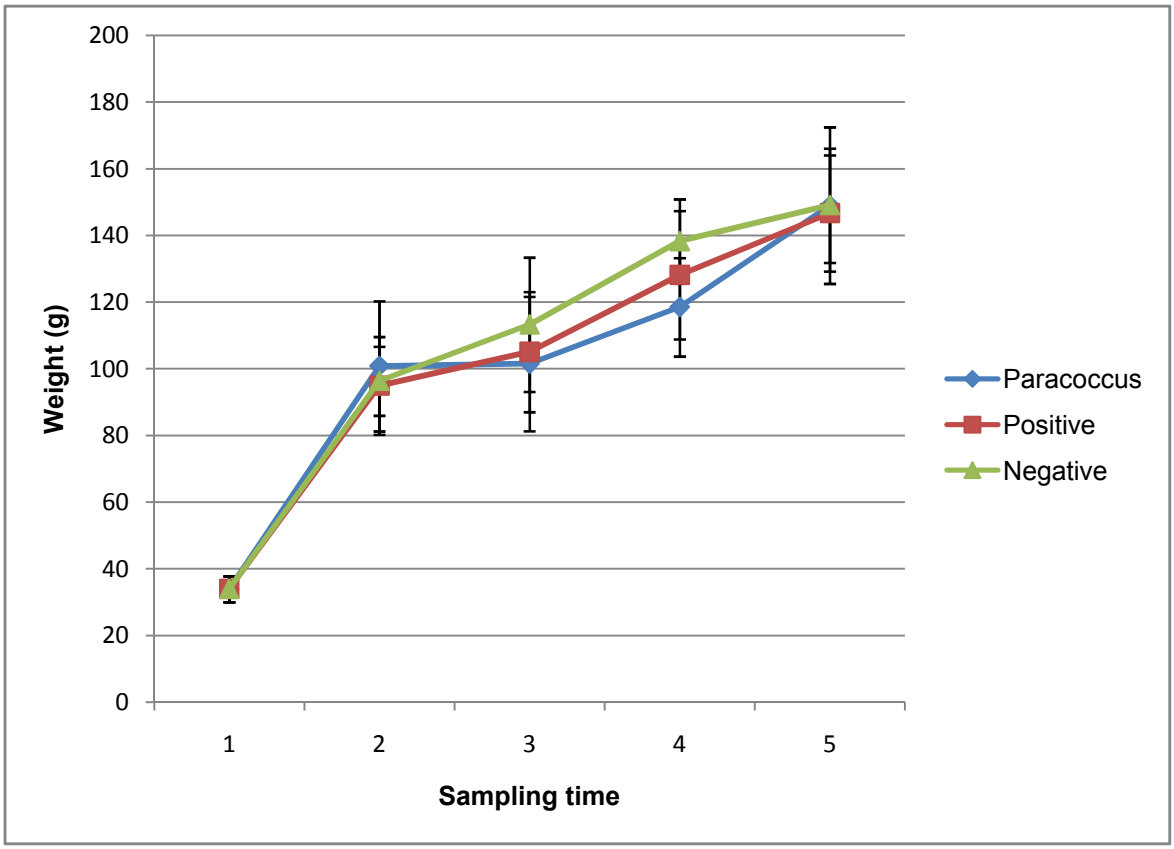

Figure 6 – Average body weight of *O. mykiss* feeding on the different treatments over the duration of the trial. Three fish from each tank were sampled every two week.

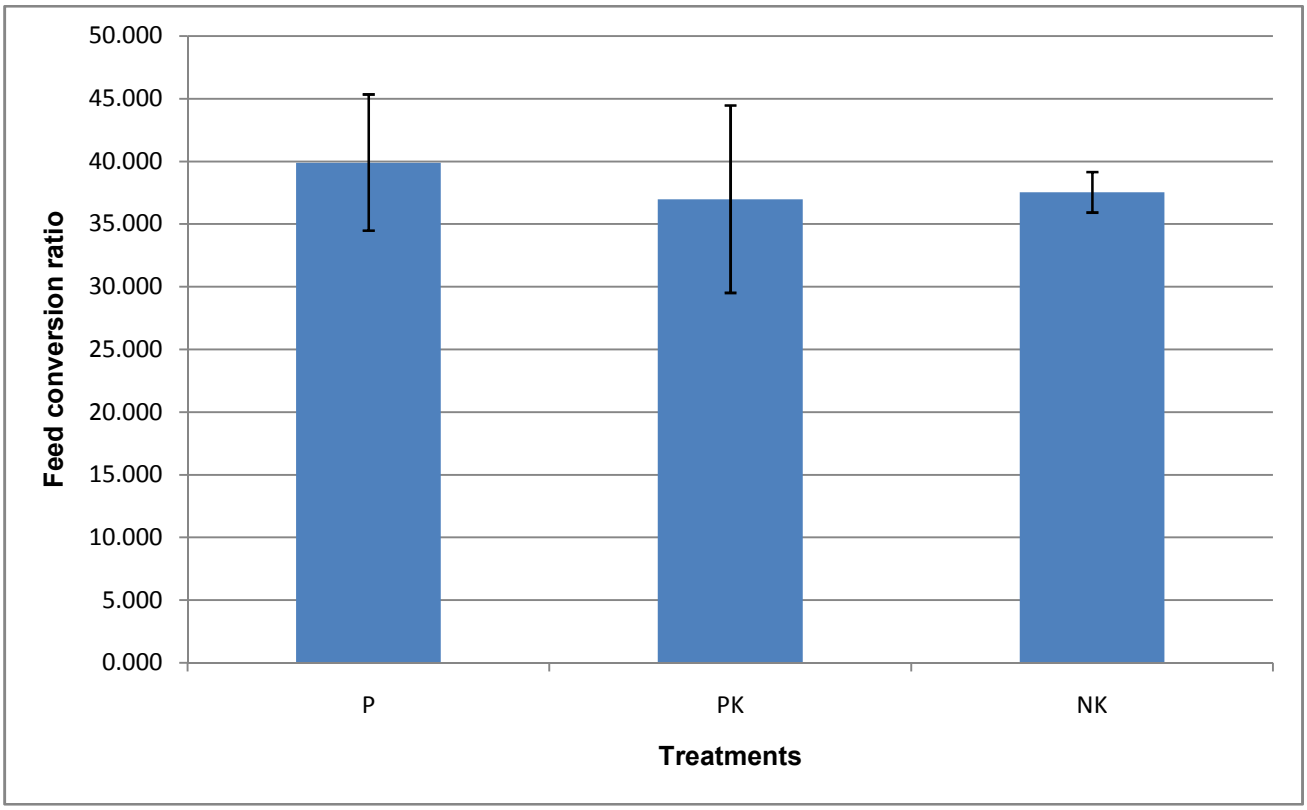

Figure 7 – Feed conversion ratio (FCR) at the end of the trial of *O. mykiss* feeding on the 3 different treatments. P= *Paracoccus marcusii*; PK= Positive control (); NK= Negative control. Total weight of dry feed given together with the initial and final mean weight of the fish were determined.

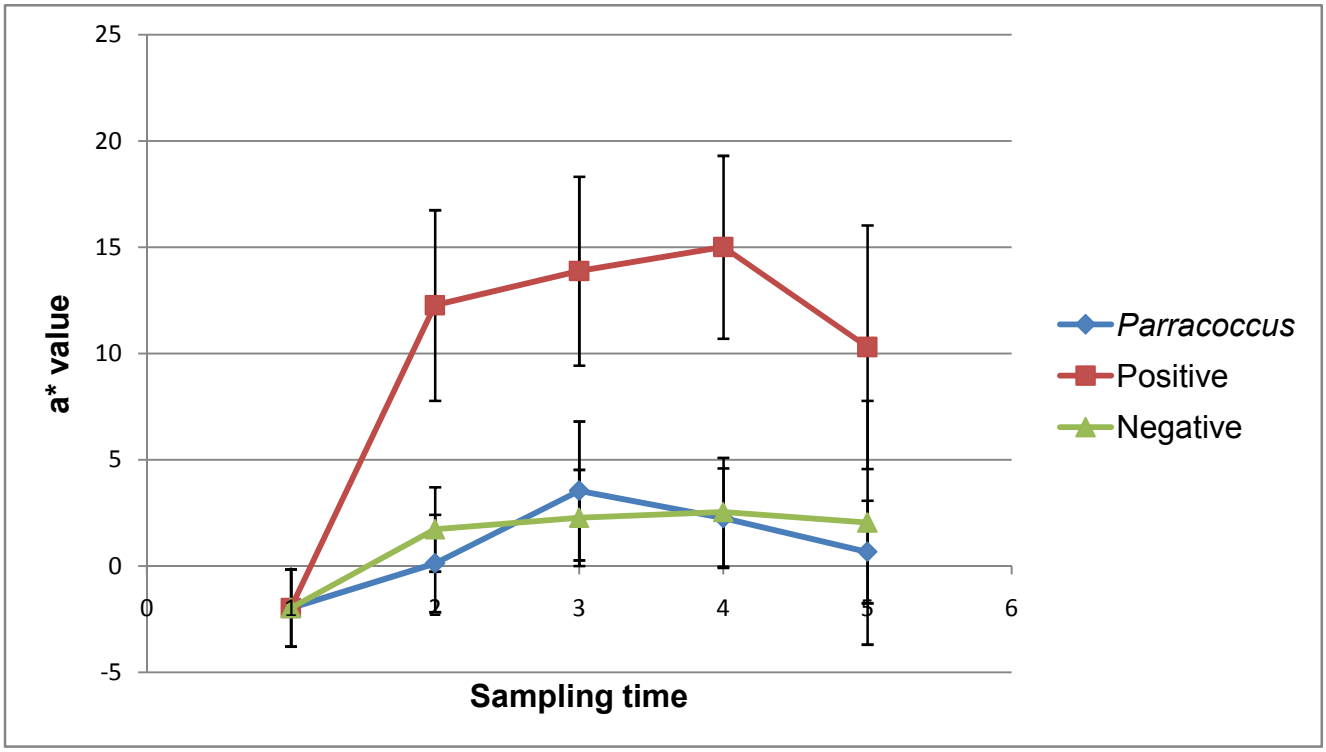

Figure 8 – a* Value of *O. mykiss* fillets sampled. This gives an indication of the redness of the fillets. Higher positive values indicate a redder colour. Three fish from each tank were sampled every 2 weeks.

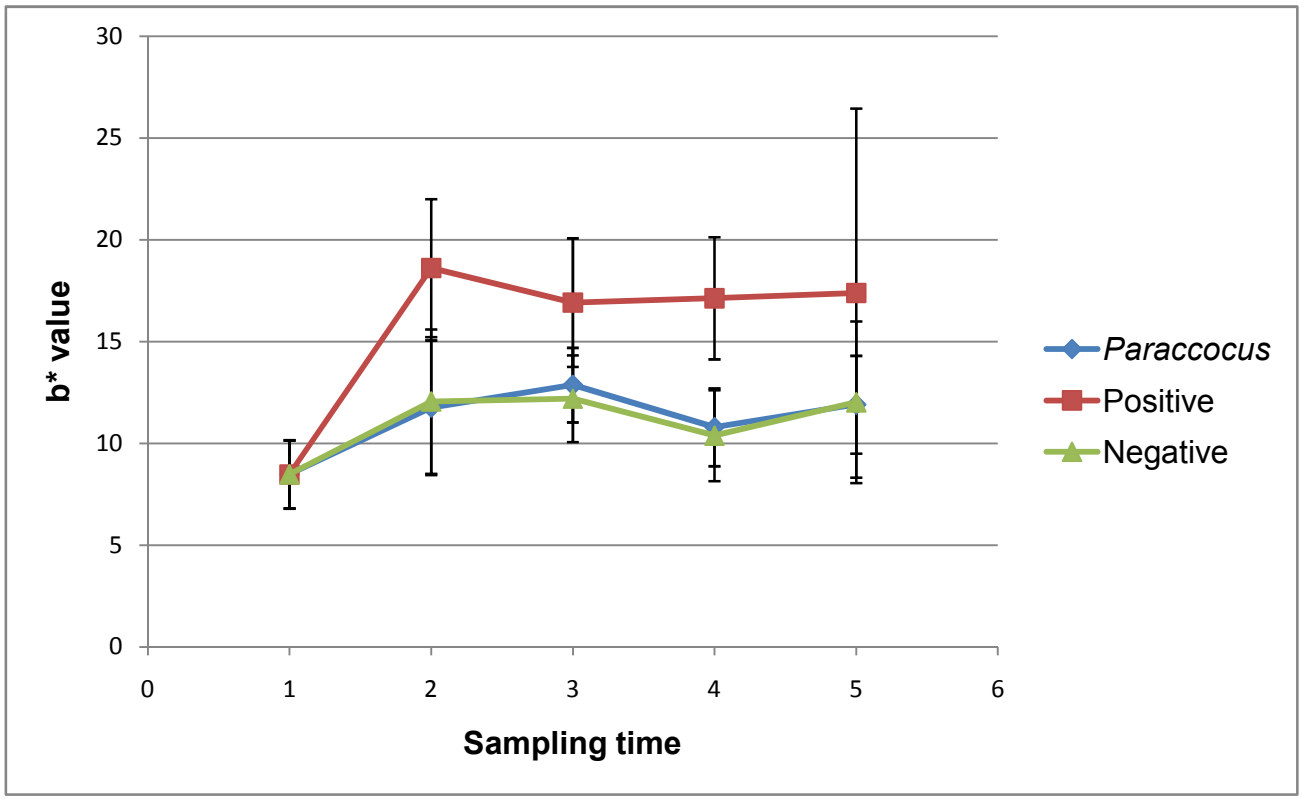

Figure 9 – b* Value of *O. mykiss* fillets. This indicates the yellow colour of the fillets. Three fish from each tank were sampled every 2 weeks.

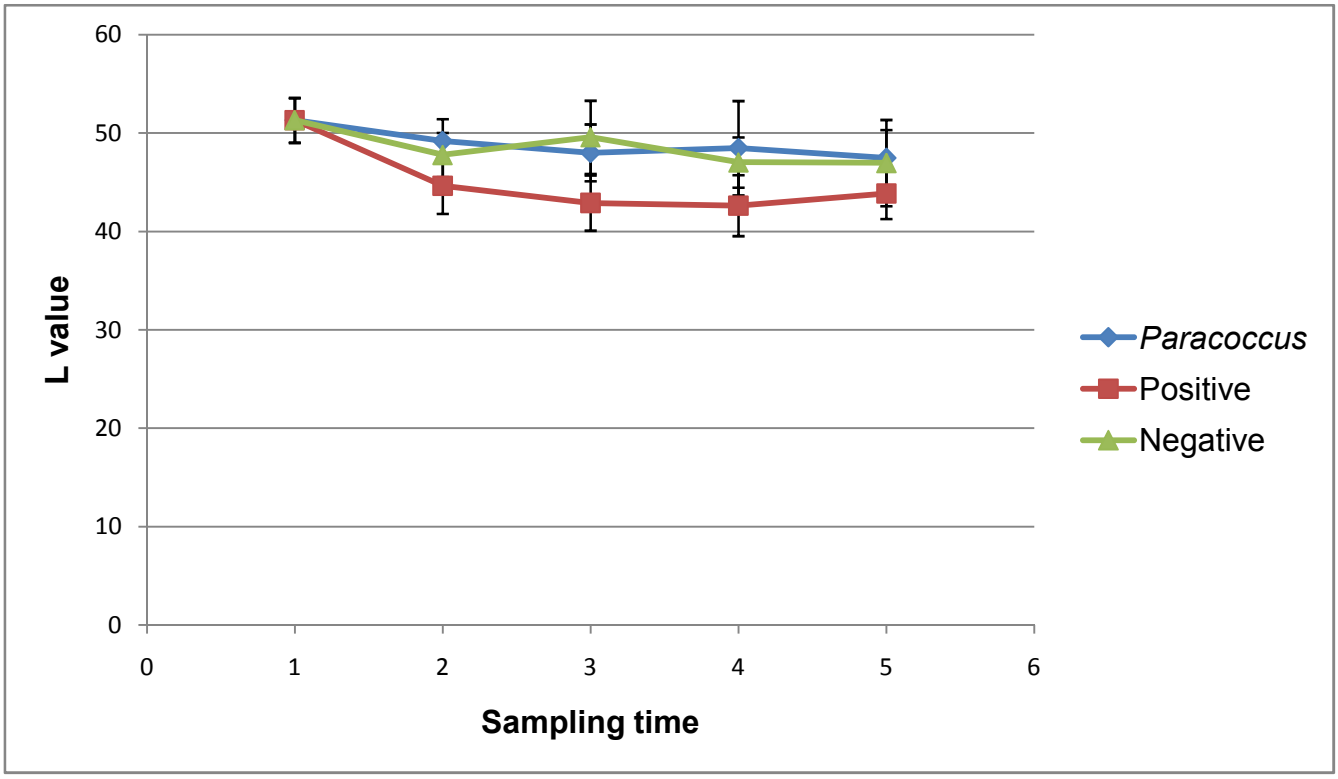

Figure 10 – L value of *O. mykiss* fillets. This indicates the lightness of the fillets. Three fish from each tank were sampled every 2 weeks.

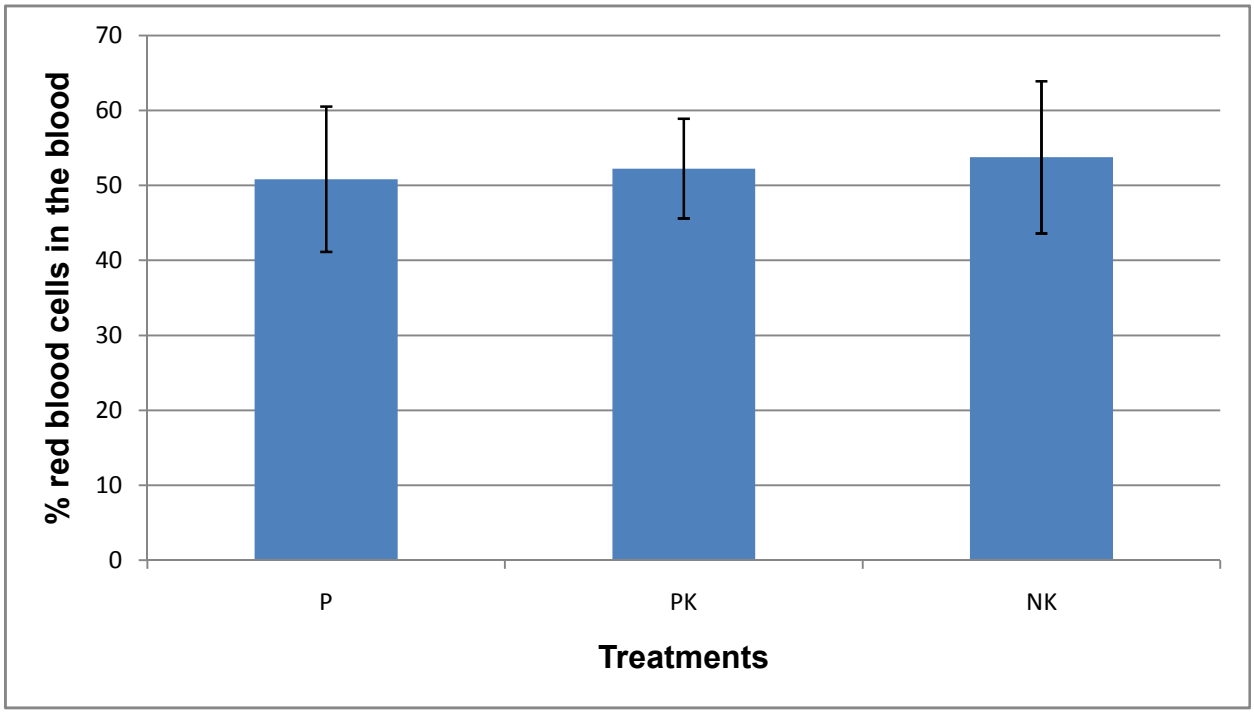

Figure 11 – Hematocrit (% of red blood cells in the blood) of blood samples collected on the last sampling (16 August 2012). P= *Paracoccus marcusii*; PK= Positive control; NK= Negative control.

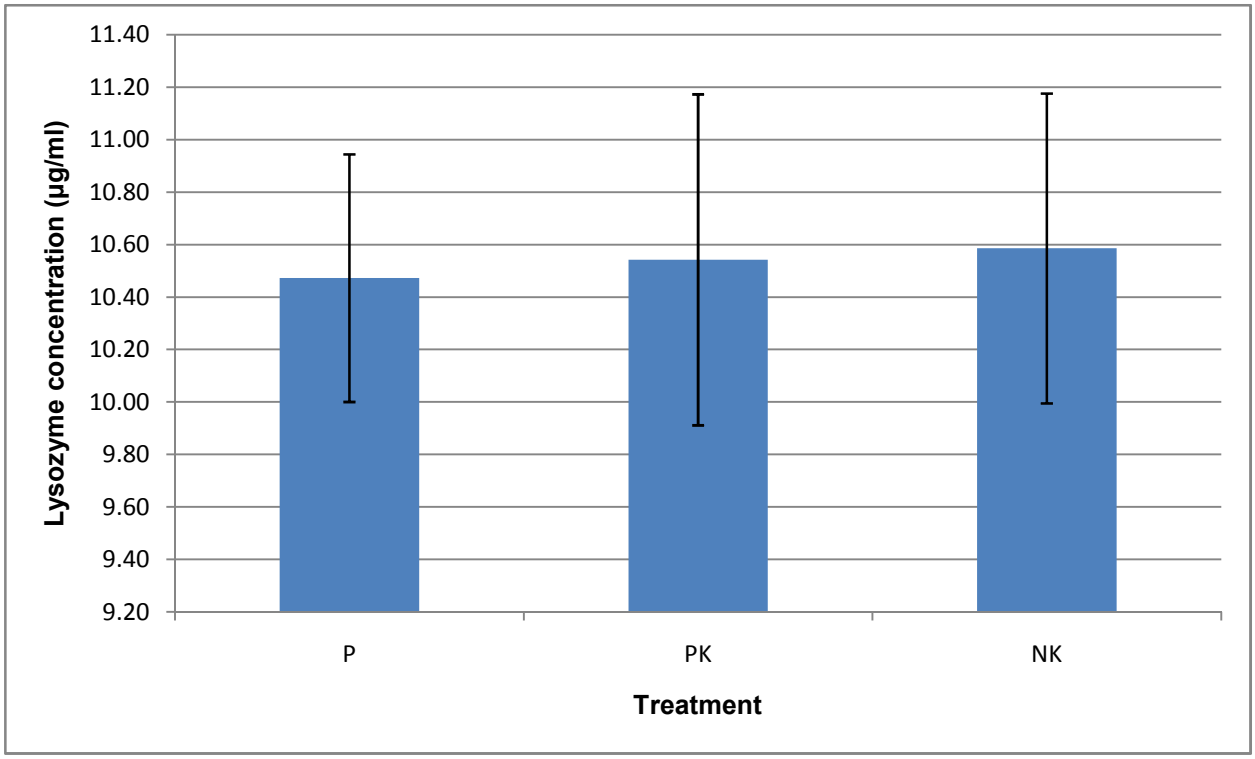

Figure 12 – Lysozyme activity in the blood. Blood samples were collected on the last sampling (16 August 2012). P= *Paracoccus marcusii*; PK= Positive control; NK= Negative control. Lysozymy activity was determined using a turbidity assay.

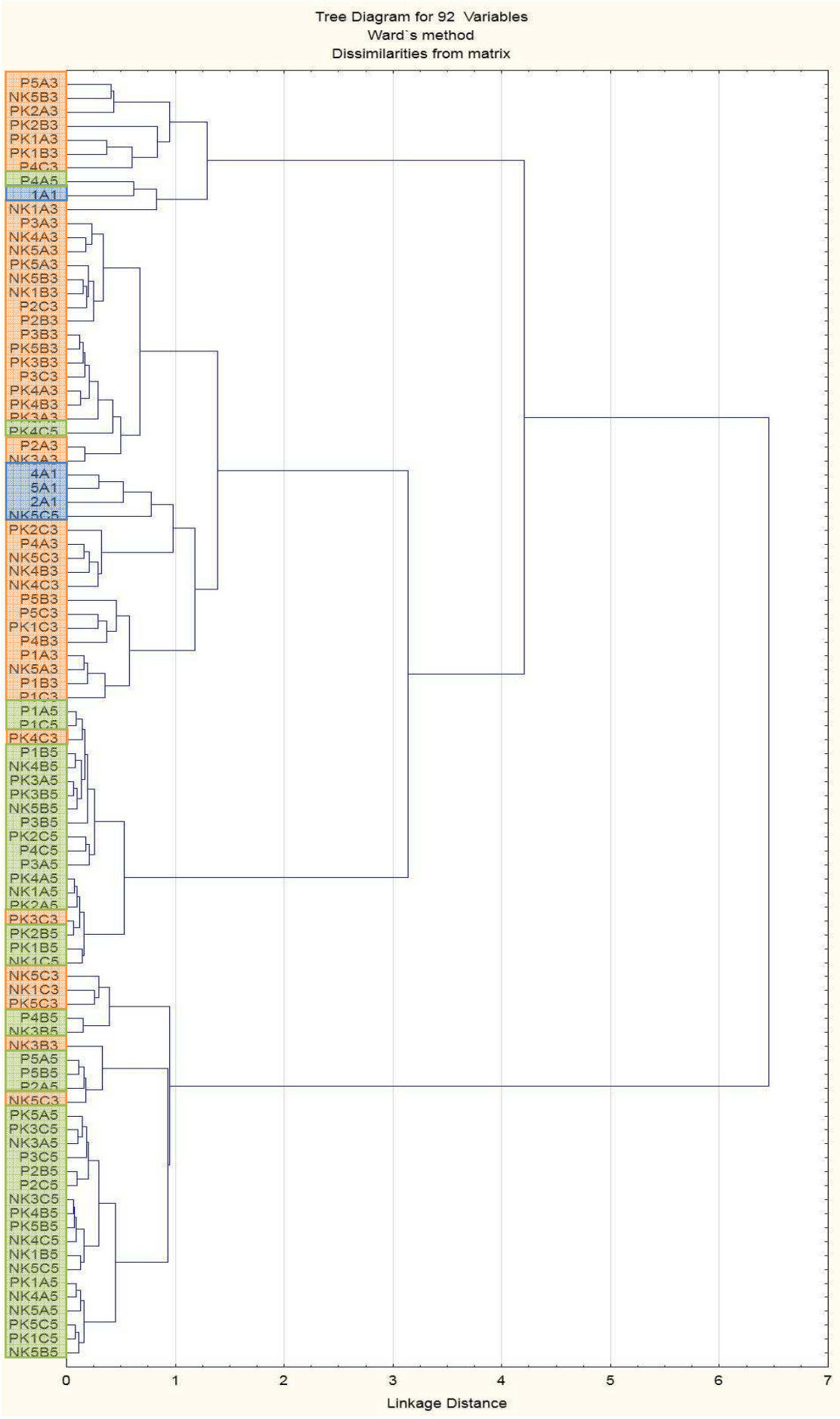

Figure 13 – Dendogram of bacterial community structure of fish feeding on the different treatments sampled at sampling time 1 (blue), 3 (orange) and 5 (green). The Whittaker index of similarity was calculated to test similarities between samples from different sampling times.

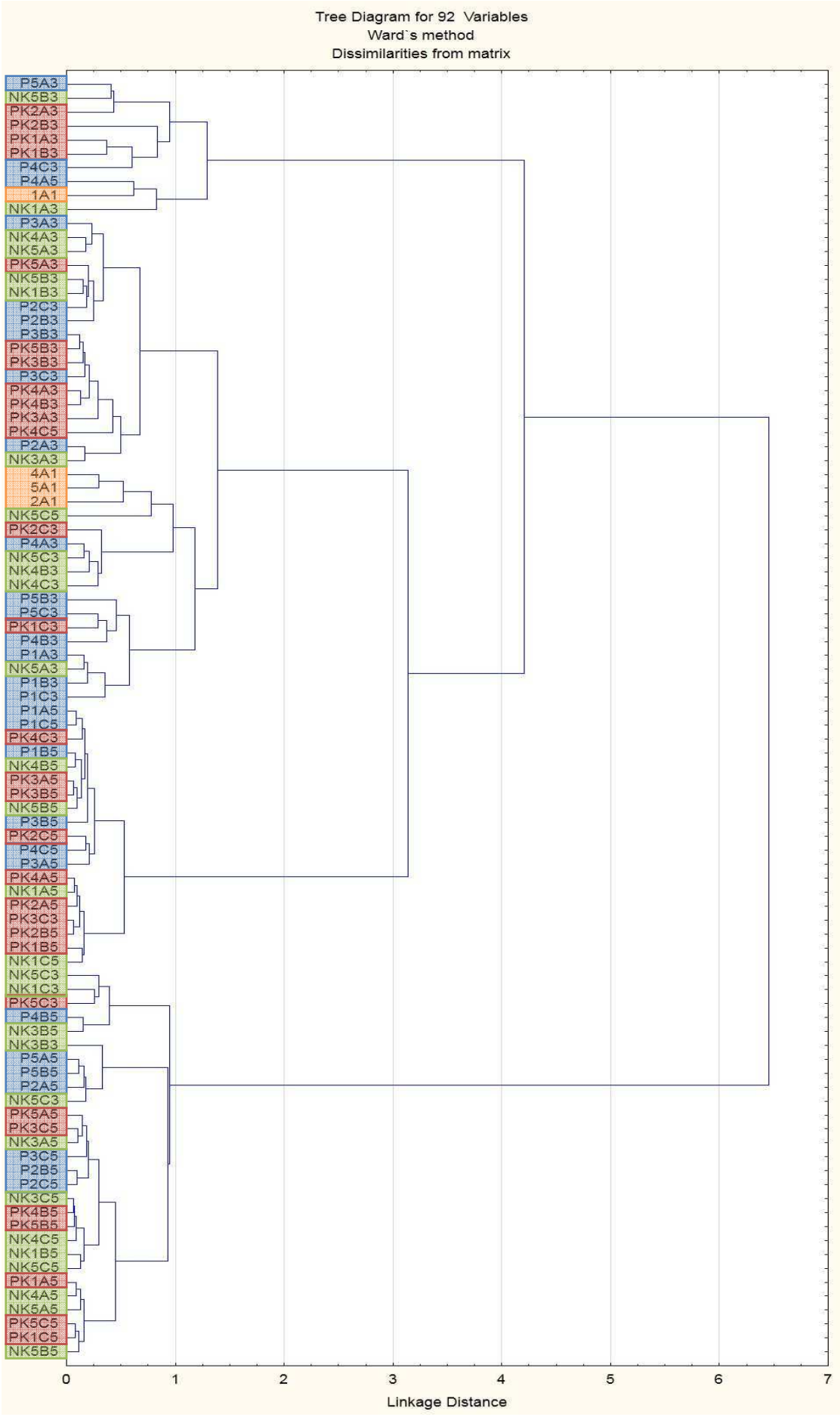

Figure 14 – Dendogram of bacterial community structure of fish feeding on the different treatments over the duration of the trial. Fish sampled at the start of the trial before starting the different treatments are indicated as orange. Treatment 1 is indicated as green, treatment 2 is red and treatment 3 is blue. The Whittaker index of similarity was calculated to test similarities between samples from different treatments.

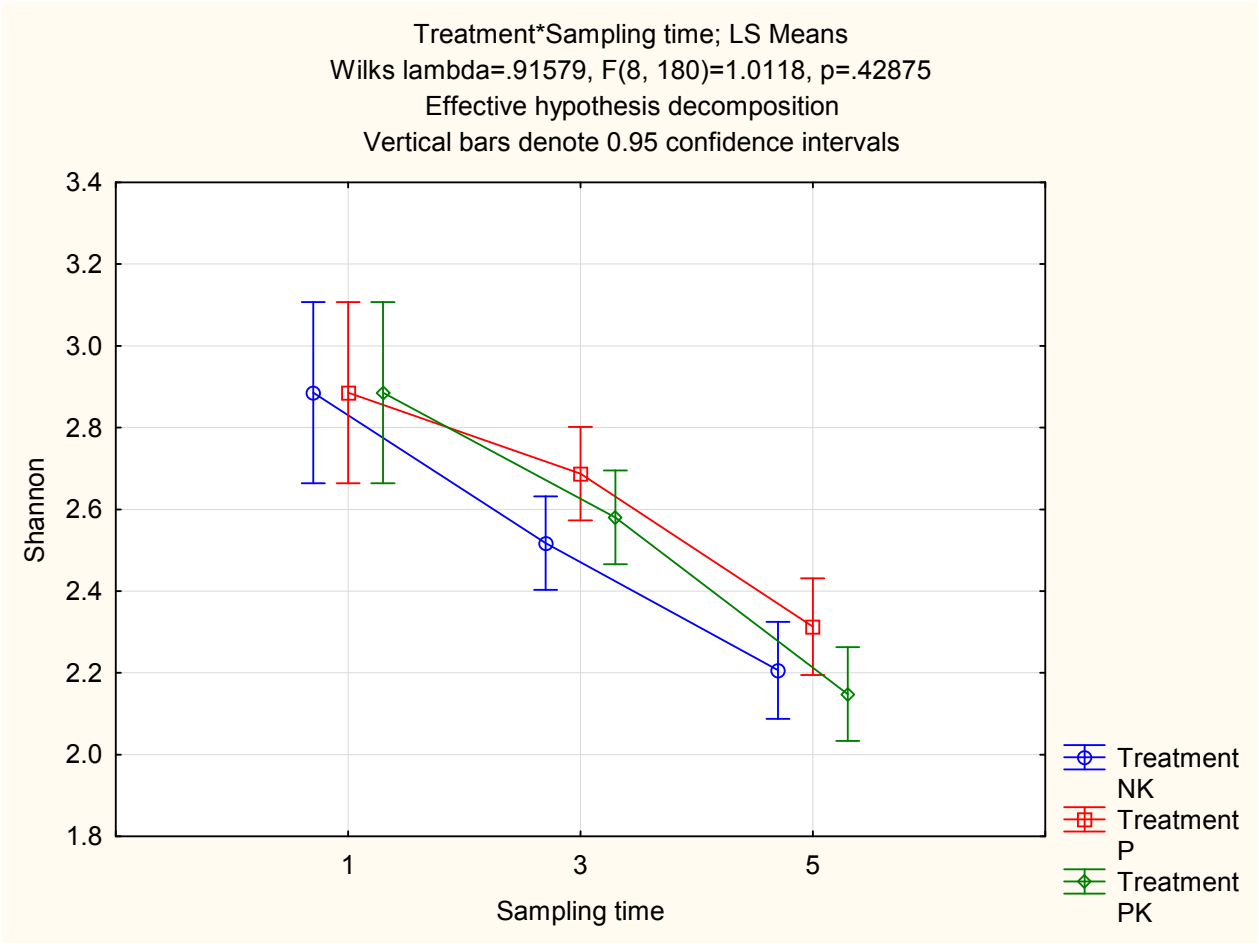

Figure 15 – ANOVA results for bacterial diversity of the three different treatments over a period time. The categorical variables are the sampling time with *H'* as the dependent variable. The Shannon diversity index was used to determing the richness and relative abundance of the operational taxanomic units (OTUs).

Tables

Table 1 : Experimental diets		
Treatment	**Diet**	**Contents**
1	Negative control (NK)	Basal diet
2	Positive control (PK)	Pellets coated with commercial pigments (Carophyll® pink - DSM)
3	Potential probiotic (P)	Pellets coated with *Paracoccus marcusii*, 1g dried cells/kg feed mixed with fish oil

Table 2 - Sampling dates 2012	
Sampling time	**Date**
1	14-Jun
2	28-Jun
3	12-Jul
4	26-Jul
5	16-Aug

Concluding remarks

Diseases in aquaculture are a big problem and the use of antibiotics to control these diseases is questioned. Research on probiotics is becoming very important in order to find a successful alternative way to control diseases in this industry. Therefore, this study aimed to isolate potential probiotic bacteria for the application in aquaculture. *Paracoccus marcusii* was isolated from the GIT of the South African abalone (*Haliotis midae*). This bacterium produces the carotenoid pigment astaxanthin, and showed potential to be used as a probiotic as well as a pigment source for fish species such as trout, salmon and shrimps (Chapter 2).

The probiotic effect of *P. marcusii* on Mozambique Tilapia (*O. mossambicus*) was evaluated in Chapter 3. Results indicated that this bacterium had a probiotic effect on tilapia, improving the FCR as well as the some immune system parameters. A pilot trial on rainbow trout (*O. mykiss*) was also done in order to evaluate the possibility of *P. marcusii* to act as a pigment source for trout. Results obtained in the pilot trial indicated that *P. marcusii* (isolate 6.15) was able to act as a pigment source. Therefore, an experimental trial was conducted in order to evaluate both, the pigmentation and probiotic effect of *P. marcusii* (Chapter 4). However, unexpected results were obtained, with no probiotic or pigmentation effects were reported. Because *P. marcusii* had a probiotic effect on tilapia and showed pigmentation effects in the pilot trial, it is strongly believe that this bacterium is able to be used as a pigmentation source and also a probiotic for rainbow trout. These unexpected results most probably were due to the decomposition of astaxanthin. This pigment is a very unstable molecule and could have been destroyed during the storage of freeze-dried cells or feed.

For future studies it is important to determine the concentration of astaxanthin produced by each *P. marcusii* cell. It is also important to determine the optimum bacterial concentration to be added to the feed. It is critical to determine the stability of the astaxanthin in the cells over time. It is also suggested is that the probiotic and pigmentation effect of *P. marcusii* on rainbow trout should be reassesed. Finally a cost beneficial analysis should be done to determine if this probiotic will be commercially viable.