The effects of probiotics on the physiological and biochemical development of the digestive tract of commercially raised dusky kob (*Argyrosomus japonicus*) larvae

by

Amy Hunter

Thesis presented in partial fulfilment of the requirements for the degree Master of Science (Agriculture) in Aquaculture at the University of Stellenbosch

Supervisor: Dr Elsje Pieterse
Co-supervisor: Prof Daniel Brink
Faculty of Agricultural Science
Department of Animal Sciences

March 2015

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 20 December 2014

Abstract

Aquaculture is one of the fastest growing food producing sectors in the world. Over the past few years, aquaculture research has focused on improving rearing protocols and standards for the culture of aquatic organisms. Probiotics are gaining increasing interest as an alternative to antibiotics to improve animal health and welfare. The effects of probiotics on the physiological and biochemical development of the digestive tract of commercially raised *Argyrosomus japonicus* (dusky kob) larvae were investigated. Two probiotic treatments were compared to a control where the standard rearing protocol was applied. The growth of the larvae and histological development of the digestive tract was studied. The specific enzyme activity of key digestive enzymes was determined. Amylase, trypsin, pepsin, alkaline phosphatase, aminopeptidase N and leucine-alanine peptidase were assayed. The mean growth of the control group of larvae differs from the CSIR and BactoSafe treatment groups ($P = <0.001$). There were no differences in the histological development between the control and two treatments. The histology did indicate a slight delay in development of the digestive tract when compared to previous studies on dusky kob. No significant differences were observed between the control and treatment groups for any of the enzyme assays. The effect of probiotics on the development of the digestive system of dusky kob larvae could not be definitively described as it was not determined to what extent the probiotics had established in the gut. What the study did conclude was that the enzyme assays need to be refined in order to determine the optimal reaction conditions required for the determination of specific enzyme activity in commercially raised dusky kob.

Uittreksel

Akwakultuur is een van die vinnigste groeiende voedsel produserende sektore in die wêreld. Oor die laaste aantal jare het akwakultuur navorsing gefokus op die verbetering van grootmaak protokolle en standaarde vir die cultuur van akwatise organismes So het die belangstelling in pro-biotika, as alternatief tot antibiotika ten einde die gesondheid en welsyn van diere te verhoog, vermeerder. Die effekte van pro-biotika op die fisiologies en biochemiese ontwikkeling van die spysverteringskanaal van kommersieel geproduseer *Argyrosomus japonicus* larwes was ondersoek. Twee probiotiese behandelings is vergelyk met ’n kontrole (standaard grootmaak protokol). Die groei van die larwes en die histologiese ontwikkeling van die spysverteringskanaal is ondersoek. Die spesifieke ensiem aktiwiteite van die sleutel verteringsensieme was ook bepaal. Amilase, tripsien, pepsien, alkaliese fosfatase, aminopeptidase N en leusien-alanien peptidase was ondersoek. Die gemiddelde groei van die kontrole groep was effe hoër as die van die ander twee behandelings. Daar was geen verskil in histologie van die groepe nie maar die tempo van ontwikkeling vir alle behandelinge blyk stadiger te wees as die wat in vorige studies vir *A. japonicus* rapporteer is. Geen betekenisvolle verskille is opgemerk tussen die kontrole en die behandelingsgroepe vir enige van die ensiem analises nie. Die invloed van die probiotika op die ontwikkeling van die spysverteringskanaal van die larwes kon nie onomwonde beskryf word nie aangesien die vestiging van die pro-biotika in die spysverteringkanaal nie omskryf is nie. Wat wel gevind was, is dat die analitiese tegnieke rakende die ensieme verfyn moet word ten einde die optimale reaksie omgewings te bepaal wat dit sal moontlik maak om spesifieke ensiemaktiwiteite in die kommersieel geproduseerde *A. japonicus* te bepaal.

Acknowledgements

I would like to express my sincerest thanks to Dr Elsje Pieterse and Prof Danie Brink, my supervisors, for their patience, guidance and support and for always making themselves available to me when I required assistance.

I would like to thank Oceanwise for providing the facilities and the animals to conduct my experiment. I would like to thank the hatchery staff for all their back breaking work and their limitless ability to step in and help.

I would like to thank the CSIR for providing me with the opportunity to conduct my laboratory work in their facilities at the Bioscience department in Pretoria.

I would like to thank THRIP for their funding and giving me the opportunity to gain valuable hands on industry experience during my study period.

I would like to express my gratitude to Gail Jordaan for all her help with the statistical analysis and interpretation of my data.

My sincerest thanks goes to Prof Pieter Swart and Stephan Hayward for allowing me to use their facilities in the Biochemistry Department at Stellenbosch University

My family deserves my greatest thanks for all the endless support they have given me. My parents have provided financial and emotional support every step of the way during my studies.

I would also like to thank Maryke Musson for her all her advice, support and kind words, Dr Niall Vine for always being prepared to take a phone call, Dr Ernst Thompson for his extensive biochemical knowledge, Varsha Chibba for her unending technical support and every other person who offered my advice and support along this journey.

Finally, I would like to thank Justin Lindsay. You have been my rock though out this process. You have been there to share the small triumphs and to pick up the pieces when things fell apart. I will be forever grateful for your unending support.

Table of Contents

List of Appreviations

A - Anus
AN – Aminopeptidase N
AP – Alkaline phosphastse
BAEE - Nα-Benzoyl-L-Arginine Ethyl Ether
BC – Buccopharyngeal cavity
CSIR – Council for Scientific and Industrial Research
DO – Dissolved oxygen
dph – Days post hatch
E - Eye
Fb – Food bolus
Gc – Goblet cells
H - Heart
HCl – Hydrochloric acid
HG – Hind gut
IL – Intestinal lumen
L – Liver
Leu-Ala – Leucine alanine peptidase
LHRH – Luteinising hormone release hormone
Mc – Mucous cells
MG – Mid gut
NaOH – Sodium hydroxide
NL – Notochord length
P - Pancreas
p-NPP – p-nitophenol phosphate
Ps – Pyloric sphincter
S – Stomach
SB – Swim bladder
SD – Standard deviation
Sv – Supranuclear vacuoles
TAN – Total ammonium nitrogen
TL – Total length

Introduction

A global trend in the decline of ocean fisheries stocks has brought forward the need for alternative sources of ocean fish for human consumption. The aquaculture industry as an alternative source of fish stocks shows promise in fulfilling this need. As the human population rapidly increases, the reliance on cultured fish production as a vital source of dietary protein will also increase (Naylor et al., 2000). The FAO State of the World Fisheries and Aquaculture (2012) states that aquaculture is the most rapidly expanding animal food producing sector globally, making fish one of the most frequently traded products on the global market. It is predicted that the trade in fish will exceed that of products such as pork, beef and poultry. The statistics published by the FAO State of the World Fisheries and Aquaculture in Rome (2012) show that fish for human consumption produced through aquaculture was reported to be about 60 million tonnes in 2010. This represents a 12 fold increase within the last three decades. These values exclude the production of aquatic plants and non-food products. Asia remains the world leader in aquaculture, securing 89% of the global market by volume in 2010. This is a result of China displaying the greatest increase in fish consumption per capita. The contribution to the market by Sub-Saharan Africa is minimal with a mere 0.5% contribution (Edwards, 2000). However, despite the aquaculture industry being relatively new in South Africa, it continues to develop with an increase in demand for food fish as well a decline in marine fish stocks in the waters surrounding the South African coastline. Prominent aquaculture species in South Africa include abalone, oysters, mussels, trout and dusky kob (Hinrichsen, 2008).

Dusky kob (*Argyrosomus japonicus*), also referred to as mulloway, or kabeljou, is a large sciaenid that is found along the coastlines of Australia, South Africa, Hong Kong, Pakistan and Japan (Fielder and Bardsley, 2007). This species is highly sought after as a table food species, as well as being a popular species targeted by recreational anglers (Ballagh et al., 2008). Many studies have suggested that dusky kob is a promising species to be considered for commercial production in the aquaculture industry. Dusky kob has been shown to have relatively rapid growth rates (Griffiths and Hecht, 1995) and a high tolerance to varying water quality conditions (Whitfield, 1999). Dusky kob is currently being commercially cultured in South Africa and Australia. The importance of the culture of dusky kob in South Africa is expected to greatly increase in the coming years (Hinrichsen, 2008). One of the drawbacks of commercially producing dusky kob is the high cost of production. This is as a result of production protocols still requiring greater research in order to improve efficiency (Ballagh et al., 2008). Studies that have been conducted on improving environmental conditions for production include salinity (Fielder and Bardsley, 2007), photoperiod and feed intervals (Ballagh et al., 2008), temperature (Collett et al., 2008) and

the effects of stocking density on food conversion ratio and survival (Collett et al., 2011). Meeting the correct dietary requirements of any species being commercially cultured is vital in optimising production efficiency and reducing production costs. One of the greatest expenses with regards to feed is the cost of providing live feed to developing larvae (Wold et al., 2007). For large yellow croaker (*Pseudosciaena crocea*), the live feed required for larval production constituted 70% of the total production costs (Ma et al., 2005).

Many studies have been conducted to determine the optimal time to wean larvae onto formulated micro diets. Digestive tract and enzyme development of larvae have been the focus of these studies with species such as large yellow croaker (*Pseudosciaena crocea)* (Ma et al., 2005), sharpsnout sea bream (*Diplodu puntazzo*) (Suzer et al., 2007; Savona et al., 2011), Atlantic cod (*Gadus morhua)* (O'Brien-MacDonald et al., 2006), white seabass (*Atractoscion nobilis*) (Galaviz et al., 2011) and meagre (*Argyrosomus regius)* (Suzer et al., 2012) being investigated. By determining the presence and the concentrations of specific digestive enzymes, the molecular development of the digestive tract of larvae can be documented (Suzer et al., 2007). The results from these studies allow one to better understand the nutrient assimilation capabilities of larvae, particularly when done in a holistic manner by combining the histological development with the biochemical development. The knowledge gained from these studies will contribute to the implementation of optimal feeding regimes. This in turn will improve production within the hatchery (Suzer et al., 2007a).

More recently, the administration of probiotics to larval rearing systems to improve water quality and the efficiency of production has received much attention. Probiotics have been shown to possess many beneficial effects on both larval growth and survival within laboratory based studies (Gomez-Gil et al., 2000). With the use of antibiotics for disease control being prohibited in many countries and increased pathogen resistance to antibiotics, the use of probiotics and their effects have started gaining increased attention in aquaculture (Suzer et al., 2008). Using the knowledge available on the development of the digestive tract and the digestive enzymes of fish larvae, studies of the effects of various enrichments, feed formulations and environmental conditions related to larval nutrition can be done (Kolkovski, 2001).

With regards to dusky kob, literature is available on the rearing conditions of juvenile and adult fish while there has been little work done on the rearing requirements for larvae. The optimal weaning period on a molecular level has not been studied. Describing the development of the digestive enzymes of dusky kob larvae, and comparing the results to a histological study, will shed light on the gut maturation process. By identifying key maturational events within the ontogeny of the digestive system, the feeding regime for

dusky kob larvae can be improved. The histology of the developing larvae will be studied and selected pancreatic and intestinal enzymes will be assayed. By evaluating the pancreatic enzymes, maturation of the pancreas can be determined (Zambonino and Cahu, 2007). These enzymes are normally expressed when the larvae begin exogenous feeding. Intestinal enzymes are comprised of brush border and cytosolic enzymes and they indicate the maturation of enterocytes in the intestinal epithelium (Zouiten et al., 2008). In this study, the pancreatic enzymes that will be evaluated are trypsin and amylase; whilst the intestinal enzymes analysed will be alkaline phosphatase, aminopeptidase N and leucine-alanine peptidase; pepsin activity will also be analysed. The effect of the addition of two probiotics during larval development will also be studied. One of the probiotics has been commercially produced in Europe and the other probiotic will be supplied by The Council for Scientific and Industrial Research (CSIR) in South Africa as an experimental probiotic. The process of gut maturation will be analysed and compared to the results where standard larval rearing protocols are implemented. Larval growth will also give an indication of the effects of the addition of a probiotic. The results of the study will hopefully enable hatcheries to improve the feeding regime of dusky kob larvae, and therefore reduce the cost of production.

Literature Review

The Sciaenidae family and their role in aquaculture

The Sciaenidae family is one of the largest and most diverse of the perciform families. Approximately 270 species have been recorded in a variety of habitats ranging from temperate to tropical waters. They display a global distribution in both coastal waters as well as estuaries. Large numbers of species in this family are found in and around the mouths of large continental rivers. The diversity in their external morphology is testament to their ability to adapt to a variety of habitats. Sciaenidae species have adapted to both benthic and pelagic environments. Their most characteristic feature is the sounds that this family of fish produce, giving them their common names of drums and croakers (Sasaki, 1989). Sciaenids have been shown to be particularly venerable to over fishing all over the world. Possible reasons for this include their large size and their schooling behaviour in estuaries during spawning periods (Gil et al., 2013). Many species of Sciaenid's are popular angling fish and are highly prized by recreational anglers and considered a good table fish (Ballagh et al., 2008). In many areas, most of the natural stocks of sciaenid's have been severely depleted. The development of aquaculture around the sciaenidae has been formed around two primary objectives: the commercial production of selected species as a food source; and the production of fish to be used for restocking programs (Hervas et al., 2010)

Dusky kob (*Argyrosomus japonicus*) is a large sciaenid species found along the southern and eastern coast of South Africa. It is popular with anglers as a favourable food species with a mild flavoured white flesh. Its popularity as an eating fish was one of the motivating factors to begin to culture the species on a commercial scale. This, together with its fairly good growth rate, highlighted the dusky kob's potential as a promising aquaculture species (Griffiths, 1996). The majority of studies conducted on the culture of *A. japonicus* have been focused on the rearing conditions for juvenile and adult fish. The larval rearing conditions for dusky kob have not been investigated in depth. The rearing conditions for dusky kob larvae in South Africa have been primarily based on existing protocols that are available for mulloway, its Australian counterpart (Fielder et al., 2010).

Ontogeny of the larval digestive tract

In order for larvae to successfully develop and increase their chances of survival, correct development of the digestive tract is essential. The capture, ingestion, digestion and absorption of food are dependent on a fully formed digestive tract (Chen et al., 2006). During the initial month of life, significant morphological and cellular changes occur within marine fish larvae. The digestive ontogeny of fish larvae includes both aspects in the process of development. It is suggested that the timing of these changes are genetically controlled

(Zambonino and; Cahu, 2001). It is vital to understand the nutritional physiology of larval fish in order to successfully time feeding regimes with physiological events to optimise feeding and nutritional assimilation. This is why it is important to study the development and differentiation of the digestive tract associated with the digestion of food and the assimilation of nutrients (Mai et al., 2005). The mechanisms of the development of the digestive tract are similar amongst all teleost fish (Zambonino et al., 2008). Three major phases exist with regards to the ontogeny of the larval digestive tract (Buddington; Diamond, 1987; Boulhic; Gabaudan, 1992; Bisbal; Bengtson, 1995). The initial phase begins when hatching occurs, and ends when endogenous feeding is complete. During this phase larvae are dependent on the yolk sac and oil globule as their sole source of energy. The transition from endogenous feeding to exogenous feeding takes place towards the end of this initial phase. Once this transition takes place, only exogenous feeding occurs where the second phase of development begins From this point in the developmental process, larvae are dependent on intracellular digestion and pinocytosis (Watanabe, 1982). At this phase, larvae normally feed on live feed such as rotifers because they are easily digested by a digestive system that lacks sufficient digestive abilities (Buddington and Diamond, 1987; Boulhic and Gabaudan, 1992). The second phase only lasts until the gastric glands in the stomach begin to develop. The third phase involves the development of the gastric glands and the pyloric caeca. This phase is indicative of the functional maturation of the digestive tract of the larvae (Govoni et al., 1986; Bisbal and Bengtson, 1995). During the third phase of development, metamorphosis occurs. The digestive system is now anatomically and physiologically equipped to accept an artificial micro pellet (Bisbal and Bengtson, 1995; Gordon and Hecht, 2002). The timing of these developmental events is different amongst various species. This timing can be affected by a variety of factors such as the general life history of a specific species, water temperature and food availability. By combining histological studies and molecular studies, the development of the gastrointestinal tract of marine fish larvae provide a fascinating model for the study of the interactions between exogenous nutrition and the process of development (Zambonino et al., 2008). An intimate understanding of the ontogenic process of the development of the digestive system of fish larvae will result in improved feeding regimes and reduce the risks and costs of weaning larvae off live feed and onto formulated diets (Galaviz et al., 2011).

Histological development of the larval digestive tract

Histological studies of the development of major organs of fish larvae have been described at length for a variety of species (Zambonino et al., 2008). Some of these species include Atlantic cod (*Gadus morhua)* (Perez-Casanova et al., 2006), European sea bass (*Dicentrarchus labrax)* (Beccaria et al., 1991; Hernández et al., 2001), the common dentex

(*Dentex dentex)* (Santamarıa et al., 2004), yellow tail kingfish (*Seriola lalandi)* (Chen et al., 2006), meagre (*Argyrosomus regius)* (Papadakis et al., 2013), yellow croaker (*Pseudosciaena crocea)* (Ma et al., 2005; Mai et al., 2005), Senegal sole (*Solea senegalensis)* (Ribeiro et al., 1999b), turbot (*Scopththalmus maximus)* (Segner et al., 1994) and the California halibut (*Paralichthys californicus)* (Gisbert et al., 2004). More recently, an initial histological study of the development of dusky kob (*A. japonicus)* has been conducted (Musson; Kaiser, 2014). Histological studies can be combined with molecular studies to provide a detailed understanding of the development of the digestive system in a holistic manner.

The major organs involved in the digestive process of larval fish include the pancreas, intestine and the stomach. The pancreas has both an endocrine and exocrine function. The endocrine section is responsible for the secretion of insulin and glucagon. These hormones are secreted into the circulatory system where they are responsible for the regulation of blood glucose levels (Schmidt-Nielsen, 1997). The exocrine function of the pancreas is responsible for the secretion of digestive enzymes involved in the intestinal digestion of large nutrient molecules (Hoehne-Reitan and Kjørsvik, 2004). The development of the exocrine pancreas can be divided into three stages. The first stage describes the appearance of a primordium at the time of hatching represented as a dorsal bud present on the digestive tract. The second stage is the differentiation of the cells of the exocrine pancreas and the development of blood vessels and an excretory duct just before mouth opening. The third stage involves the enlargement of the pancreas during larval and juvenile development. During this stage, the frequency of zymogen granules and an increase in digestive enzyme secretion occurs. Acidophilic zymogen granules are considered to be precursors for pancreatic enzymes. These can normally be detected a day before exogenous feeding begins. The numbers of these granules tend to increase with the age of the larvae. They perform a vital role in pancreatic enzyme secretions during the agastric period of development. These are genetically determined as opposed to being induced by dietary components (Zambonino and Cahu, 2001). At hatching and mouth opening, the larval exocrine pancreas is fully differentiated and functioning (Zambonino et al., 2008). In summer flounder (Bisbal and Bengtson, 1995), haddock (Perez-Casanova et al., 2006) and Atlantic cod (Park et al., 2006), acidophilic zymogen granules are present in the pancreas and the exocrine cells are organised and arranged in acini soon after hatching. On the other hand, for species such as Senegal sole (Ribeiro et al., 1999b), California halibut (Gisbert et al., 2004), common dentex (Santamarıa et al., 2004) and gilthead sea bream (Sarasquete et al., 1995), the acinar structure will only develop at the time of mouth opening.

All species of marine fish start off with a simple, undifferentiated digestive tract with differentiation only occurring at around three days post hatch (dph) (Galaviz et al., 2011). The epithelium of the early intestine consists of a simple columnar epithelium with microvilli being present (Zambonino et al., 2008). The intestinal epithelium has a fairly regular surface with the apical differentiation of enterocytes being initiated. This is the beginning of the establishment of the brush border membrane. When this epithelial layer begins to thicken and fold, digestive enzyme activity that is specific to this area begins to increase (Zambonino and Cahu, 2001). The differentiation of the cytosol of the enterocytes is an indicator of intestinal maturation. This coincides with exogenous feeding and indicates that the intestine has matured to allow for the absorption of nutrients (Segner et al., 1994). As the digestive tract develops, the intestine loops within the visceral cavity due to its increasing size. This allows for the intestine to fit within the visceral cavity. Three clearly separate sections begin to form. They are formed according to their histological development. The prevulvular section forms, which includes the pancreatic segment and is characterised by the presence of columnar epithelium with obvious microvilli and many enterocytes. This section is the primary site for lipid absorption. The postvulvular section shows little histological difference to the prevulvular section except for the number of mucosal fold being higher and their size and depth are greater. The last section is a short rectal section which displays different cell types according to different species. Dover sole have columnar epithelium with a few enterocytes present, while European sea bass have simple cuboidal epithelium (Zambonino et al., 2008).

In many species of fish, a rudimentary stomach can be observed shortly after hatching. The stomach appears to be the last organ to develop in the ontogeny of the digestive tract. The stomach begins as a small cluster of cuboidal and columnar epithelium. These eventually form gastric pits as the gastric mucosa folds to form the stomach in the later stages of development. The gastric glands develop from the gastric pits and the development of the gastric glands indicates the transition from a larval mode of digestion to an adult one (Zambonino et al., 2008). Studies have shown that the number and the size of the gastric glands increases as the larvae age (Galaviz et al., 2011). Studies on yellow croaker (*Pseudosciaena crocea*) showed that the stomach was the last digestive organ to differentiate with the first gastric glands being observed at 21 dph (Mai et al., 2005). For white seabass (*Atractoscion nobilis*), the gastric glands were first observed at 16 dph and the digestive tract was shown to be fully developed by 32 dph (Galaviz et al., 2011).In Senegalese sole (*Solea senegalensis*), the differentiation of the gastric glands was noted between 24 dph and 27 dph. This was marked by the appearance of many transverse canals within the epithelium in the stomach and the formation of cuboidal epithelium of the stomach

mucosa (Ribeiro et al., 1999). In yellowtail (*Seriola lalandi*), gastric glands began to appear at 15 dph and the stomach was fully differentiated by 36 dph (Chen et al., 2006).

Digestive enzyme development of fish larvae

It has been suggested that the larvae of marine fish do not possess sufficient digestive enzymes to be able to cope with feeding on compound diets before developing into juveniles (Zambonino et al., 2008). Previously, it was thought that the live feed presented to fish larvae contributed to larval digestive enzyme activity by "giving" the larvae their own digestive enzymes when they are consumed. This is thought to occur by either autolysis or zymogens that trigger larval endogenous digestive enzymes (Kolkovski, 2001). In the last 20 years, many studies have been conducted on the development of the larval digestive enzymes and these studies have revealed that pancreatic and digestive enzymes have been present at first feeding (Wold et al., 2007). This is due to the fact that during the first few days of life, the digestive tract is largely undifferentiated and lacking a stomach. This results in the digestion of food occurring in the larval intestine (Kolkovski, 2001). The activity of digestive enzymes differs between species and feeding regimes. Larvae fed live feed as opposed to larvae fed compound feeds show differences in digestive enzyme levels. A possible explanation for the difference is that there is a degree of enzymatic adaptation to a particular nutritional substrate present in the compound feed. Studies have shown that insufficient weaning diet may delay or halt the maturation process of digestive enzymes in fish larvae (Zambonino et al., 1996). Therefore, the process of maturation of digestive enzymes of fish larvae may be manipulated by diet composition. The effects of diet manipulation my either enhance, stop or delay the maturation process (Zouiten et al., 2008). By assessing the presence and activity of particular digestive enzymes, an indication of larval development can be given and larval survival can be predicted. The appearance of digestive enzyme activity can be utilized as an indicator of food acceptance by larvae as well as the potential digestive ability relative to the type of feed presented to the larvae (Suzer et al., 2007).

Pancreatic enzymes

The secretion of pancreatic enzymes is indicative of the first steps in the process of maturation of the digestive tract in fish larvae (Zouiten et al., 2008). The developments of the enzymes are a result of genetic programming which can be mildly modified by the composition of the diet (Wold et al., 2007). For most species, pancreatic enzyme activity occurs before mouth opening and continues to increase post-mouth opening. This increase in pancreatic enzyme activity has been shown to coincide when the zymogen granules are

first secreted. Once this developmental event has occurred, it can be said that the pancreatic secretory function has been achieved (Zambonino et al., 2008).

Trypsin secretion is activated in response to food ingestion and is present in the pancreas as inactive trypsinogen (Suzer et al., 2007b). Trypsin is also the primary enzyme responsible for protein digestion (Zambonino et al., 2008). Prior to mouth opening, Trypsin is regulated by a hormonal regulator thought to be cholecystokinin. This mechanism is modulated by the concentrations of dietary proteins (Zambonino and Cahu, 2007). Trypsin activity shows an increase during the first few days after hatching and mouth opening. This trend has been noted amongst most species of marine fish (Zambonino and Cahu, 2001). In white seabass (Galaviz et al., 2011), Trypsin specific activity was detected from one day post hatch with a gradual increase occurring, especially after the first exogenous feeding event. This increase occurred up until 14 dph. A slight decrease was noted thereafter. The trypsin specific activity fluctuated until it displayed a steady decline until the end of the experiment at 40 dph. For large yellow croaker (Ma et al., 2005), the specific trypsin activity in the pancreatic segment showed a similar pattern to that displayed in white seabass with an increase in specific activity occurring in the first few days of development. A decrease was observed from 5 dph to 25 dph. After this period, a low level of specific activity was maintained until the end of the experiment a 40 dph For sharpsnout seabream (Suzer et al., 2007), trypsin activity was also detected at hatching with an increase in specific activity occurring, especially around the time of mouth opening. After 28 dph, a decrease in specific activity was observed and fluctuations of the specific activity were measured until the end of the experiment at 40 dph. Similar profiles were observed for thick lipped grey mullet (Zouiten et al., 2008) and red drum (Zambonino and Cahu, 2001).

In carnivorous species of fish, amylase forms an integral part of the enzymatic capabilities of developing larvae. Similarly to tryptic activities, amylase specific activities do not seem to be triggered by food and is also mediated by gene expression (Suzer et al., 2007). For most marine fish species, amylase activity has been shown to be high during the initial stages of larval development and gradually decrease as the larvae develop (Zouiten et al., 2008). The stimulation of amylase is normally stimulated by starch, glycogen and polysaccharide chains. The starch content in compound feeds may have an effect on the specific activity of amylase at the time of weaning (Savona et al., 2011). For sharpsnout seabream (Suzer et al., 2007), amylase specific activity was first measured on 2 dph and an increase was noted during the initial week of development. The specific activity continued to increase until 10 dph and then began to decline from 22 dph. For large yellow croaker (Ma et al., 2005), the specific activity of amylase was measurable on 1 dph and showed an increase

in activity up until 15 dph. A decrease in activity was noted from 15 dph until 25 dph with an increase until the end of the experiment. The specific activity of amylase followed a similar pattern to that of trypsin. Similar patterns have been observed in yellowtail kingfish (Chen et al., 2006) and european seabass (Zambonino and Cahu, 1994).

Intestinal enzymes

The epithelium in the intestine is thought to be the main site for the digestion of peptides present in the lumen in vertebrates and the appearance of the microvillus membrane in the enterocytes is indicative of a vital step in the maturation of the digestive system. This step represents the transition of the digestive process from a larval one to an adult one (Zambonino et al., 2008). The peptide hydrolases are located in two subcellular localities: the cytosol and the brush border membranes of the enterocytes. Enzymes located in the cytosol of the enterocytes are primarily di- and tripeptidases. Their function is to complete the hydrolysis of proteins by reducing peptides to free amino acids (Zambonino and Cahu, 2007). When the enterocytes mature, the cytosolic enzyme activity begins to decrease while brush border enzyme activity begins to increase (Ma et al., 2005). Early maturation of the enterocytes always results in an increase in larval survival (Zambonino and Cahu, 2007). Dietary factors can influence the maturational process of the enterocytes. Polyamines are found in many foods, particularly in fish meal. Polyamines are low molecular weight biogenic amines that can be found in all living cells. Spermine and spermidine have a positive effect on the maturation of the gastrointestinal tract of mammals and birds. Studies have shown that the addition of 0.33% of spermine to marine fish larval diets produce similar results to those for mammals and birds (Zambonino and Cahu, 2007). In studies of the intestinal enzyme activity for fish larvae, the ratio of brush border membrane enzyme activity to the cytosolic enzyme activity is calculated. This ratio is considered to be a good indicator of intestinal digestion development (Zouiten et al., 2008).

Alkaline phosphatase (AP) is a brush border membrane enzyme. The activity of this enzyme depicts the development of the brush border membrane of the enterocytes (Kolkovski, 2001). It is produced in the enterocyte's Golgi apparatus. Alkaline phosphatase then migrates to the intestinal brush border membrane, which is its primary site of activity. The phosphatase group of enzymes are involved in intestinal transport of phosphorylated proteins, as well as the mineralization and hydrolysis of those proteins (Martinez et al., 1999). An increase in the amount of AP in the intestinal membrane indicates that the enterocytes are more functionally developed (Suzer et al., 2007). A decrease in AP activity is normally associated with the use of inadequate feeds or starvation (Wold et al., 2007). For sharpsnout seabream (Suzer et al., 2007), the AP activity showed a sudden increase in

activity in relation to larval age. For large yellow croaker (Ma et al., 2005), the activity of AP in relation to protein content, remained low during the development of the larvae. From 21 dph until 25 dph, the activity sharply increased and then reached a plateau until the end of the experiment. For Senegalese sole (Martinez et al., 1999), a very similar profile was observed (Ribeiro et al., 1999). A sharp increase in AP activity was noted around the time of metamorphosis. For European seabass (Zambonino and Cahu, 1994), the same profile was observed as described above. With regards to meagre (Suzer et al., 2012), AP activity showed a slight decrease until 15 dph and then fluctuated until 20 dph. AP activity then increased until the end of the experiment.

Aminopeptidase N (AN) is a brush border enzyme that is distributed in a variety of tissues such as blood, kidneys and the liver. This makes it difficult to establish when the intestine begins to secrete aminopeptidase when studying whole larvae samples. This is why it is more effective to study the intestinal segment specifically (Suzer et al., 2007). The function of AN is to hydrolyse peptides to amino acids during the final process of the digestion of proteins. It is also associated with the completion of the digestive process through luminal proteases. A high level of activity of AN is shown throughout the larval phase (Kurokawa and Suzuki, 1998). The study of the activity of AN in conjunction with AP gives a good indication of rate of digestion at a membrane level. This provides a clear indication of the transition from an initial larval mode of digestion to an adult mode of digestion (Zambonino and Cahu, 2001). For sharpsnout seabream (Suzer et al., 2007a), the activity of AN was similar to that of AP where it was relatively low before 20 dph but then showed a dramatic increase until 50 dph when the experiment was concluded. The pattern of activity of AN was equivalent for a variety of species including Senegalese sole (Martinez et al., 1999), large yellow croaker (Ma et al., 2005), meagre (Suzer et al., 2012) and European seabass (Zambonino and Cahu, 1994).

Leucine-alanine peptidase (Leu-ala peptidase) is a cytosolic peptidase (Suzer et al., 2012) that is found in the brush border membrane of the enterocytes. Leu-ala peptidase has been detected from one dph for a variety of species (Galaviz et al., 2011) which suggests that its presence is genetically programmed. This allows the yolk nutrients to be absorbed before exogenous feeding commences. Leu-ala peptidase digests intracellular peptides (Naz and Turkmen, 2008). The activity of leu-ala peptidase has been shown to decrease during larval ontogeny. This is due to the decrease in cytosolic enzymes normally decreasing with an increase in brush border membrane enzymes. This occurs around the third week of development and when acid protease activity commences. This process indicates the maturation of the enterocytes during larval development (Suzer et al., 2007). This pattern of

activity has been seen for meagre (Suzer et al., 2012), sharpsnout seabream (Suzer et al., 2007), Senegalese sole (Ribeiro et al., 1999) and large yellow croaker

Pepsin is an important enzyme involved in the digestion of proteins. Together with HCl, pepsin forms part of the gastric juice secreted by the gastric glands in the mucosa of the stomach (Zambonino et al., 2008). Pepsin does not seem to be influenced by dietary proteins and therefore does not appear to be a pre-requisite for protein digestion. This enzyme is of great importance when identifying the transition from larval digestive morphology to juvenile digestive morphology (Zambonino and Cahu, 2007). A study conducted on white seabass (*Atractoscion nobilis*) showed that pepsin activity began at 10 dph (Galaviz et al., 2011), while for red porgy (*Pagrus pagrus*) pepsin was detected at 28 dph (Suzer et al., 2007b). When the pepsin activity in sharpsnout seabream (*Diplodus puntazzo*) was studied, pepsin was first detected at 32 dph (Suzer et al., 2007). In European seabass (*Dicentrarchus labrax*), pepsin activity was first detected at 24 dph, which coincided with the formation of a fully functioning stomach (Zambonino and Cahu, 1994).

The role of probiotics in aquaculture

The use of probiotics initially became the focus of attention as an alternative to antibiotics. The use of some antibiotics, such as virginiamycin, spiramycin and bacitracin, has been banned in many European countries (Kesarcodi-Watson et. al, 2008). The appearance of drug resistant strains of antibiotics has also driven research towards the use of an alternative method of disease control (Suzer et al., 2008). The term probiotics has loosely been described as relatively harmless intestinal bacteria that result in improved health in host organisms. The substances produced by the microorganisms are said to promote growth and survival of another organism (Balcázar et al., 2006). Lactic acid bacteria have been used extensively for human and animal health and lactic acid bacteria have been shown to be present in the intestines of fish. Many companies in Europe are marketing probiotics as “wonder products” that improve larval performance in an easy and cost effective manner. The most common strains fall within the lactobacilli and bifido-bacteria. The storage potential of *Lactobacillus* spp. makes it an appealing choice. *Lactobacillus* spp. can be stored in its spore form indefinitely without sophisticated storage equipment (Kesarcodi-Watson et al., 2008). Probiotics provide protection to the host organism by interrupting the cellular functions of pathogenic bacteria and are able to protect the host organism by improving water quality and promoting an enhanced immune response (Suzer et al., 2008). They also protect the host by producing metabolic by-products that prevent invading bacteria from colonising the host organisms. Probiotics are also able to compete with neighbouring bacterial colonies for resources, including nutrients and space (Kesarcodi-

Watson et al., 2008). The benefits of probiotics in animal husbandry include increased feed efficiency and growth rates, the prevention of possible intestinal disorders and the pre-digestion of anti-nutritional factors that can be present in feed. Studies of recent interest have included the effects of probiotics on feed utilization, growth and increases in the efficiency of feeding (Suzer et al., 2008). The use of probiotics in aquaculture has suggested that there is a degree of host specificity of probiotic strains. However, further studies have to be conducted to prove this. Probiotics have been administered through compound feeds for weaned fish and in live feeds (*Artemia* and rotifers) for larval fish. Probiotics have also been administered in the tank water of larval rearing tanks (Irianto and Austin, 2002).

With the multitude of probiotic products available on the market, researchers and farmers alike are curious as to how probiotics affect larval performance on a molecular and cellular level. The primary questions surrounding probiotics are firstly, do probiotics actually work? And secondly, exactly what effects do probiotics have on the physiological development and nutrient assimilation capabilities of fish larvae? A few studies have been conducted on freshwater species including the common carp (*Cyprinus carpio*). These initial studies were on adult fish with a *Bacillus* based probiotic supplemented into the feed. The results shows a remarkable significant improvement in the growth and feed conversion ratios (FCR's) of the adults being fed the supplemented feed in contrast to the fish being fed the control feed that was free of probiotics. The digestive enzymes also showed significant differences between the treatments with significant increases in protease, amylase and lipase activities. This study also evaluated the differences in performance of the fish being fed two different mixes of probiotics. The results also indicate that the formulation of the probiotic itself also has a significant effect on the performance and FCR of adult carp (Yanbo and Zirong, 2006). Another study was conducted on the effects of *Lactobacillus* as a probiotic in the gilthead seabream (Suzer et al., 2008). The results of the study showed that the administration of a probiotic had a positive effect on the activity of all digestive enzymes assayed. The result from the study does suggest that the addition of the probiotic can enhance digestion and increase the rate of nutrient absorption. This would result in an increase in larval growth and survival. The effect of probiotics on the growth and survival of Atlantic cod has also been described with similar results being shown (Gildberg et al., 1997).

The purpose of this study on *A. japonicus* larvae will assess whether probiotics present a promising aspect to be included into the larviculture phase of the commercial production of *A. japonicus*. Initial studies show that not only does the addition of a probiotic have a significant effect on the performance of both adult and larval fish, but the combination of probiotic strains are also an important aspect to consider when looking to improving

performance and survival. The possibility of improving digestive performance and survival of fish larvae by using a relatively harmless probiotic additive is an exciting prospect when considering the immense costs and expertise required to optimise larval nutrition to improve growth and survival. This thesis will answer the questions of whether probiotics have a significant effect on larval growth and digestive performance in a commercial hatchery, as well as what effects they have at the molecular and cellular level.

Materials and Methods

The ethical approval application form for this project was reviewed and approved on 18 September 2013 by the Stellenbosch University's Research Ethics Committee: Animal Care and Use. The protocol number is SU-ACUM13-00033.

Broodstock and spawning

Wild caught, mature *Argyrosomus japonicus* broodstock that had been conditioned in captivity at Oceanwise were used for spawning. These fish were caught along the Eastern Cape coast of South Africa. The broodstock were given a diet of sardines, hake and squid. This diet was supplemented with vitamins. Photoperiod and temperature were used to induce maturation of the oocytes. After being anesthetised with AquiS™, the broodstock were then checked by canulation and the biopsied oocytes were evaluated for their developmental stage under a dissection microscope. The females with oocytes larger than 400µm and ripe running males were then induced to spawn using an injectable LHRH analogue. The LHRH analogue promotes the maturation of the gametes and the subsequent release of gametes within a period of 48 to 72 hours. One female and two males were injected. After 48 hours, eggs were collected from the spawning tank by an egg collector attached to the spawning tank. The eggs were disinfected using 160ppm formalin and transferred into 180L incubators. The incubators were filled with sterilized sea water at a water temperature of 27°C. The incubators were stocked with around 50 000 eggs per litre. The eggs were allowed to hatch for 6 hours. Once the eggs had hatched, the larvae were transferred to the larval rearing tanks.

Larval rearing

Larvae were reared in three 6000 liter fibreglass tanks and six 2800 liter fibreglass tanks. Each larval rearing tanks was stocked with 78 larvae per liter from the eggs that had hatched in incubators,The larval rearing tanks remained flow through systems for the duration of the experiment with a flow rate of approximately 1.2 liters per minute. Each tank was equipped with an air stone which was set to release oxygen at a rate of 8 mg per liter. This was controlled using oxygen regulators. The water temperature in each tank was controlled using 100 watt thermostat controlled aquarium heaters. The environment in the hatchery was partially controlled by insulated walls and roof.

Live cultures of *Nanochloropsis* sp. and ω3 Algae™ (manufactured by BernAqua, NV Hagelberg 3 B-2250, Olen, Belgium, www.bernaqua.eu) were used for the green water technique in the larval rearing tanks. Figure 1 indicates the larval feeding regime followed by Oceanwise. Larvae relied on endogenous feeding from 1 dph until 3 dph. Once exogenous feeding began, which was easily observed in the larval rearing tanks, the larvae began to

feed on rotifers. Rotifers were added to the larval rearing tanks from 1 dph until 6 dph and maintained at a density of around 18 rotifers per millilitre. Rotifers were cultured in 1000L tanks that were filled with sterilized sea water and live *Nannochloropsis* spp. A mixture of yeast and commercially produced rotifer enrichments were added to the culture tanks twice a day. Rotifers were added to the larval rearing tanks on a daily basis. *Artemia* were culture in 180L conical tanks and were not enriched. 1st nauplius stage *Artemia* were added to the larval rearing tanks. From 4 dph until 21 dph, *Artemia* nauplii were added to the tanks. This was done on a daily basis at a density of about 1 000 000 artemia per 1000 liters of water. From 7 dph until the end of the study, a formulated commercially available microdiet was fed to the larvae.

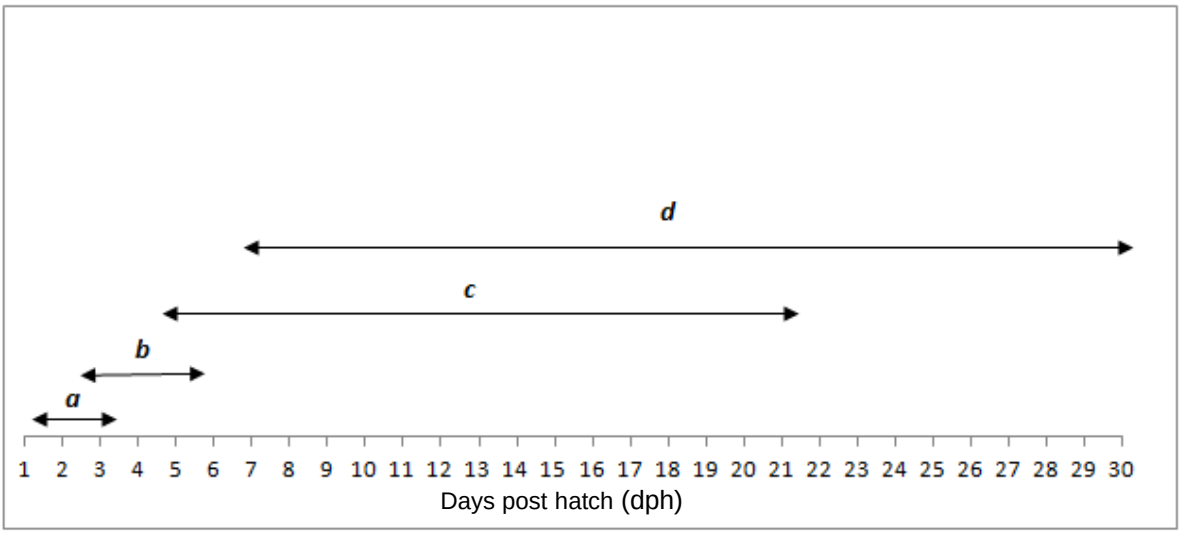

Figure 1 A summary of the feeding protocol of *A. japonicus* larvae (dph) at Oceanwise where *a* is endogenous feeding; *b* is rotifers; *c* is *Artemia* nauplii; and *d* is the formulated diet

Dissolved oxygen (% and mg/mL) and water temperature (°C) were measured twice a day with measurements being taken at 09:00 and 15:00. pH was measured once a day and total ammonium nitrogen (TAN, mg/L) was measured once a week. Table 1 summarises the average values for each of the water quality parameters for the study.

Table 1 The total mean (± SD) values for the water quality parameters that were measured in the larval rearing system for the duration of the study

Water quality parameter	Value
Temperature (°C) AM	24.58 ± 1.20
Temperature (°C) PM	25.30 ± 1.11
pH	7.02 ± 0.23
Nitrite (mg/L)	0.08 ± 0.03
TAN (mg/L)	1.03 ± 0.79
DO (mg/L) AM	7.56 ± 2.05
DO (mg/L) PM	7.35 ± 2.24
DO (%) AM	111.15 ± 30.12
DO (%) PM	110.39 ± 32.75

Experimental design and sampling

Two different probiotics and a control, where no probiotics were added, were used in the study. A total of six 2800L fibreglass tanks and three 6000L fibreglass tanks were selected. Each treatment was done in triplicate. As a result of the different tank sizes, a randomised block design was used to allocate treatments to the tanks. The three larger tanks were blocked together while the remaining six smaller tanks were randomly allocated into another two blocks. Each treatment was randomly assigned to a tank in each block. The experiment was scheduled to run for 30 days from the moment the larvae were stocked into the larval rearing tanks from the incubators at 1dph. Figure 2 shows a floor plan of the layout of the tanks and the assignment of the treatments to the tanks.

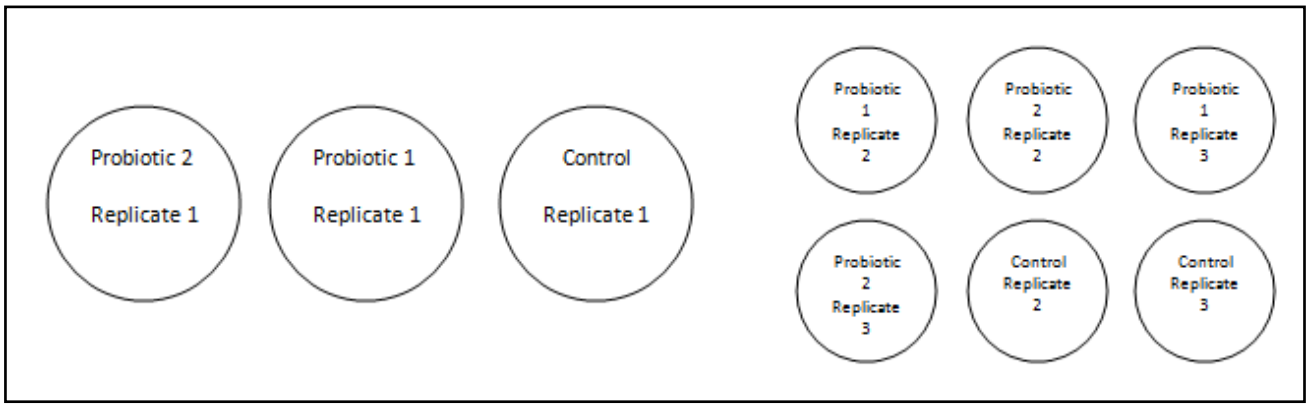

Figure 2 A schematic diagram illustrating the set up the larval rearing tanks in the hatchery at Oceanwise used for the two treatments groups and the control group. Each treatment was replicated three times

The tanks remained closed systems for the first 3 days. From 4 dph, the tanks became flow through systems. A flow meter was used to ensure that the flow rate was maintained at 1.2 liters per minute. Each tank was assigned its own daily maintenance equipment, which includes siphons, filters, heaters, lamps and water collection jugs, to prevent cross contamination between tanks. All equipment was sterilized on a daily basis in hypochlorite then stored in a disinfectant called Virkon S™ (DuPont Chemical Solutions Enterprise, www.dupont.com). All equipment was rinsed thoroughly with freshwater before being used in the larval rearing tanks.

Larvae were sampled on predetermined days by concentrating them in a large net and randomly collecting the required amounts of larvae from the concentrated larvae. Table 2 contains the number of larvae collected for the enzyme analysis, histological analysis and the growth study. For the enzyme analysis, 1 dph, 5 dph and 10 dph required that larvae were collected to fill a volume of 0.2mL. The larvae collected from the growth study were also used for the histological study. The number of sampling days and the number of larvae collected was sufficient for statistical analysis.

Table 2 The number of larvae sampled per tank on predetermined days for the enzyme analysis, histological analysis and growth study

Days post hatch	Enzyme analysis	Histology and growth
1	0.2mL	10
5	0.2mL	10
10	0.2mL	10
14	100	10
15	100	10
16	80	10
17	80	10
18	80	10
19	80	10

Preparation and administration of selected probiotics

Two probiotic products were used in the study. The first probiotic was a product supplied by Bern Aqua (Bernaqua NV Hagelberg 3 B-2250, Olen, Belgium www.bernaqua.eu). The product, called BactoSafe, is composed of four strains of probiotic bacteria, namely *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilus* and *Pediococcus acidilactici* For the tanks assigned to BactoSafe, the instructions provided by the manufacturer were carefully followed so that a 1x10exp9 CFU/g of bacteria were added to each tank. A 1% solution was brewed by mixing a ratio of 1kg of the probiotic in 100L of fresh water. This mixture was allowed to stand for a minimum of 12 hours to allow the bacteria to be activated. The pH was checked regularly until a pH of 5.5 was reached, indicating that the probiotic was active. After 12 hours, 1L of the brewed suspension was used per m^3 of water in the live feed tanks, while 1L per $10m^3$ of the brewed suspension was added per $10m^3$ in the larval rearing tanks.

The second probiotic was supplied by the Council for Scientific and Industrial Research (CSIR, Meiring Naude Road, Pretoria) in South Africa. This was an experimental probiotic that had been largely tested in freshwater systems. For the tanks where the CSIR probiotic was added, 10g of the product was directly added per m^3 for the live food tanks, while 1g of the product was directly added per m^3 of water in the larval rearing tanks. No brewing or activation steps were required for probiotic 2, allowing the probiotic to be used immediately. Both the probiotics were added to their respective tanks on a daily basis at the same time every day. The CFU/mg (colony forming units per mg) was determined for each

of the probiotics before the start of the trial to ensure that the strains in the probiotics were viable.

Larval growth of Argyrosomus japonicus

To determine the growth of the larvae during the experiment, 10 larvae were taken from each tank on 1, 5, 10, 14, 15, 16 17, 18 and 19 dph. Larvae were exposed to a lethal dose of 500µL/L of phenoxy ethanol to minimise stress. The larvae were then measured under a dissection microscope using an ocular micrometer. From 1 dph to 16 dph, the notochord length was measured, as shown in Figure 3. For larvae older than 16 dph, total length was recorded.

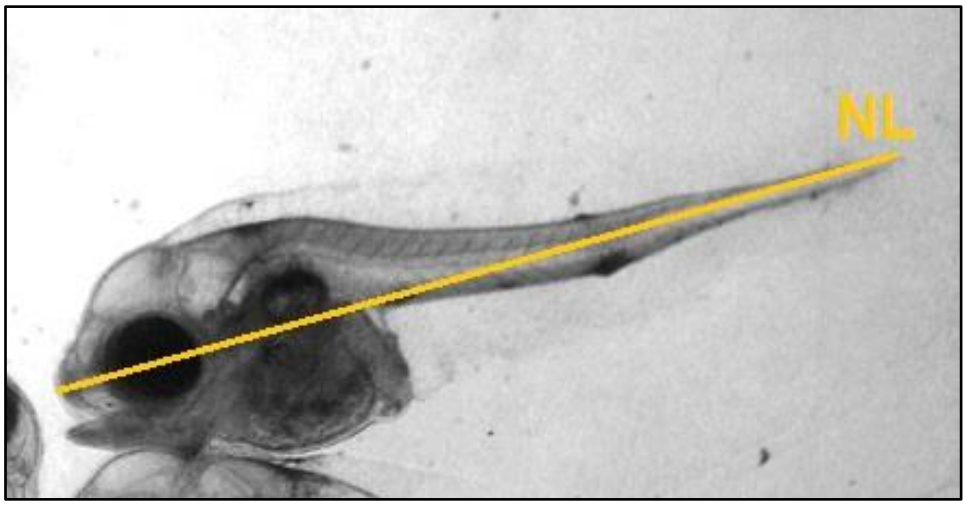

Figure 3 A photomicrograph depicting the notochord length (NL) of an *A. japonicus* larvae at 10dph. (10x magnification)

Histological preparation of Argyrosomus japonicus larvae

In order to prepare the larvae for histological analysis, 10 larvae were sampled from each tank on predetermined days. The sampling days included 1, 5 10, 14, 15, 16, 17, 18 and 19 dph. Larvae were euthanized using a lethal dose of phenoxy ethanol. The phenoxy ethanol was administered at 500µL/L. The larvae were then rinsed with distilled water and placed directly into 10% neutral buffered formalin until further processing. The larvae were sent to the Stellenbosch University Tygerburg Medical Campus to be processed for histological analysis. The larvae were dehydrated using a graded series of ethanol, followed by clearing with xylene. The cleared larvae were embedded in paraffin wax and then sagittally sectioned into 5um thick slices using a microtome. The sectioned larvae were mounted onto glass slides and stained using hemotoxylin and eosin (H&E).

The determination of specific enzyme activity of larval digestive enzymes

Preparation of larval tissue

Whole body homogenates were prepared for all larvae: 45.5±0.39 mg/mL of larvae were homogenised in cold 50mM Tris-HCL (pH 8) (Suzer et al., 2012).The larvae were homogenized using an IKA Ultra Turrax TubeDrive™ control homogenizer with IKA BMT-20MS™ tubes for 5 minutes at 4000 rpm. The samples were then centrifuged at 13 000xg for 30 minutes at 4°C. The supernatant was collected, alloquotted into 150uL subsets and stored at -80°C until used for further analysis.

Amylase

Amylase activity was determined using the dinitrosalicylic acid (DNS) method (Métais and Bieth, 1968). The assay was run in a BioRad Thermo Cycler. A program was set where the Thermo Cycler would run at 50°C for 10 minutes, 95°C for 15 minutes and then cooled down to 4°C indefinitely. 25uL of the sample and 0.5% soluble starch substrate were pipette into each well. After 10 minutes at 50°C, 1% DNS was pipette into each well to stop the reaction. After 15 minutes at 95°C, the samples were cooled to 4°C and 50uL of the reaction mixture was removed and pipette into the 96 well flat bottomed plate. 20mM phosphate buffer was added to each well and the absorbance was measured at 540nm using a PowerWaveHT-1 microplate reader. A blank was prepared in the same way as the samples but using 20mM phosphate buffer. A positive enzyme control was also included using 1mg/mL P500 enzyme fungal amylase prepared in 20mM phosphate buffer. A standard curve for amylase was created using maltose as the standard. A maltose stock solution of 0.9mM was made up and a series of standards were created from the stock solution. The concentration range was between 0.090mM and 0.450mM. The specific enzyme activity was calculated using the following equation:

$$\text{Specific amylase activity (mU/mg prot)} = \frac{\text{umol of maltose liberated per minute}}{\text{mg of protein}}$$

Trypsin

Tryptic activity was determined using a standardized protocol developed by Sigma-Aldrich. This protocol was adapted from the one described by Holm et al. (1988). The method required the use of a 0.25mM Nα-Benzoyl-L-Arginine Ethyl Ether (BAEE) substrate solution (Sigman-Aldrich, B4500), which was prepared in a 67mM sodium phosphate buffer; pH 7.6. The substrate and enzyme solutions were pipette into quartz cuvettes. A blank was also prepared by adding the enzyme diluent (50mM Tris-HCl; pH8) to the substrate. A

positive control was included which was made up using Trypsin from porcine pancreas (Sigma-Aldrich, T0303). After exactly 5 minutes, the absorbance was read at 253nm using a Beckman-Coulter DU800 spectrophotometer. One BAEE unit produced a ΔA_{253} of 0.001 per minute at pH 7.6 at 25°C in a reaction volume of 3.20mL. The specific activity of Trypsin was calculated using the following equation:

$$\text{Specific Trypsin activity (mU/mg prot)} = \frac{\text{BAEE units per mL per minute}}{\text{mg of protein}}$$

Pepsin

The enzyme activity of pepsin was determined using bovine haemoglobin as the substrate (Anson, 1938). A 2.5% bovine haemoglobin substrate was prepared in deionized water. The substrate solution was filtered using Whatman No. 1 filter paper. The filtered haemoglobin solution was diluted using 0.3N HCl in a ratio 1:4. A 5% trichloracetic Acid solution was prepared in ultrapure water. Folin and Ciocalteu's phenol reagent was also prepared according to the method described by Sigma-Aldrich (Catalogue number F9252). An L-tyrosine standard curve was prepared using a 3mg/mL stock solution. A concentration series was used ranging from 2mM to 16.5mM. 0.5N NaOH and the phenol reagent were added to the standard and after 5 minutes, the absorbance was read at 555nm. The blank was prepared with the 2.5% haemoglobin substrate, 50mM Tris-HCL at pH 8 and 5% trichloracetic acid. The colour was developed using 0.5N NaOH and the phenol reagent and read after 5 minutes against the standard. The positive control was made up using pepsin powder from Sigma Aldrich (P052500). Digestion was carried out at 25°C where the enzyme and the haemoglobin substrate were added together in a 1:5 ratio. After 10 minutes the reaction was stopped using 5% trichloracetic acid. Thereafter 0.5N NaOH and the phenol reagent were added and the absorbance was read after 5 minutes at 555nm. The specific activity of pepsin was calculated using the following equation:

$$\text{Specific pepsin activity (mU/mg prot)} = \frac{\text{umol of L-tyrosine liberated per minute}}{\text{mg of protein}}$$

Alkaline phosphatase

To determine the alkaline phosphatase (AP) activity, a p-nitrophenol phosphate substrate was used (Bessey et al., 1946). The substrate was a pre prepared p-nitrophenol phosphate (p-NPP) liquid buffer system from Sigma-Aldrich (N7653). A positive control was also included where AP from bovine intestine (Sigma-Aldrich, P0114) was used. The enzyme and the p-NPP were pipette into a 96 well microtitre plate and incubated at room

temperature for 25 minutes. Since the p-NPP was light sensitive, the assay was done in the dark. After 25 minute, the absorbance was read at 405nm using a PoweWaveHT-1 microplate reader. The specific activity of AP was calculated using the following equation:

$$\text{Specific AP activity (mU/mg prot)} = \frac{\text{umol of p-nitrophenol liberated per minute}}{\text{mg of protein}}$$

Aminopeptidase N

The Aminopeptidase N (AN) activity was determined using L-leucine p-nitroanilide as the substrate (Maroux et al., 1973). A 0.1mM L-leucine p-nitroanilide (Sigma-Aldrich, L-9125) substrate solution was prepared in a 1mM tricine buffer (pH 8). The reaction cocktail was prepared by adding the 0.1mM L-leucine p-nitroanilide solution, 200mM tricine buffer and deionized water. The positive control was prepared using Leucine Aminopeptidase from porcine (Sigma-Aldrich, L5006). The blank was prepared using a 20mM tricine buffer (pH8) and 0.05% (w/v) bovine serum albumin (Sigma-Aldrich; A7030). The solutions were pipette into a 96 well microtitre plate and incubated at 25°C for 60 minutes. The absorbance was read at 405nm using a PowerWaveHT-1 microplate reader. The specific activity of AN was calculated using the following equation:

$$\text{Specific AN activity (mU/mg prot)} = \frac{\text{umol of p-nitroaniline liberated per minute}}{\text{mg of protein}}$$

Leucine-alanine peptidase

The method using leucine-alanine dipeptide as the substrate was used to determine the Leucine-alanine peptidase (Leu-Ala) activity (Nicholson and Kim, 1975). The L-amino acid oxidase (LAOR) substrate solution was prepared using L-amino oxidase from *Crotalus atrox* (Sigma-Aldrich, A5147), horse radish peroxidise (Sigma-Aldrich, P-8375) and o-dianiside (Sigma-Aldrich, D9143) in a 1M tris-HCl buffer (pH 8). The Leucine-Alanine dipeptide was prepared in 50mM Tris-HCl buffer (pH 8) at a concentration of 5µM. The standard curve was created using L-leucine as the standard. The LAOR, homogenate and dipeptide were pipette into a 96 well microtitre plate and incubated at 20°C for 20 minutes. After 20 minutes, 50% H_2SO_4 was added to stop the reaction. The absorbance was then measured using a PowerWaveHT-1 microplate reader at an absorbance of 530nm. The standard was prepared in the same way, except 25µL of 50mM Tris-HCl (pH 8) buffer was added instead of the homogenate. The specific activity of Leu-Ala was calculated using the following equation:

$$\text{Specific Leu-Ala activity (mU/mg prot)} = \frac{\text{umol of L-leucine liberated per minute}}{\text{mg of protein}}$$

Total protein

The total protein content of the homogenate was determined using the Bradford assay (Bradford, A, 1976). Bovine serum albumin was used to create the standard curve. The assay was performed at room temperature (25°C) in a 96-well plate and the absorbance was measured at 595nm using a PowerWave-HT multi-well plate reader. This total protein value was used in all the above calculations.

Statistical analysis

Statistical Analysis Software (SAS) Enterprise Guide 5.1 was used to perform an analysis of variance (ANOVA) where linear models were generated. Any significant difference between the control group and the two treatment groups for the larval growth and specific enzyme activities was determined and a significance level of $P< 0.05$ was utilized. ANOVA was used for each separate enzyme analysis. Multiple linear regressions were also performed for the growth curves for the control group and each treatment group. Standard deviations of the means were also calculated.

Results

On day 18 of the study, one of the three replicate tanks for the BactoSafe™ treatment crashed due to insufficient oxygen. At the time when this was noticed, the mg/L oxygen and % oxygen level was 76.1mg/L and 3.51% respectively. These values fell below the minimum levels required to sustain life. This resulted in all the larvae in that tank dying. Therefore, from 18 dph, the BactoSafe™ treatment was excluded from the results. The oxygen levels in each of the tanks showed some fluctuation on a daily basis. Figure 4, Figure 5 and Figure 6 show the daily average oxygen levels (mg/L) that were recorded for the duration of the study for each of the tanks for the control and BactoSafe and CSIR treatment groups. Standard deviations have been indicated on each day for each tank..

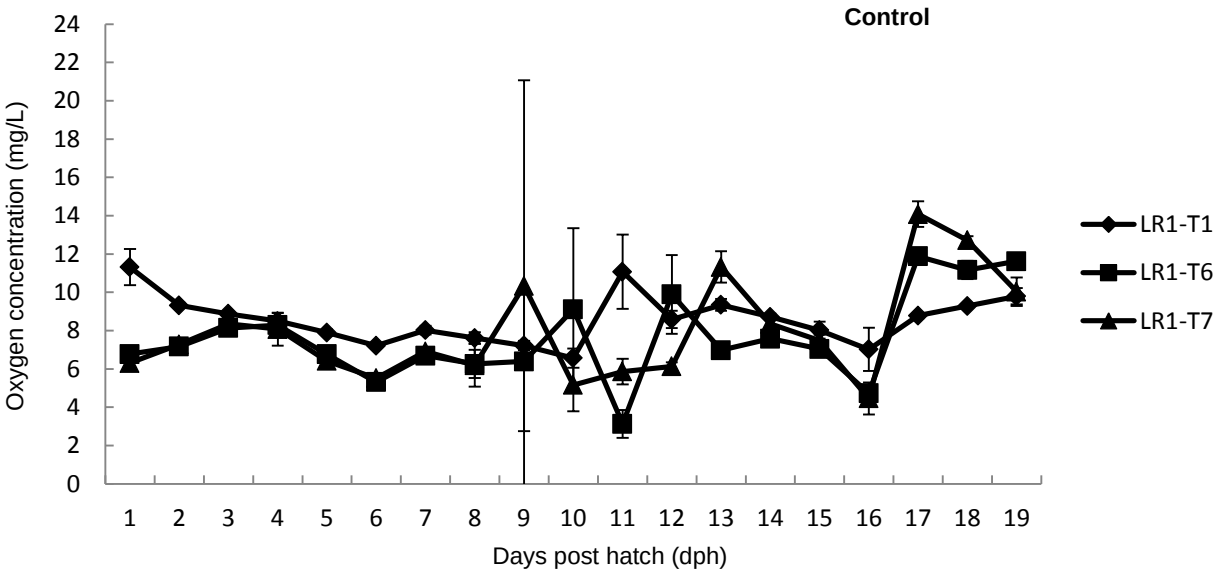

Figure 4 The daily average oxygen concentrations (mg/L ± SD) of each tank for the control group

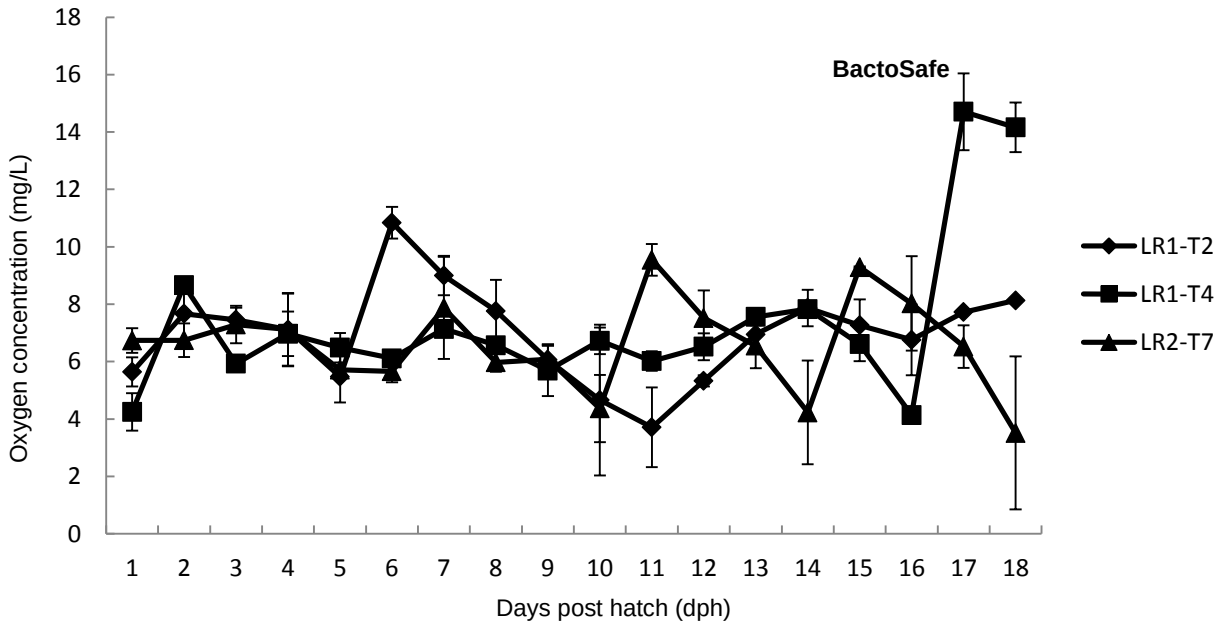

Figure 5 The daily average oxygen concentrations (mg/L ±SD) of each tank of the BactoSafe treatment

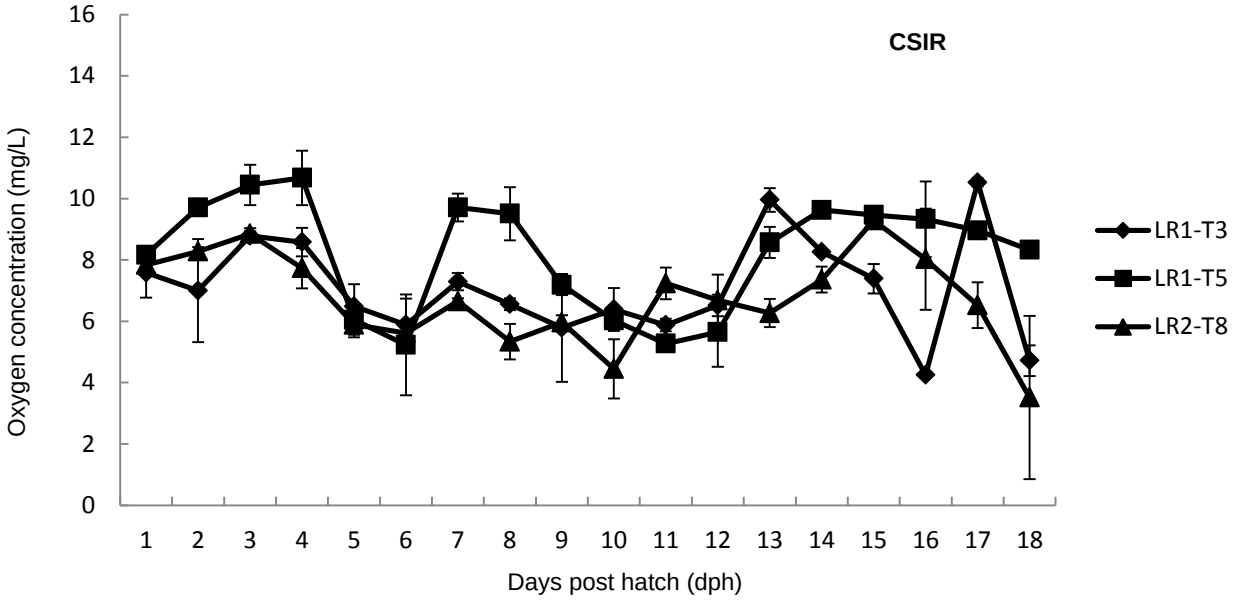

Figure 6 The daily average oxygen concentrations (mg/L ± SD) of each tank of the CSIR treatment

The effects of probiotics on the growth of A. japonicus larvae.

Notochord length was measured for larvae on 1 dph, 5 dph and 10 dph. From 14 dph, total length was measured. Figure 7 represents the difference in notochord length between the control and two treatment groups.

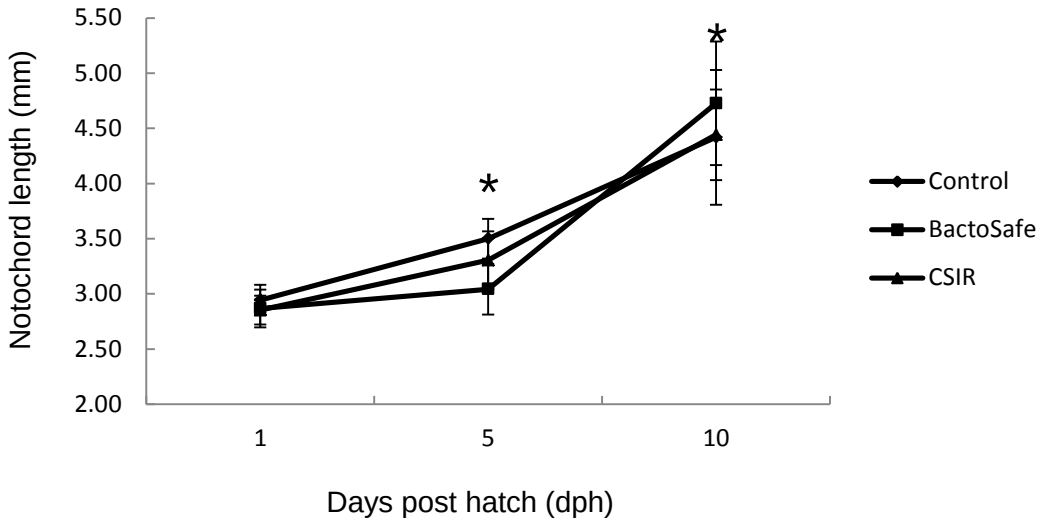

Figure 7 The average notochord length (mm ± SD) of *A. japonicus* larvae from 1dph to 10 dph from the control and two treatments groups. The asterisk represents significant differences (P<0.05)(n=3)

The larval growth on 5 dph results in a significant difference between the control and the treatment groups with the average notochord length of the control being greater than the CSIR (P = 0.0013) and BactoSafe (P <0.001) treatment groups. All three differ significantly from each other. On 10 dph, the control and the BactoSafe treatment group differ significantly (P = 0.015) from each other with the notochord length for BactoSafe being greater than the control. A linear regression was fitted which showed that slopes of the mean of the control group differed from the CSIR and BactoSafe group (<0.001). Figure 8 shows the total length of the *A. japonicus* from 14 dph until 19 dph. A clear increasing trend in the average total length for the control group and the two treatment groups can be observed. A regression model was fitted, which shows that there is also a difference between the means of the slope of the control group and the CSIR and BactoSafe group

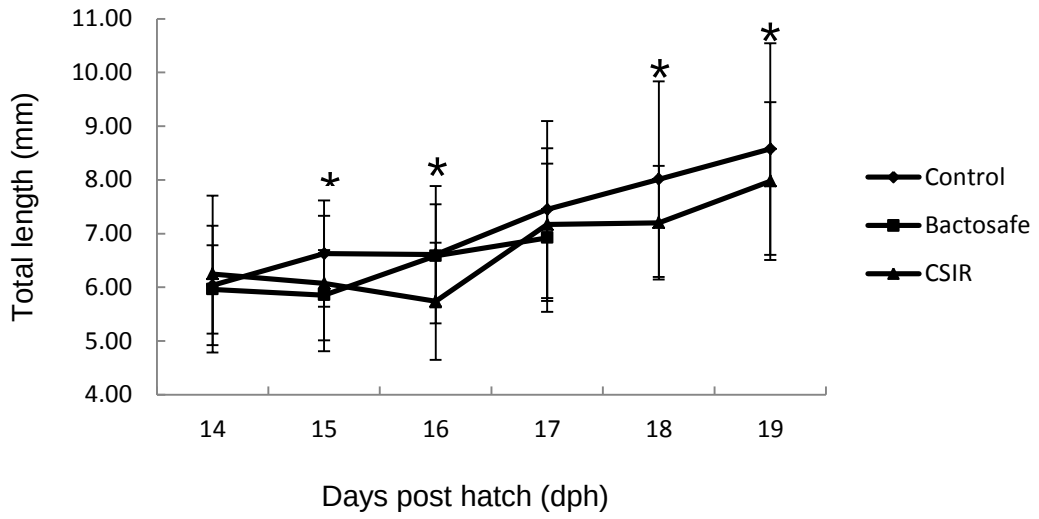

Figure 8 The average total length (mm ±SD) of A. japonicus larvae from 14 dph until 19 dph from the control group and two treatment groups. The asterisk represents significant difference between the control and two treatment groups (P<0.05)(n=3)

Figure 8 clearly indicates an increasing trend in the average total length of the larvae from all groups over time. On 15 dph, the average total length of the control group is significantly greater than that of the BactoSafe group (P = 0.002) but does not differ from the CSIR group. On 16 dph, the mean length of the control group and the BactoSafe group do not significantly differ from each other but their means are significantly greater than that of the CSIR group (0.0473). On 18dph and 19 dph, the mean total length of the control group is significantly greater than those of the CSIR group (P = 0.0028 and 0.0497 respectively). The results obtained from both the means of the notochord length and total length suggests that the overall average length is greater for the control group than the two treatment groups.

The effect of probiotics on the histological development of A. japonicus larvae

. Table 3 is a summary of the observations made regarding the development of the digestive tract of the control group and two treatment groups.

Table 3 A summary of the development of the digestive tract of A. japonicus larvae in the control, BactoSafe™ and BioBac™ groups

Histology	Control	BactoSafe™	BioBac™
Pancreas: • First visible • Zymogen granules	• 5 dph • 5 dph – 19 dph	• 5 dph • 5 dph - 17 dph	• 5 dph • 5 dph – 19 dph
Intestine: • Differentiation • Pyloric sphincter • Appearance of mucous cells in mid gut • Vacuoles in hind gut	• 5 dph • 5 dph • 15 dph - 19 dph • 5 dph – 19 dph	• 5 dph • 5 dph • 15 dph - 19 dph • 5 dph – 17 dph	• 5 dph • 5 dph • 15 dph – 19 dph • 5 dph – 15 dph
Stomach: • Differentiation between cardiac and pyloric stomach • Appearance of mucous cells in cardiac stomach • Appearance of gastric glands in pyloric stomach	• 14 dph • 14 dph • 17 dph	• 14 dph • 14 dph • 17 dph	• 14 dph • 14 dph • 17 dph

Pancreas

In this study, the pancreas was clearly visible by 5 dph in the control group as well as both treatment groups. The same was observed for the control and two treatment groups. As the larvae developed, the pancreas began to surround the anterior intestine and was positioned in close proximity to the liver. Figure 9A shows the position of the pancreas around the midgut as well as the acini with the acidophilic zymogen granules (Figure 9B). The structure of the pancreas did not differ as the larvae developed and the same cellular structures were observed until the end of the sampling period at 19 dph. All three groups showed little differences between them in the development of the pancreas and the presence of zymogen granules.

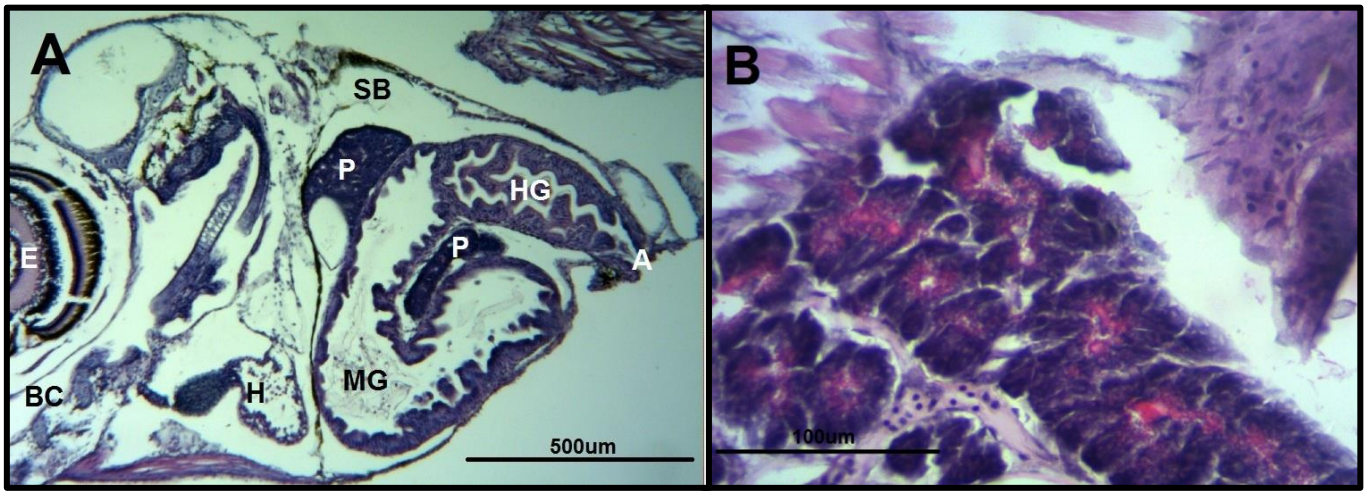

Figure 9 The position of the pancreas in 5 dph *A. japonicus* larvae (A), the acidophilic zymogen granules in the basophilic acini of the pancreas in larvae 16 dph (B). (P: pancreas; SB: swim bladder; MG: mid gut; HG: hind gut; A: anus; H: heart; SB: swimbladder; E: eye; BC: buccopharyngeal cavity)

Intestine

The intestine on 5 dph was definitively separated into the anterior intestine (mid gut) and the hind gut. A 90 degree loop in the intestinal section was clearly observed and this was seen for all larvae from the control group and the two treatment groups until the end of the study. The epithelium of the mid gut consisted of simple columnar epithelium with basal nuclei. Microvilli could clearly be seen on the apical surface of the epithelial cells. Mucosal folds were evident at this stage, increasing the surface area for absorption of nutrients. The mid gut was separated from the stomach by the pyloric sphincter. The hind gut was also comprised of columnar epithelium but with supranuclear lipid vacuoles being present.

Microvillli were observed on the apical surface of the epithelial cells. Figure 10 shows the 90 degree loop between the mid gut and the hind gut of the larvae on 5dph. The pyloric sphincter can be seen between the mid gut and the stomach.

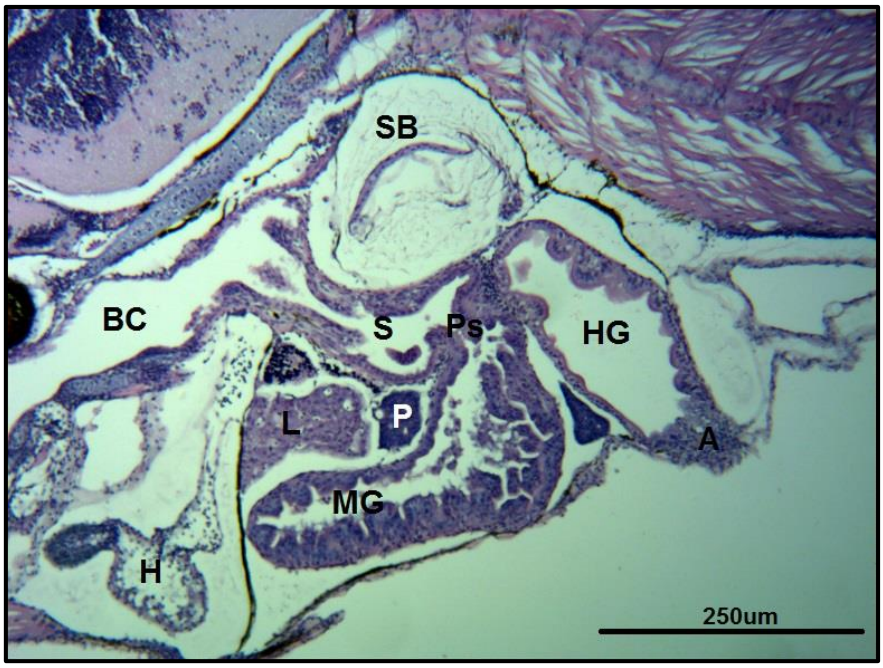

Figure 10 The 90 degree loop between the mid gut and hind gut of 5 dph *A. japonicus* larvae. (S: stomach; Ps: pyloric sphincter; L: liver; MG: mid gut; P: pancreas; BC: buccalpharygeal cavity; SB: swim bladder; HG: hind gut; H: heart)

Goblet cells in the mid gut were first observed on 15 dph in the control group as well as the two treatment groups. The number of goblet cells in the mid gut showed a slight increase until 19 dph. Figure 1111 shows goblet cells in the epithelial layer of the mid gut mucosa. The supra nuclear lipid vacuoles in the epithelium of the hind gut were observed in high numbers from 5 dph until 19 dph for the control group and the two treatment groups. The number of supra nuclear lipid vacuoles present did not decrease as the larvae developed. Figure 12A shows the supra nuclear lipid vacuoles of the hind gut of 19 dph larvae while Figure 12B shows the epithelium on the mid gut in contrast to the epithelium of the hind gut of 10 dph larvae. The brush border on the apical surface of the epithelial cells in both the mid gut and hind gut was observed in larvae from 5 dph until 19 dph. The brush border is clearly seen in Figure 12A and Figure 12B.

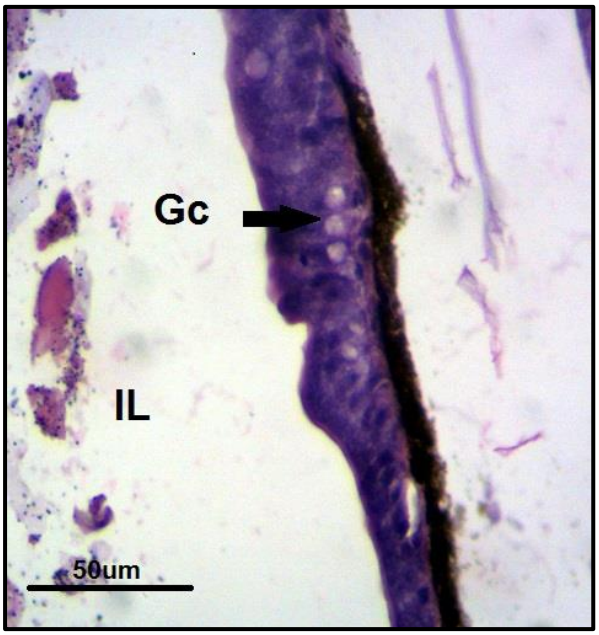

Figure 11 The goblet cells of the epithelium of the mid gut of 15 dph larvae. (Gc: goblet cell; IL: intestinal lumen)

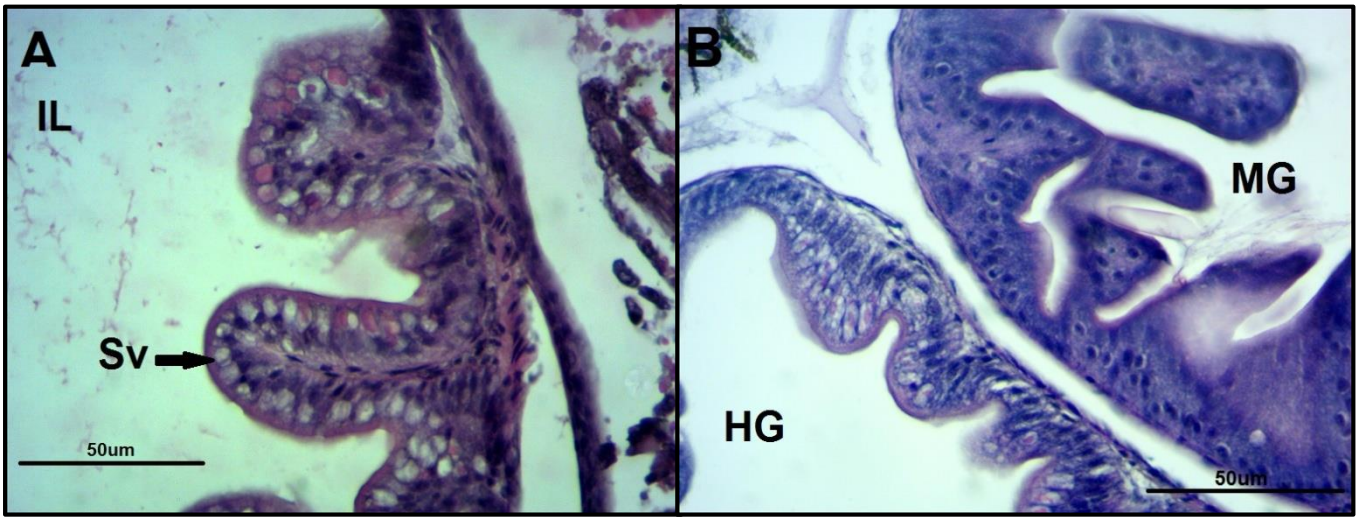

Figure 12 The supra nuclear vacuoles of the hind gut of 19 dph larvae (A), the difference between the hind gut and mid gut epithelium in 10 dph larvae(B) (IL: intestinal lumen; Sv: Supra nuclear vacuoles; MG: mid gut; HG: hind gut)

Stomach

On 5 dph in this study, the stomach was present as a simple sac-like structure with simple cuboidal epithelium and basal nuclei. Microvilli were also present on the apical surface of the stomach epithelium. The pyloric sphincter was visible, which separates the stomach from the anterior intestine and mid gut. From 10 dph, glandular cells (gastric glands) were observed in the oesophagus (Figure 13A and Figure 13B) but had not been observed in the stomach. The stomach was still a simple sac-like structure with simple cuboidal epithelium (Figure 13C).

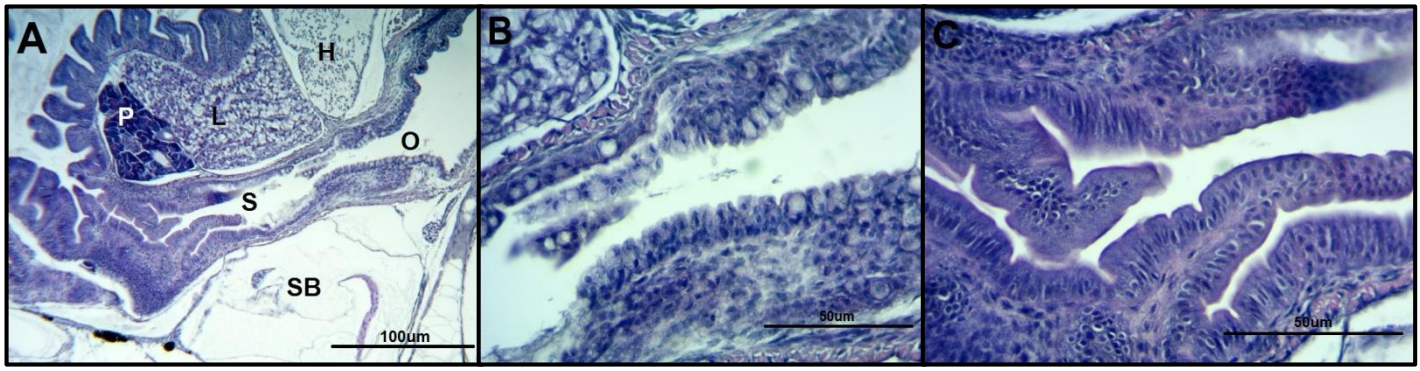

Figure 13 A cross section of the stomach of 10 dph larvae (A), the glandular cells that are found in the oesophagus of 10 dph larvae (B), and the simple cuboidal epithelium of the stomach of 10 dph larvae (C). (P: pancreas; L: liver; H: heart; O: oesophagus; S: swim bladder; S: stomach)

By 14 dph, the stomach was divided into the cardiac stomach and the pyloric stomach. Mucous cells started to appear in the cardiac stomach (Figure 14A) while the epithelium in the pyloric stomach remained as a simple cuboidal epithelium with microvilli in the apical surface of the cells (Figure 14B).

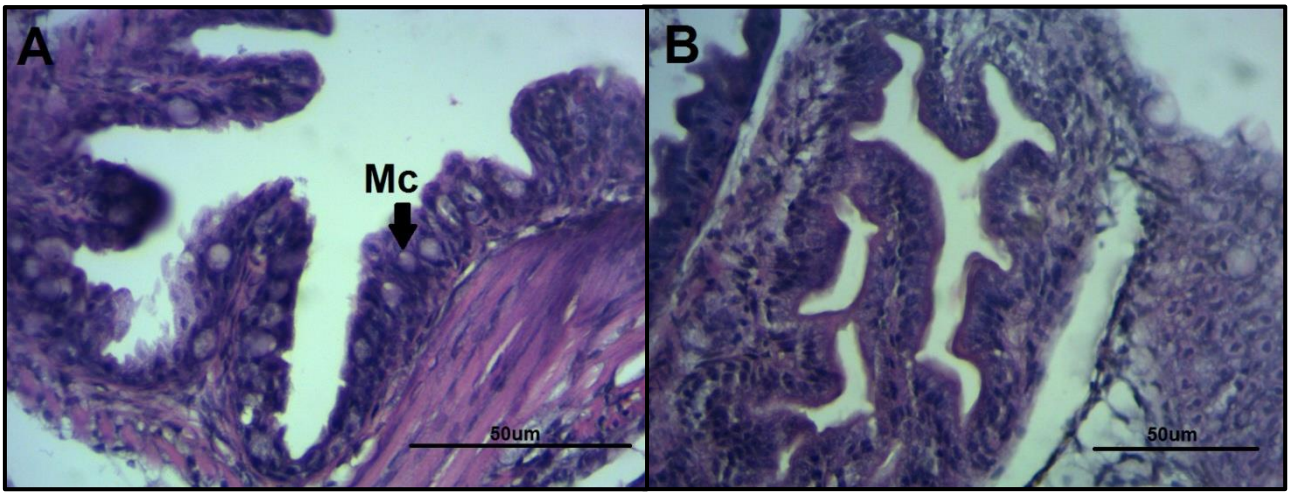

Figure 14 The cardiac stomach at 14 dph containing mucous cells within the epithelium (A) and the pyloric stomach with simple cuboidal epithelium without any mucous cells (B) (Mc: mucous cells)

By 17 dph, mucous cells were still present in the cardiac stomach and gastric glands started to develop in the pyloric stomach (Figure 15A). The number of gastric glands in the pyloric stomach increased slightly up until 19 dph (Figure 15B). The presence of gastric glands in the stomach is indicative of the maturation of the stomach and the consequent ability for the larvae to fully digest proteins. The development of the stomach was observed to be the same between the control group and the two treatment groups.

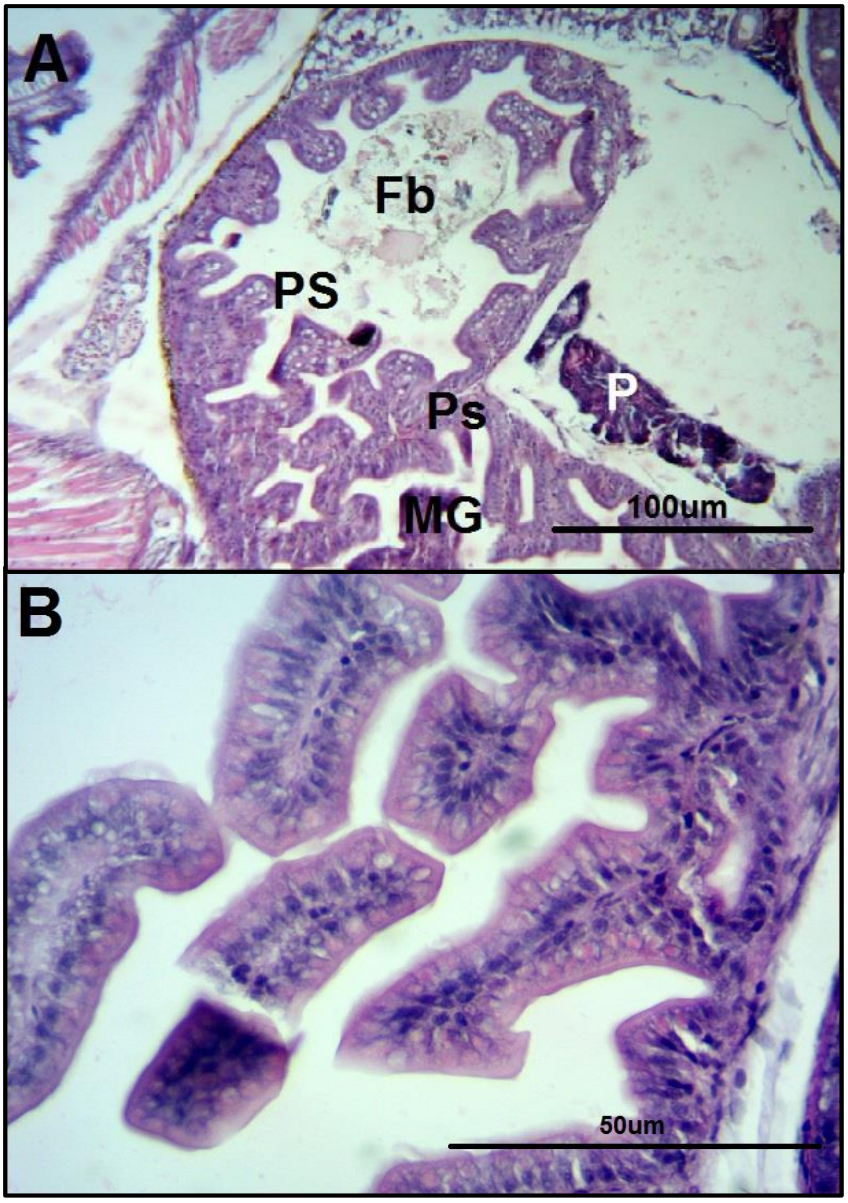

Figure 15 A sagital section of the pyloric stomach at 17 dph with the appearance of gastric glands (A) and the gastric glands of the pyloric stomach of 19 dph larvae (B) (Fb: food bolus; PS: pyloric stomach; Ps: pyloric sphincter; MG: mid gut; P: pancreas)

The observations that were made regarding the development of the digestive tract on a cellular level, suggest that the addition of probiotics have no observable effect on the cellular development of *A. japonicus* larvae. No differences in the key developmental phases

of important digestive organs were seen between the control group and the two treatment groups.

Enzyme analysis

Six critical digestive enzymes were analysed in whole body homogenates, including total soluble protein. The results are expressed as specific enzyme activity (U/mg protein) over time (dph).

Figure 16 shows the specific amylase activity of the control, CSIR and BactoSafe

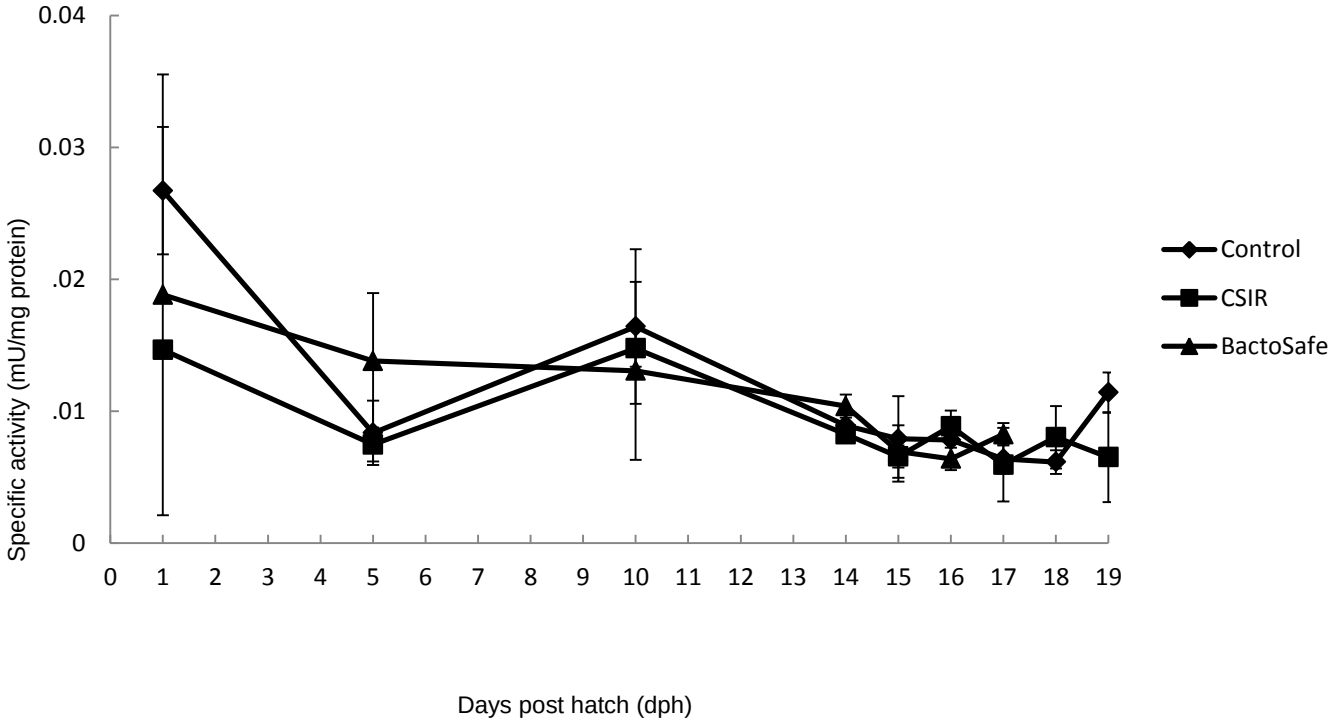

Figure 16 The specific activity (mU/mg protein) of amylase with error bars depicting standard deviation (n=3)

Although not statistically compared, the trends observed between the control and the two treatments are similar with higher specific activity levels being detected on 1 dph and a gradual decrease in activity until the end of the study. The control and CSIR treatment show some fluctuation of activity from 5 dph until the end of the study. There are no significant differences in specific enzyme activity of amylase between the control and two treatments. The lack of significance between the control and treatments suggests the probiotics do not have a significant effect on the specific amylase activity.

Figure , shows the results obtained for the specific Trypsin activity (U/mg protein) of the control, CSIR and BactoSafe treatments respectively.

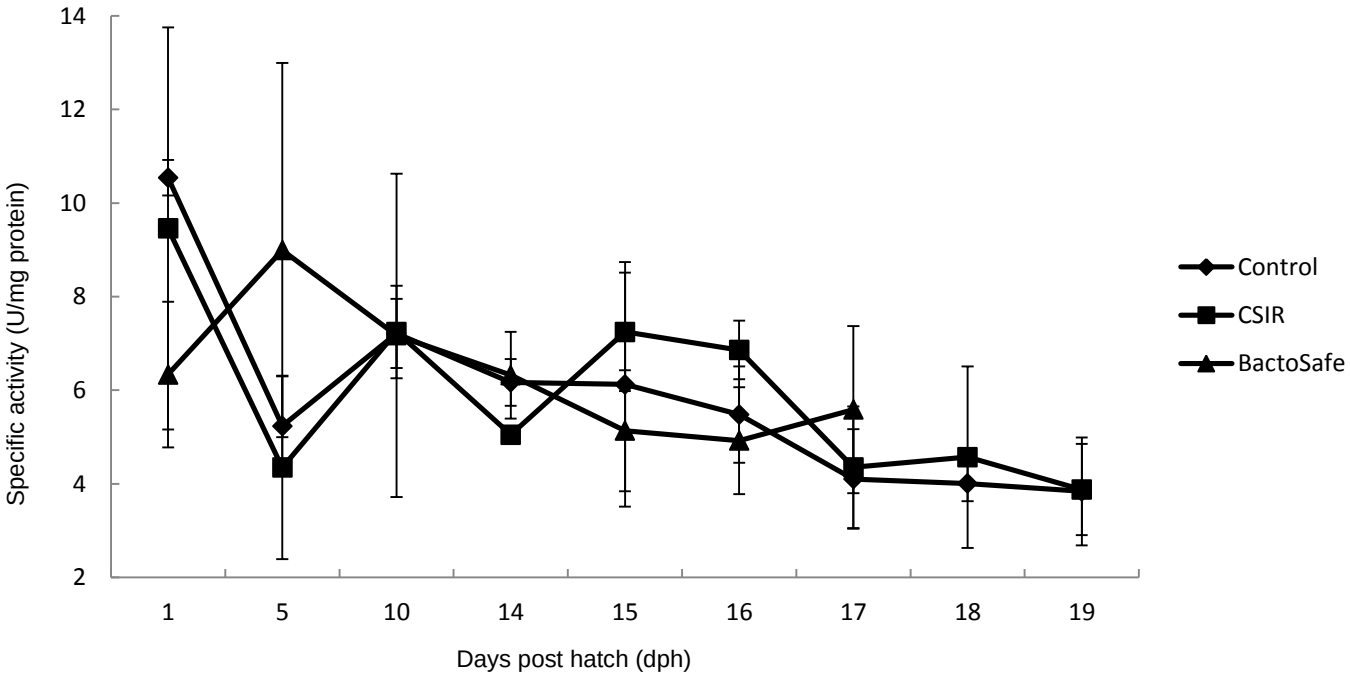

Figure 17 The specific Trypsin activity (U/mg protein) for the control, CSIR and BactoSafe treatments over time (dph) with error bars depicting standard deviation (n=3)

The specific trypsin activity for the control shows an initial reading on 1 dph and then decreases on 5 dhp. After 5 dph the activity increases slightly and gradually decreases again until the end of the study. For the CSIR treatment, a similar tend can be observed as that seen with the control. With the CSIR group however, a slight increase in activity can be seen on 15 dph with a gradual decrease in activity until 19 dph. The BactoSafe treatment shows a lower initial activity with an increase in activity occurring on 5 dph. The activity then gradually decreases until 16 dph with a slight increase in activity on 17 dph. There is no significant difference between the control and two treatments suggesting that the treatments have no significant effect on the specific trypsin activity.

Figure 18 illustrates the results from the specific enzyme activity assay for pepsin. Error bars depicting standard deviation are also presented.

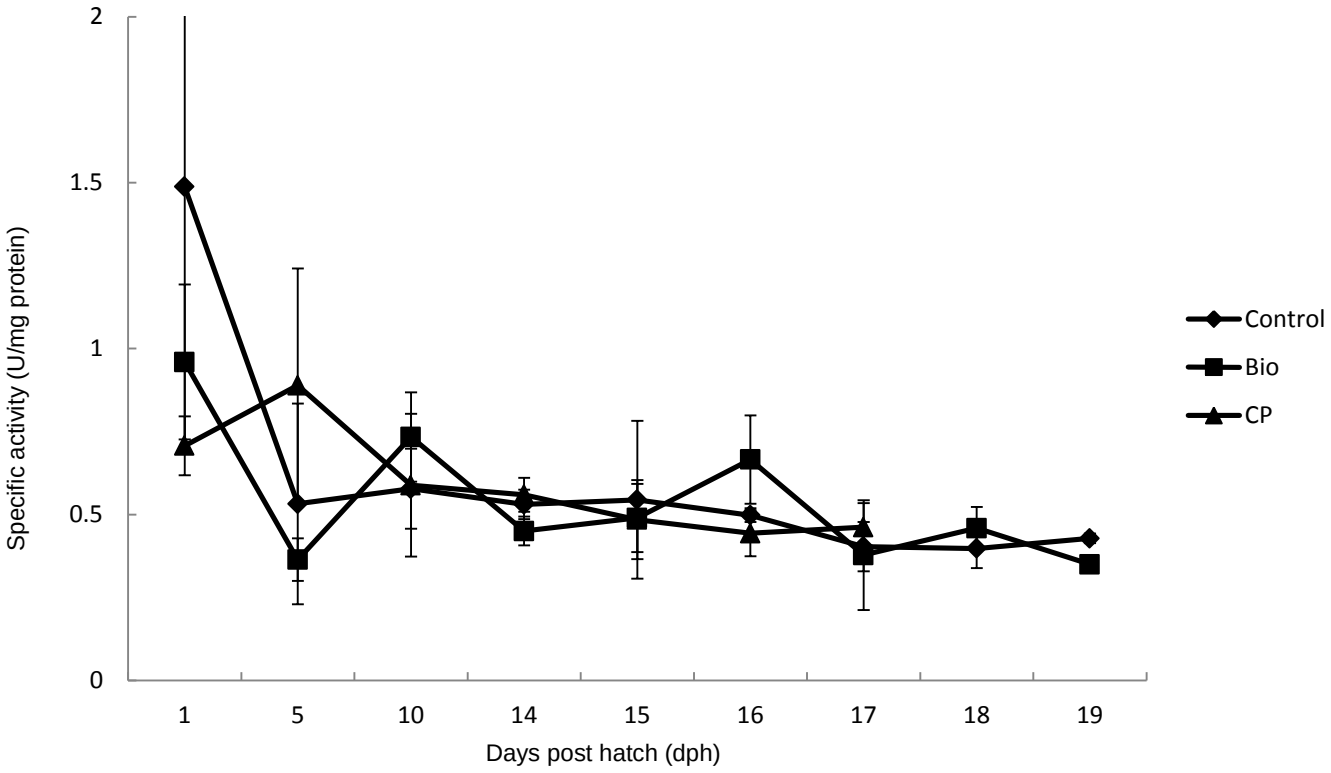

Figure 18 The specific pepsin activity (U/mg protein) of the control, CSIR and BactoSafe treatments over time (dph) with error bars depicting standard deviation (n=3)

Both the control and CSIR treatment showed relatively high pepsin activity levels on 1 dph (between 1 and 1.5 U/mg protein). While the control gradually decreased over the study period, the CSIR treatment showed a decrease in activity on 5 dph with an increase on 10 dph. The activity decreased slightly before showing another increase on 16 dph. The specific activity fluctuated slightly until the end of the study. The specific activity of the larvae exposed to the BactoSafe treatment showed a lower initial activity then the control and CSIR treatment. A slight increase in activity was observed on 5 dph with a gradual decrease until 17 dph. There is a significant difference between specific pepsin activity of the control and CSIR treatment on 19 dph ($P = 0.016$). The specific pepsin activity of the control is significantly higher than that of the CSIR treatment. No significant differences exist between the control and two treatments for the other days during the study. The results suggest that the neither of the treatments have a significant overall effect on the specific pepsin activity of developing larvae.

Figure shows the results from the assay for the specific alkaline phosphatase activity for larvae exposed to the control and two treatments. The standard deviation of the means of the control and treatments is illustrated by the errors bars on the graphs.

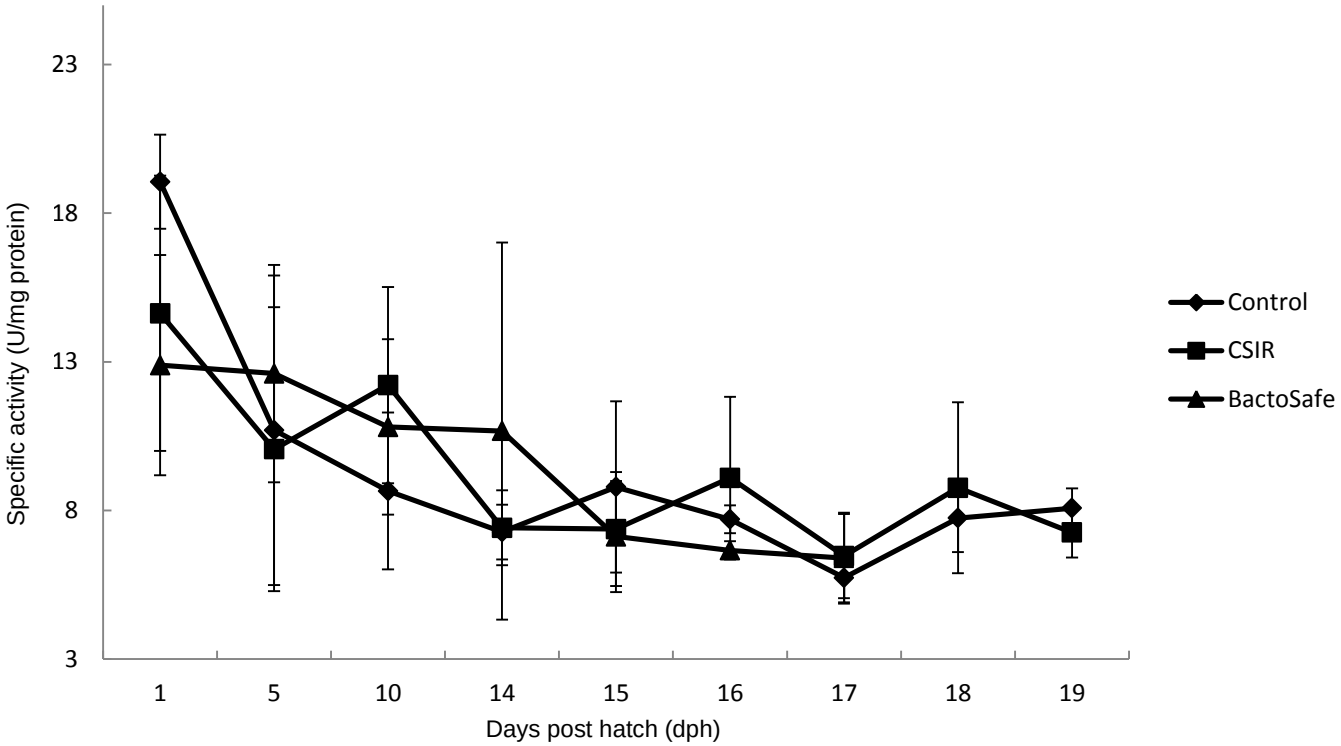

Figure 19 The specific alkaline phosphatase activity (U/mg protein) of the control, CSIR and BactoSafe treatments over time (dph) with error bars depicting standard deviation (n=3)

The control and both treatments show similar initial alkaline phosphatase activities on 1 dph. The control and the BactoSafe treatment show a gradual decrease in activity for the duration of the study. The control shows some fluctuation from 14 dph until 19 dph. The CSIR treatment shows a fluctuation in enzyme activity throughout the duration of the study with the enzyme activity reaching its lowest point on 19 dph. No statistical difference between the control and two treatments is observed, which suggests that there is no significant effect of probiotics on the specific alkaline phosphatase activity of larvae from 1 dph until 19 dph.

The results for the specific aminopeptidase N activities for the control and two treatments are represented in Figure . Error bars depicting standard deviation of the means have been included in the graphs.

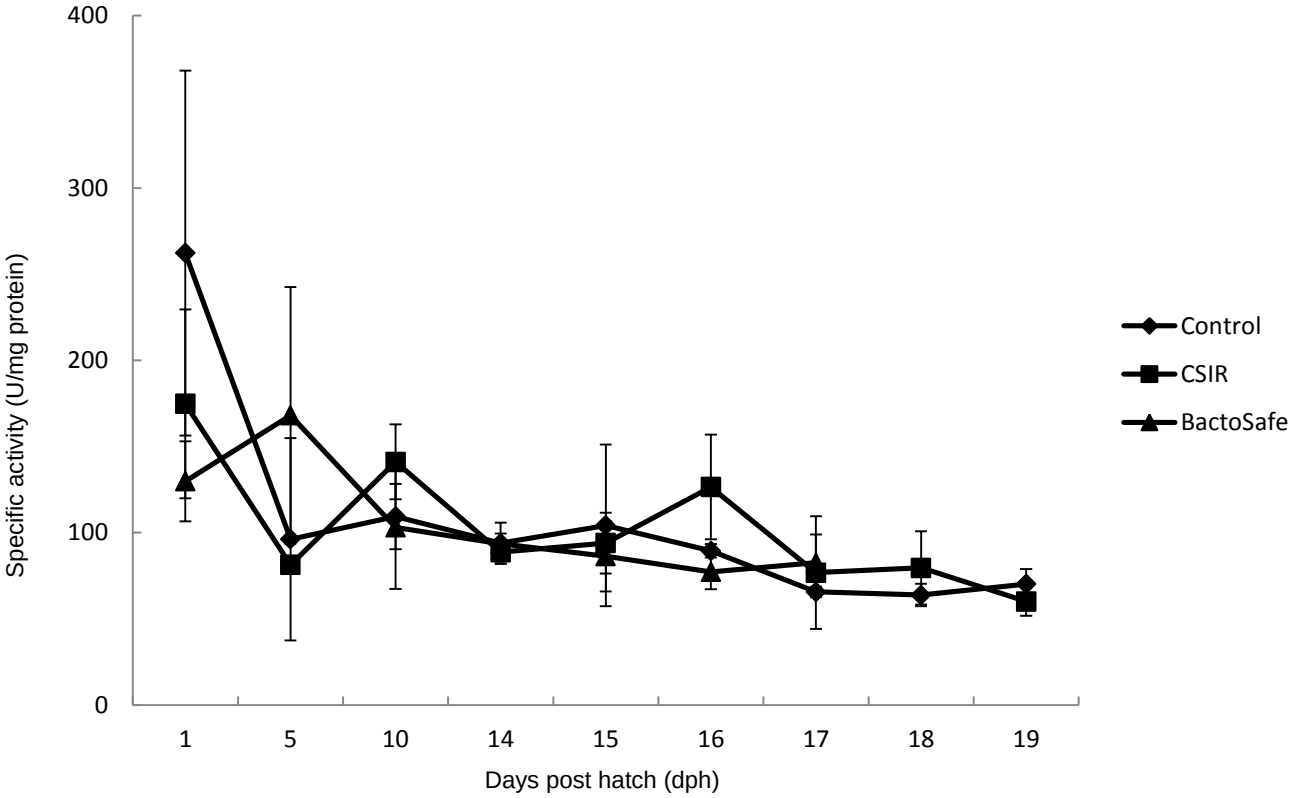

Figure 17 The specific aminopeptidase N activity (U/mg protein) of the control, CSIR and BactoSafe treatments over time (dph) with error bars depicting standard deviation (n=3)

The results for the specific aminopeptidase N specific activity of the control show a high initial activity followed by a sharp decline on 5 dph. The activity then fluctuates around the 100 U.mg protein mark until 19 dph. The results for the CSIR treatment show a similar trend with the initial activity being relatively high with a decrease in activity at 5 dph. The specific activity also fluctuated for the remainder of the study. However, the fluctuation in activity is slightly more pronounced than that of the control. With regards to the BactoSafe treatment, the initial specific activity is relatively low and shows a slight increase in activity at 5 dph. The activity then decreases at 10 dph and continues to show a slight decreasing trend until 17 dph. Similar to the other enzymes, no significant difference between the means of the control and the two treatments was observed. It can be suggested that there is no significant effect of the treatments on the specific aminopeptidase N activity of larvae during the first few weeks of development.

The specific leucine-alanine peptidase activity was determined and the results of the assay are shown in Figure 18. The standard deviation is shown as error bars in each of the graphs.

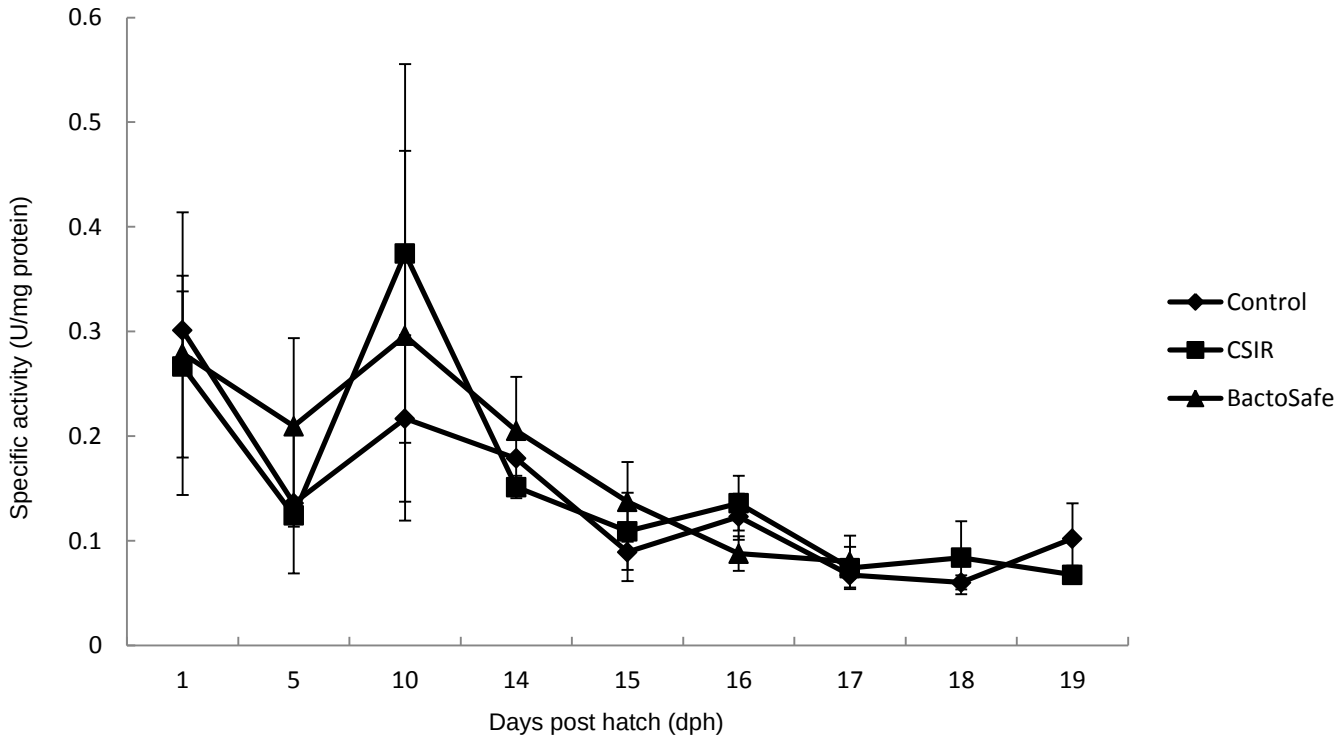

Figure 18 The specific leucine-alanine peptidase activity (U/mg protein) of the control over time (dph) with error bars depicting standard deviation (n=3)

For the control and both treatments, the initial specific enzyme activity was very similar, sitting at approximately 0.3 U/mg protein. Both the control and CSIR treatment showed a decrease in activity at 5 dph with an increase at 10 dph. The increase at 10 dph appeared to be greater than that of the control however did not show a significant difference. Yet after this point, the specific activity of both the control and CSIR treatment showed a very similar pattern until the end of the study at 19 dph. The BactoSafe treatment also showed a decrease in activity at 5 dph with a slight increase at 10 dph. After 10 dph, the activity shows a gradual decrease until 17 dph, by which stage the activity has seemed to reach a constant level at around 0.1 U/mg protein. No significant difference exists between the specific enzyme activity of the control and two treatments. This again suggests that probiotics do not have a significant effect on the specific leucine-alanine peptidase activity of *A. japonicus* larvae.

Discussion

The use of probiotics in animal production has gained increasing popularity due to a greater awareness in the general public of the negative implications associated with the widespread use of antibiotics (Verschuere et al., 2000). Probiotics have been shown to provide a variety of benefits to chickens, pigs, goats, cattle and horses, including the improvement of growth, performance and disease resistance to pathogens such as *Vibrio* spp. (Musa et al., 2009). A wide variety of probiotics have been considered as potential candidates for use in aquaculture. These include species within the *Bacillus* genus, *Lactobacillus* genus and *Pseudomonas* genus (Irianto and Austin, 2002). Both of the treatments administered during this study were primarily *Bacillus* based probiotics. Probiotics have been shown to have a variety of mechanisms in which they affect the host organism. These mechanisms include the competitive exclusion of pathogenic bacteria (Vine et al., 2006).

Probiotics also provide a source of nutrients and some contribution to the digestive enzyme assemblage of their host (Balcázar et al., 2006) and the potential to improve the host's immune capabilities against pathogens (Vine et al., 2006). The BactoSafe comprised of four different probiotic species, namely *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilus* and *Pediococcus acidilactici*. The CSIR product also contained a mixture of *Bacillus* species but the exact composition of the product was not disclosed. *Bacillus cereus* has been shown to have a fairly long persistence in the gastrointestinal tract of humans (Duc et al., 2004). In silver catfish (*Rhamdia quelen*), the addition of *B. cereus* showed immunogenic properties where an inhibitory effect against *Vibrio carchariae* was observed; *V. carchariae* is a pathogen that causes gastroenteritis in fish. *B. licheniformis* and *B. subtilus* have been linked to water quality improvement in larval rearing systems and, when administered together, do not display any antagonistic effects towards each other. Studies conducted on sea bream and rainbow trout (*Onchorynchus mykiss*) revealed that *B. cereus* and *Lactobacillus* spp. are linked to the decrease in the number of Glucocorticoid Receptors (GR). GR's are transcription factors that are induced by the presence and binding of ligands. This ligand-induced mechanism either activates or suppresses the expression of certain target genes. Glucocorticoid-mediated gene expression has an effect on a whole host of physiological mechanisms. These mechanisms include metabolism, immunity, behaviour and osmoregulation. Cortisol is produced and released when this glucocorticoid expression mechanism is activated. Cortisol functions as an immuno-stimulant. By measuring the concentration of cortisol, one can determine the amount of stress experience by the organism. A decrease in GR's indicate an increase in fish health (Avella et al., 2010).

Pediococcus acidilactici, which is present in BactoSafe, has been shown to improve larval growth, development, FCR and survival of *O. mykiss* (Merrifield et al., 2011).

During this study the notochord length of the larvae was measured from 1 dph to 10 dph due to the caudual fin of the larvae not being fully developed at this stage making it is very difficult to accurately measure total length. Total length is measured from the tip of the nose to the end of the tail. The results show an increasing trend in size over time for the control and two probiotic treatments with significant differences between the means of the control and two treatment groups existing on 15 dph, 16 dph, 18 dph and 19 dph (Figure 8). Studies performed on sea bass (Avella et al., 2010), silver catfish (Souza et al., 2012), shrimp (*Penaeus vannamei*) (Wang, 2007) and gilthead seabream (Suzer et al., 2008) resulted in a significant increase in the average growth of the larvae that were treated with probiotics in comparison with the control group. It has been suggested that the dose of the probiotic that is administered is the limiting factor when wanting to achieve the optimal benefits from the probiotic (Souza et al., 2012). The slightly lower average growth for the two probiotic treatments during this trial could therefore be a result of the dose being the limiting factor. The addition of the probiotics could have increased the overall bacterial load in the system, resulting in a bacterial load that would have been detrimental to larval health and welfare. This could have resulted in a slightly poorer growth performance in the treatment groups as opposed to the control group. Since this outcome was not expected the bacterial load before and after the study was not determined, it is thus recommended that further studies should include this parameter. In a study conducted with sea bream, it was found that the bio-encapsulation of the probiotic within the live feed reduced the overall bacterial load (Avella et al., 2010). In this study, probiotics were administered directly into the tank water as well as being bio-encapsulated in the live food (i.e. rotifers and *Artemia*). This was done to ensure that the larvae received the optimal exposure to the probiotics. However, this approach could also have resulted in an increase in the overall bacterial load to levels that could have inhibited the beneficial effect of the probiotic dose on the larvae but since the initial and final bacterial loads in the systems were not determined, this cannot be confirmed.

The recent introduction of Dusky kob into commercial aquaculture has not allowed for domestication and adaptation to intensive aquaculture conditions with broodstock predominantly obtained from wild populations. Domestication can be defined as adaptation and improvement of desired traits through natural and artificial selection over continuous generations through controlled breeding (Bilio, 2008). The undomesticated status of dusky kob and the consequent high levels of genetic diversity amongst commercial populations result in varied growth rates and development among individual fish (personal

communication from. Professor Danie Brink, Stellenbosch University). This naturally occurring variation contributed to the high standard deviation that has been observed between individuals within each of the treatment groups when studying the growth of the larvae over time. The standard deviation within each of the treatment groups on 1 dph and 5 dph remained low as mouth opening only occurs on 2-3 dph (Musson and Kaiser, 2014). The larvae are entirely reliant on their oil globules as a source of nutrients up until this point. Larvae from a particular spawning event and homogeneous egg quality are expected to display fairly similar growth rates during these early developmental stages based on the similar source of nutrition. Once mouth opening occurs, larvae would have to actively seek out their prey at which stage various factors could potentially affect the success of the larvae obtaining sufficient food. These factors include the availability of food such as live feed or formulated diets, as well as genetic factors including swimming ability, behavioural differences and mouth gap width (Duray et al., 1996). Larvae used in this study are expected to display large genetic diversity due the undomesticated status of the wild parent stock, which would start to affect individual growth and development particularly onwards from the first feeding stages. From 14 dph, the standard deviation within each treatment group was fairly high. The feeding regime within the hatchery may also have contributed to this as it was altered by adding *Artemia* nauplii earlier than the standard operating procedure dictated. The reason for this being that the rotifer cultures that were available for the early stage larvae had collapsed due to high ciliate loading and high total ammonia nitrate (TAN) levels. By 4 dph, no rotifers were available for the larvae; therefore *Artemia* nauplii had to be added. *Artemia* have a hard chitinous exoskeleton that can be difficult for early stage larvae to digest. This is why rotifers are utilized in the early larval stages as they do not have a chitinous exoskeleton making the digestion and assimilation of nutrients more efficient.

The histological study provides insight into the physiological development of the digestive tract of the early larval development stages at a cellular level. Key developmental milestones occur during the development of the larval digestive tract from a primitive and rudimentary mode of digestion to a more adult and complex mode of digestion. The first key developmental mile stone is the transition from an endogenous mode of feeding to an exogenous mode of feeding (Zambonino and Cahu, 2001). This transition occurs when the mouth of the larvae open. At this stage the digestive tract is exposed to the microbial flora present in the environment and would be the moment when microbes would have the first opportunity to colonize the digestive tract (Schulze et al., 2006). It would be at this stage that the probiotics in the water would colonize the digestive tract of the larvae in the two treatment groups.

On 1 dph, the intestine in dusky kob larvae is a straight, undifferentiated tube but by 4 dph, when exogenous feeding begins, the digestive tract is differentiated into the buccopharyngeal cavity, oesophagus, intestine and hindgut (Musson and Kaiser, 2014). On 5 dph, supranuclear vacuoles were observed in the hindgut of the larvae of the control and the BactoSafe and CSIR treatment groups (Figure 10), which indicates that the larvae have the ability to metabolize intracellular proteins (Watanabe, 1982). In previous studies conducted on dusky kob (Musson and Kaiser, 2014), the larvae showed a decrease of supranuclear vacuoles from 9 to 11 dph as the brush border membrane developed and the gastric glands in the stomach developed. However, the presence of supranuclear vacuoles in the control and two probiotic treatment groups remained constant until the end of the study (19 dph). Mucous cells also only became apparent in the midgut around 15 dph while in a previous study of dusky kob indicated the presence of mucous cells in the midgut from 9 dph (Musson and Kaiser, 2014). Goblet cells only began to appear in the midgut in the control and the BactoSafe and CSIR treatment groups from 15 dph while in previous studies of dusky kob where no probiotics were added, they began to appear from 9 dph (Musson and Kaiser, 2014). This suggests that there is a delay in the development of the intestine in this study and that the addition of probiotics has no observable effect on the development of the intestinal tract at a cellular level. A delay in development of the stomach was also observed. The gastric glands in the stomach only began to appear around 17 dph in the control and the BactoSafe and CSIR treatments groups, as opposed to 9 dph in previous dusky kob studies where no probiotics were added (Musson and Kaiser, 2014) and 16 dph in white sea bass (Galaviz et al., 2011).

The pancreas is one of the first organs observed during the development of the digestive tract that exhibits an endocrine function. The pancreas in *A. japonicus* was visible as early as 1 dph in a previous study done on *A. japonicus* by Musson and Kaizer. The pancreas had developed from a small cluster of undifferentiated calls that was positioned behind the yolk sac. It can be assumed that the larvae in this study would have shown a similar developmental pattern as the study conducted by Musson and Kaizer. The pancreas of the larvae was visible on 5 dph for the control and two treatment groups. In previous studies conducted on dusky kob (Musson and Kaiser, 2014), a well-developed pancreas was observed from 4 dph while in white sea bass, the well-developed pancreas was observed from 3 dph (Galaviz et al., 2011). As the larvae developed, the concentration of zymogen granules increased. This increase in zymogen granules in the developing pancreas highlights the importance of the pancreas secretory function during the agastric stage of development (Ribeiro et al., 1999). The pancreas is responsible for the secretion of protease enzymes, such as trypsin, to enable the developing larvae to digest proteins without the

presence of other proteases secreted by the gastric glands in the stomach (Beccaria et al., 1991). The probiotics populate the intestinal wall and would therefore not necessarily have a direct effect on the development of the pancreas.

The suggested delay in development of key aspects of digestive tract could be attributed to the change in feeding regime. Rotifer cultures had crashed after 6 dph and the larvae were fed *Artemia* from day 5 as opposed to the standard regime where they would have only started feeding on *Artemia* from day 10. *Artemia* possess a hard exoskeleton made from chitin and this chitinous layer is difficult for larvae to digest with their simple digestive tract in the early stages. The difficulties in digesting the chitinous exoskeleton would have resulted in the larvae not being able to acquire sufficient nutrients to allow for proper development. Daily fluctuations in the oxygen levels that were observed in the commercial hatchery (Figure 4, Figure 5 and Figure 6) would also have resulted in physiological stress experience by the larvae (Avella et al., 2010). Such physiological stress could have resulted in the delay of the development of the digestive tract. With regards to the effect of probiotics on the digestive development of the larvae, it is difficult to conclude with conviction that the probiotics had no effect on development because the microbial flora of the digestive tract was never determined during this study. By categorizing the intestinal microflora of the larvae, it could be determined whether the desired strains had colonized in the digestive tract or not. However, since no difference was observed between the development of the digestive tract of the control and two treatments, it can be assumed that if the probiotics had established that they had no effect on the physiological development of dusky kob larvae.

Early stage fish larvae have been shown to possess an assemblage of digestive enzymes, essential for the utilization of nutrients from their yolk globule during endogenous feeding and from live food during exogenous feeding (Zambonino and Cahu, 2007). The use of probiotics in larval rearing has been motivated by the ability of probiotic bacteria to enhance digestive enzyme development by contributing their own digestive enzymes to the digestive enzyme assemblage of the host organism (Vine et al., 2006). Key digestive enzymes were chosen as the focus of this study and to determine whether the addition of probiotics enhances the digestive capability of dusky kob larvae.

Amylase is a key digestive enzyme responsible for the digestion of carbohydrates into simple sugars. Amylase has been detected in larvae from 1 dph in a variety of species, including large yellow croaker (Ma et al., 2005), meagre (Suzer et al., 2012), red drum (Lazo et al., 2007), shi drum (Parillo et al., 2004) and white sea bream (Cara et al., 2003). Amylase is present in both carnivorous and herbivorous fish, and is genetically modulated and not

induced by the presence of food (Ma et al., 2005). On 1 dph, the specific amylase activity was fairly high for the control and two probiotic treatments, which confirms the theory that amylase secretion is not induced by the presence of food. In this study, the specific amylase activity shows a slight increase on 10 dph. This increase could be explained by the availability of formulated feed, which contains a 30% carbohydrate component. The decrease in specific activity after this point may not necessarily indicate a decrease in activity (Ma et al., 2005). Since whole body homogenates were used, the protein component increases as the larvae increase in size. Specific activity is the ration of enzyme units per mg of protein and therefore the specific activity will appear to decrease as the larvae get larger in size. No significant differences were observed between the control and two probiotic treatments. Again, it is difficult to conclude with certainty that probiotics do not have an effect on larval digestive development since it was never determined whether the probiotic strains did, in fact, establish in the digestive tract. The trend observed in other similar species such as large yellow croaker (Ma et al., 2005) and meagre (Suzer et al., 2012) show a similar trend with the activity being relatively high on 1 dph with a slight decrease in activity on 5 dph and a sharp increase in activity on 10 dph. The activity in larvae yellow croaker shows a trend very similar to dusky kob but the mU/mg protein values are substantially higher than those recorded for dusky kob larvae.

Trypsin is secreted by the pancreas in developing larvae and is detected from as early as 1 dph in a variety of species. These species include large yellow croaker, white sea bream, gilthead sea bream and red drum. In a previous study, the pancreas was first seen from 1 dph in dusky kob larvae (Musson and Kaiser, 2014) which explains the presence of trypsin from the moment of hatching. As the pancreas develops with increase in concentration in zymogen granules, so does the enzyme activity of trypsin. However, the results from this study show a gradual decrease in activity during the course of the study. Other species such as large yellow croaker and meagre show an increase in activity from 1 dph until 15 dph with the activity declining as the larvae develop. Again this decline in activity may not represent a loss in activity but an increase in the protein content of the larvae as discussed earlier.

Pepsin activity is associated with the appearance of gastric glands in the stomach. The secretion of pepsin indicated that the stomach is fully formed and the larvae are able to fully digest proteins. No studies have detected the presence of pepsin in the first few days of life. In meagre, pepsin was first detected on 15 dph; in sharp snout sea bream, pepsin was first detected on 30 dph; and in common pandora (*Pagellus erythrinus*), pepsin was first detected on 20 dph. In this study, pepsin was detected from 1 dph however; the gastric glands were

first visible on 17 dph which would mean that pepsin would only be detected on 17 dph. The detection of pepsin so early on in the study could indicate that there was contamination from another source or that the assay used to determine the pepsin activity was not optimal for dusky kob larvae and that the assay was giving false positive results. Hemoglobin was used as the substrate and is subject to hydrolyzation by general protease activity (Lazo et al., 2007). Since trypsin is considered a protease, and was detected from 1 dph, and a whole host of other acid proteases may exist in the homogenate, the non-specific assay could be detecting products released by general protease activity. A more specific substrate would have to be found in order to determine specific pepsin activity.

Alkaline phosphatase is an enzyme that is specific to the brush border membrane in the intestine of larvae (Zambonino and Cahu, 2007). In the early stages, before the maturation of the intestinal tract, alkaline phosphatase is produced in the Golgi apparatus of the enterocytes. Alkaline phosphatase is also considered an indicator of the maturation of the enterocytes. As the enterocytes develop and mature, the alkaline phosphatase activity increases (Suzer et al., 2007). In meagre and large yellow croaker, the alkaline phosphatase activity increases over time, indicating the maturation of the brush border of the intestine. In this study, the alkaline phosphatase activity starts off relatively high and gradually decreases over time. This same trend was observed for the control and two probiotic treatments. This same trend can be seen for the Aminopeptidase N activity for dusky kob larvae. Aminopeptidase N is also a brush border membrane enzyme and is also indicative of enterocyte maturation. Previous studies have shown high levels of Aminopeptidase N throughout the larval stage (Suzer et al., 2012). The activity of alkaline phosphatase and Aminopeptidase N is coupled with the activity of leucine-alanine peptidase. Leucine-alanine peptidase is a cytosolic intestinal enzyme. The presence of leucine-alanine peptidase in the early stages of larval development suggests that it is genetically modulated and not initially activated by the presence of food (Zambonino and Cahu, 2001). Many studies have reported that the activity of leucine-alanine peptidase is high in the initial development of larvae and gradually decreases as the brush border membrane enzyme activities increase. The decrease of leucine-alanine peptidase activity is an indication of the maturation of the intestine of larvae as the brush border membrane enzymes begin to take over protease function (Suzer et al., 2012). In this study, the specific activities for leucine-alanine peptidase show the same trend that was observed for all the other enzymes that were assayed. The general trend for leucine-alanine peptidase does, however, follow the trend described in literature for other scianidae species, including meagre (Suzer et al., 2012) and large yellow croaker (Ma et al., 2005). Again there was no significant difference of the specific enzyme activity between the control and two treatments. The results indicate that the addition of

probiotics may not have an effect on the specific enzyme activity of leucine-alanine peptidase. As discussed before, it is difficult to confirm that the probiotics had no effect since no testing was performed to ascertain whether the probiotics had colonized the digestive tract or not.

A similar trend was observed for all enzymes assayed in this study. According to the literature that is available for the specific activity of all the enzymes assayed for a variety of fish species, each enzyme would produce its own unique trend that is directly linked to the development of the digestive tract. The fact that the same trend was observed in this study suggests that the enzyme assays might not have been successfully implemented for the selected enzymes. The possible explanations for the unsuccessful assays include a loss of enzyme activity in the larval homogenates due to storage or the homogenization process itself, as well as the enzyme assays not having been optimized for dusky kob larvae.

Enzyme activity may also have been lost during the storage of the larvae before tissue processing. Enzymes are sensitive to high temperatures and mechanical degradation (Voet et al., 2013). Studies have shown that a fast freezing and slow thawing method results in severe degradation of proteins if cryoprotectants have not been used (Cao et al., 2003). Fast freezing has been described as a change in temperature that is greater than -20°C per minute. The larval samples would have been exposed to a freezing rate such as this since they were stored in liquid nitrogen which is -192°C. The long term storage of samples at this low temperature can result in a decrease in enzyme activity due to the denaturing of the enzymes (Gilliland and Lara, 1988). The larval samples were stored in liquid nitrogen for one month before they were transferred into the -80°C freezer. No literature is available to suggest the effect of storage time in liquid nitrogen on the viability of digestive enzymes. A common cryoprotectant that is utilized to maintain cellular integrity during freezing is glycerol (Diener et al., 1993). Glycerol was not used in this study as it acts as an inhibitor for amylase activity (Darias et al., 2006). The homogenization process could also have resulted in the loss of enzyme activity. The homogenization process entailed the mechanical disruption of the larval tissue using stainless steel ball bearings. A low temperature could not be maintained during the homogenization process due to heat being generated by friction as the steel ball bearings collided with each other. Voet et.al. (2006) describe how increasing temperatures result in the denaturation of proteins. Since most enzymes are proteins (including the enzymes assayed in this study), an increase in temperature will result in structural changes of the enzyme and therefore resulting in a reduction in enzyme activity. The combination of heat and mechanical disruption could have compromised the integrity of the enzyme structure. A freeze-thaw cycle also took place during the homogenization

process as the whole larvae, which had been stored at -80°C, had to be thawed in order to homogenize the tissue. After homogenization and centrifugation, the supernatants were alloquotted into smaller volumes and placed back into the -80°C freezer. This freeze thaw cycle could have resulted in a decrease in enzyme activity as the enzyme structure could have been degraded (Pearce et al., 1996).

The protocols that were followed for each of the enzyme assays were not specifically adapted for dusky kob enzymes. An online information system called BRENDA (www.brenda-enzymes.org) is one of the most comprehensive enzyme repositories currently available. BRENDA was consulted when information was required for a variety of factors specific to particular enzymes. Amylase, for example, has a wide range of optimum pH conditions that are specific to different species. The optimum pH for each of the enzymes specific to dusky kob would have to be determined. The possibility exists that compounds were present in the homogenates acted as inhibitors of some of the enzymes. For example, in yellowfin tuna (*Thunnus albacores*), an increase in the concentration of sodium chloride results in a decrease in trypsin activity by up to 30% (Klomklao et al., 2006). Some amino acid sterioisomers may act as inhibitors as in the inhibitory effect of poly L-lysine on pepsin in Atlantic salmon (Fruton, 1971). Urea has been shown to inhibit alkaline phosphatase activity in Atlantic cod (Ásgeirsson and Guðjónsdóttir, 2006). It is also important to identify any cofactors that enzymes require for optimal enzyme activity and to ensure that those cofactors are available in the reaction mixture. Aminopeptidase N in red sea bream, for example requires the presence of Zn^{2+} as a cofactor (Wu et al., 2008). Optimal incubation temperature would also have to be determined. The optimal temperature for many enzymes differed from species to species. With regards to aminopeptidase N, for example, the optimal temperature for Atlantic cod is 47°C (Kvåle et al., 2007) and 45°C for red sea bream (Wu et al., 2008). For gilthead sea bream, the optimal temperature for aminopeptidase N activity is 37°C (Dópido et al., 2004). The incubation temperature that was used for the dusky kob aminopeptidase assay was room temperature (25°C), which was automatically controlled by air conditioning units. The ambient temperature was constantly monitored by digital thermometers that were placed in the work space where the assay was performed. This temperature may not be the optimal temperature for this species and would therefore have to be verified. The buffers that were used for each assay would also have to be tested to determine their effect on enzyme activity. Tris-HCl (pH 8) was used as the buffer for the homogenate solution. In humans, Cl^- acts as an inhibitor of amylase activity (Rauscher et al., 1991) and could therefore also act as in inhibitor for amylase in dusky kob. This demonstrates the importance of understanding the characteristics and optimal conditions required for each enzyme that is being assayed.

The assays, however, did pick up some enzyme activity which suggests that total activity was not lost. With optimization of the enzyme assays and the careful storage and processing of the samples, a more definitive description of the enzyme activity of dusky kob digestive enzymes would be produced. In future studies where the effects of probiotic are to be studied in aquaculture, the characterization of the existing microflora needs to be conducted. By comparing the intestinal microflora of the treatment groups with that of the control group, the effective colonization of the digestive tract by the probiotics can be determined. Only once this has been done can one describe with confidence the effect that probiotics have on the physiological and biochemical development of dusky kob larvae.

Conclusion

The aim of the study was to determine the effects of probiotics on the physiological and biochemical development of commercially raised dusky kob larvae. The results for the growth study indicated a significant difference in the mean body length between the control and the two probiotic treatments; the mean body length of the control was higher than that of the two treatment groups. The possible explanation for the slightly higher average growth of the control is the increased bacterial load of the two treatment groups as a result of adding more bacteria (the probiotics) to the system. Probiotic dose has been suggested to be a limiting factor for achieving optimal benefits of the probiotic, and by overloading the system, the larvae may have experienced detrimental effects. These detrimental effects could have manifested themselves in the reduced growth of the larvae. As for the histological study, a slight delay in development was observed in comparison to previous studies conducted on dusky kob. This slight delay in development could be attributed to the change in feeding regime where *Artemia* were fed early on in the larval development. *Artemia* are more difficult for larvae to digest and therefore the larvae do not acquire the optimal amount of nutrients to ensure optimal development. The specific enzyme analysis of the key digestive enzymes showed little difference between the control and treatment groups as well as between the different assays themselves. The enzyme assays for dusky kob larvae need to be refined by determining the optimal reaction conditions to ensure an accurate representation of the enzymatic development of dusky kob larvae. In terms of determining the effects of probiotics on the digestive development of dusky kob larvae, it is difficult to draw a confident conclusion from this study as to whether they enhance or delay digestive development. The study did not include a test to determine whether the probiotics had established in the digestive tract of the larvae. Therefore, any results obtained from the study could be influenced by a variety of biophysical and chemical factors. However, the study of the larval development in dusky kob is in its very early stages. No previous studies have been conducted on the biochemical development of dusky kob and this study serves as one of the initial attempts to describe the enzymatic development of the digestive tract of dusky kob larvae. Further studies should be conducted to optimise the rearing conditions of dusky kob larvae and to refine the protocols that are to be applied to accurately describe the biochemical development.

References

Anson, M. L. 1938. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. Journal of General Physiology. 22(1): 79-89.

Ásgeirsson, B., and Guðjónsdóttir K. 2006. Reversible inactivation of alkaline phosphatase from atlantic cod (*Gadus morhua*) in urea. Biochimica Et Biophysica Acta - Proteins and Proteomics. 1764(2): 190-198.

Avella, M. A., Gioacchini G., Decamp O., Makridis P., Bracciatelli C., and Carnevali O. 2010. Application of multi-species of *Bacillus* in sea bream larviculture. Aquaculture. 305(1): 12-19.

Balcázar, J. L., Blas I. d., Ruiz-Zarzuela I., Cunningham D., Vendrell D., and Múzquiz J. L. 2006. The role of probiotics in aquaculture. Veterinary Microbiology. 114(3): 173-186.

Ballagh, D., Pankhurst P., and Fielder D. 2008. Photoperiod and feeding interval requirements of juvenile mulloway, *Argyrosomus japonicus*. Aquaculture. 277(1): 52-57.

Beccaria, C., Diaz J., Connes R., and Chatain B. 1991. Organogenesis of the exocrine pancreas in the sea bass, *Dicentrarchus labrax*., reared extensively and intensively. Aquaculture. 99(3): 339-354.

Bessey, O., Lowry O., and Brock M. 1946. Rapid coloric method for the determination of alkaline phosphatase in five cubic millilitres of serum. Journal of Biological Chemistry. 164: 321-329.

Bilio, M. 2008. Controlled reproduction and domestication in aquaculture. European Aquaculture Society, Oostende.

Bisbal, G., and Bengtson D. 1995. Development of the digestive tract in larval summer flounder. Journal of Fish Biology. 47(2): 277-291.

Boulhic, M., and Gabaudan J. 1992. Histological study of the organogenesis of the digestive system and swim bladder of the dover sole, *Solea solea* (Linnaeus 1758). Aquaculture. 102(4): 373-396.

Bradford, A. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical. Biochemistry. 72(7): 248-254.

Buddington, R. K., and Diamond J. M. 1987. Pyloric ceca of fish: A" new" absorptive organ. American Journal of Physiology-Gastrointestinal and Liver Physiology. 252(1): G65-G76.

Cao, E., Chen Y., Cui Z., and Foster P. R. 2003. Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. Biotechnology and Bioengineering. 82(6): 684-690.

Cara, J., Moyano F., Cárdenas S., Fernández-Díaz C., and Yúfera M. 2003. Assessment of digestive enzyme activities during larval development of white bream. Journal of Fish Biology. 63(1): 48-58.

Chen, B. N., Qin J. G., Kumar M. S., Hutchinson W., and Clarke S. 2006. Ontogenetic development of the digestive system in yellowtail kingfish *Seriola lalandi* larvae. Aquaculture. 256(1): 489-501.

Collett, P. D., Vine N. G., Kaiser H., and Baxter J. 2008. Determination of the optimal water temperature for the culture of juvenile dusky kob *Argyrosomus japonicus* Temminck and Schlegel 1843. Aquaculture Research. 39(9): 979-985.

Collett, P., Kaiser H., and Vine N. 2011. The effect of crowding density on growth, food conversion ratio and survival of juvenile dusky kob *Argyrosomus japonicus* (teleostei: Sciaenidae). African Journal of Aquatic Science. 36(2): 155-158.

Darias, M., Murray H. M., Gallant J. W., Astola A., Douglas S. E., Yúfera M., and Martínez-Rodríguez G. 2006. Characterization of a partial α-amylase clone from red porgy (*Pagrus pagrus*): Expression during larval development. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 143(2): 209-218.

Diener, B., Utesch D., Beer N., Dürk H., and Oesch F. 1993. A method for the cryopreservation of liver parenchymal cells for studies of xenobiotics. Cryobiology. 30(2): 116-127.

Dópido, R., Rodríguez C., Gómez T., Acosta N. G., and Díaz M. 2004. Isolation and characterization of enterocytes along the intestinal tract of the gilthead seabream

(*Sparus aurata* L.). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 139(1): 21-31.

Duc, L. H., Hong H. A., Barbosa T. M., Henriques A. O., and Cutting S. M. 2004. Characterization of *Bacillus* probiotics available for human use. Applied Environmental Microbiology. 70(4): 2161-2171.

Duray, M. N., Estudillo C. B., and Alpasan L. G. 1996. The effect of background color and rotifer density on rotifer intake, growth and survival of the grouper (*Epinephelus suillus*) larvae. Aquaculture. 146(3): 217-224.

Edwards, P. 2000. Aquaculture, poverty impacts and livelihoods. Natural Resources Perspective. 55: 1-4..

Fielder, D. S., Ballagh D., Booth M. A., Pankhurst P. M., Becker C. E., and Allan G. L. 2010. Aquafin Crc Project 1b5: Feed Technology For Temperate Fish Species Volume 1: Feeding Strategies.

Fielder, D., and Bardsley W. 2007. A preliminary study on the effects of salinity on growth and survival of mulloway *Argyrosomus japonicus* larvae and juveniles. J. World Aquaculture Society. 30(3): 380-387.

Fruton, J. S. 1971. 4 pepsin. The Enzymes. 3: 119-164.

Galaviz, M. A., García-Gasca A., Drawbridge M., Álvarez-González C. A., and López L. M. 2011. Ontogeny of the digestive tract and enzymatic activity in white seabass,*Atractoscion nobilis*, larvae. Aquaculture. 318(1): 162-168.

Gil, M. D. M., Grau A., Basilone G., Ferreri R., and Palmer M. 2013. Reproductive strategy and fecundity of meagre *Argyrosomus regius* asso, 1801 (pisces: Sciaenidae): Implications for restocking programs. Scientia Marina. 77(1): 105-118.

Gildberg, A., Mikkelsen H., Sandaker E., and Ringø E. 1997. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of atlantic cod (*Gadus morhua*). Hydrobiologia. 352(1): 279-285.

Gilliland, S. E., and Lara R. C. 1988. Influence of storage at freezing and subsequent refrigeration temperatures on beta-galactosidase activity of lactobacillus acidophilus. Applied Environmental Microbiology. 54(4): 898-902.

Gisbert, E., Piedrahita R. H., and Conklin D. E. 2004. Ontogenetic development of the digestive system in California halibut (*Paralichthys californicus*) with notes on feeding practices. Aquaculture. 232(1): 455-470.

Gomez-Gil, B., Roque A., and Turnbull J. F. 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture. 191(1): 259-270.

Gordon, A., and Hecht T. 2002. Histological studies on the development of the digestive system of the clownfish *Amphiprion percula* and the time of weaning. Journal of Applied Ichthyology. 18(2): 113-117.

Govoni, J. J., Boehlert G. W., and Watanabe Y. 1986. The physiology of digestion in fish larvae. Environmental Biology of Fishes. 16(1-3): 59-77.

Griffiths, M. 1996. Life history of the dusky kob *Argyrosomus japonicus* (Sciaenidae) off the east coast of South Africa. South African Journal of Marine Science. 17(1): 135-154.

Griffiths, M., and Hecht T. 1995. Age and growth of south african dusky kob *Argyrosomus japonicus* (Sciaenidae) based on otoliths. South African Journal of Marine Science. 16(1): 119-128.

Hernández, M. G., Lozano M., Elbal M., and Agulleiro B. 2001. Development of the digestive tract of sea bass (*Dicentrarchus labrax* L). light and electron microscopic studies. Journal of Anatomy and Embryology. 204(1): 39-57.

Hervas, S., Lorenzen K., Shane M. A., and Drawbridge M. A. 2010. Quantitative assessment of a white seabass (*Atractoscion nobilis*) stock enhancement program in California: Post-release dispersal, growth and survival. Fisheries Research. 105(3): 237-243.

Hinrichsen, E. 2008. Introduction to Aquaculture in the Eastern Cape: Edition 1. Division of Aquaculture, Stellenbosch University Report. Republic of South Africa, Provincial Government of the Eastern Cape, Department of Economic Development and Environmental Affairs, Bhisho.

Hoehne-Reitan, K., and E. Kjørsvik. 2004. Functional development of the liver and exocrine pancreas in teleost fish. American Fisheries Society Symposium,

Holm, H., Hanssen L. E., Krogdahl A., and Florholmen J. 1988. High and low inhibitor soybean meals affect human duodenal proteinase activity differently: In vivo comparison with bovine serum albumin. Journal of Nutrition. 118(4): 515-520.

Irianto, A., and Austin B. 2002. Probiotics in aquaculture. Journal of Fish Disease. 25(11): 633-642.

Kesarcodi-Watson, A, a,□, Kaspar, H a, Lategan, JM and b, Gibson, L. 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. Aquaculture. 274: 1-14.

Kesarcodi-Watson, A., Kaspar H., Lategan M. J., and Gibson L. 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. Aquaculture. 274(1): 1-14.

Klomklao, S., Benjakul S., Visessanguan W., Kishimura H., Simpson B. K., and Saeki H. 2006. Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: Purification and characterization. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 144(1): 47-56.

Kolkovski, S. 2001. Digestive enzymes in fish larvae and juveniles—implications and applications to formulated diets. Aquaculture. 200(1): 181-201.

Kurokawa, T., and Suzuki T. 1998. Development of intestinal brush border aminopeptidase in the larval japanese flounder *Paralichthys olivaceus*. Aquaculture. 162(1): 113-124.

Kvåle, A., Mangor-Jensen A., Moren M., Espe M., and Hamre K. 2007. Development and characterisation of some intestinal enzymes in atlantic cod (*Gadus morhua* L.) and atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. Aquaculture. 264(1): 457-468.

Lazo, J., Mendoza R., Holt G., Aguilera C., and Arnold C. 2007. Characterization of digestive enzymes during larval development of red drum (*Sciaenops ocellatus*). Aquaculture. 265(1): 194-205.

Ma, H., Cahu C., Zambonino J., Yu H., Duan Q., Le Gall M., and Mai K. 2005. Activities of selected digestive enzymes during larval development of large yellow croaker (*Pseudosciaena crocea*). Aquaculture. 245(1): 239-248.

Mai, K., Yu H., Ma H., Duan Q., Gisbert E., Infante J., and Cahu C. L. 2005. A histological study on the development of the digestive system of *Pseudosciaena crocea* larvae and juveniles. Journal of Fish Biology. 67(4): 1094-1106.

Maroux, S., Louvard D., and Barath J. 1973. The aminopeptidase from hog intestinal brush border. Biochimica Et Biophysica Acta (BBA)-Enzymology. 321(1): 282-295.

Martinez, I., Moyano F., Fernández-Díaz C., and Yúfera M. 1999. Digestive enzyme activity during larval development of the senegal sole (*Solea senegalensis*). Fish Physiology and Biochemistry. 21(4): 317-323.

Merrifield, D., Bradley G., Harper G., Baker R., Munn C., and Davies S. 2011. Assessment of the effects of vegetative and lyophilized *Pediococcus acidilactici* on growth, feed utilization, intestinal colonization and health parameters of rainbow trout (*Oncorhynchus mykiss* walbaum). Aquaculture Nutrition. 17(1): 73-79.

Métais, P., and Bieth J. 1968. Détermination de l'α-amylase par une microtechnique. Annales Biologie Clinique. 26: 133-142.

Musa, H., Wu S., Zhu C., Seri H., and Zhu G. 2009. The potential benefits of probiotics in animal production and health. Journal of Animal and Veterinary Advances. 8(2): 313-321.

Musson, M., and Kaiser H. 2014. Development of the digestive system in dusky kob, *Argyrosomus japonicus*, larvae. Aquaculture International. 22(2): 783-796.

Naylor, R. L., Goldburg R. J., Primavera J. H., Kautsky N., Beveridge M. C., Clay J., Folke C., Lubchenco J., Mooney H., and Troell M. 2000. Effect of aquaculture on world fish supplies. Nature. 405(6790): 1017-1024.

Naz, M., and Turkmen M. 2008. Digestive enzymes and hormones in gilthead seabream larvae (*Sparus aurata*) fed *Artemia* nauplii enriched with free histidine. Israeli Journal of Aquaculture. 60(4): 230-236.

Nicholson, J. A., and Kim Y. S. 1975. A one-step L-amino acid oxidase assay for intestinal peptide hydrolase activity. Analytical Biochemistry. 63(1): 110-117.

O'Brien-MacDonald, K., Brown J. A., and Parrish C. C. 2006. Growth, behaviour, and digestive enzyme activity in larval atlantic cod (*Gadus morhua*) in relation to rotifer lipid. ICES Journal of Marine Science: Journal Du Conseil. 63(2): 275-284.

Papadakis, I. E., Kentouri M., Divanach P., and Mylonas C. C. 2013. Ontogeny of the digestive system of meagre *Argyrosomus regius* reared in a mesocosm, and quantitative changes of lipids in the liver from hatching to juvenile. Aquaculture.

Parillo, F., Gargiulo A., and Fagioli O. 2004. Complex carbohydrates occurring in the digestive apparatus of *Umbrina cirrosa* (L.) fry. Veterinary Research Communications. 28(4): 267-278.

Park, H. G., Puvanendran V., Kellett A., Parrish C. C., and Brown J. A. 2006. Effect of enriched rotifers on growth, survival, and composition of larval atlantic cod (G*adus morhua*). ICES Journal of Marine Science: Journal Du Conseil. 63(2): 285-295.

Pearce, R. E., McIntyre C. J., Madan A., Sanzgiri U., Draper A. J., Bullock P. L., Cook D. C., Burton L. A., Latham J., and Nevins C. 1996. Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. Archives of Biochemistry and Biophysics. 331(2): 145-169.

Perez-Casanova, J., Murray H., Gallant J., Ross N., Douglas S., and Johnson S. 2006. Development of the digestive capacity in larvae of haddock (*Melanogrammus aeglefinus*) and atlantic cod (*Gadus morhua*). Aquaculture. 251(2): 377-401.

Rauscher, E., Schmidt A., and von der Eltz H. 1991. Substrates and Reagents Useful in Determining A-Amylase and a Method for Determining A-Amylase.

Ribeiro, L., Sarasquete C., and Dinis M. 1999b. Histological and histochemical development of the digestive system of *Solea senegalensis* (Kaup, 1858) larvae. Aquaculture. 171(3): 293-308.

Ribeiro, L., Zambonino J., Cahu C., and Dinis M. 1999. Development of digestive enzymes in larvae of *Solea senegalensis*, Kaup 1858. Aquaculture. 179(1): 465-473.

Santamarıa, C., Marın de Mateo M., Traveset R., Sala R., Grau A., Pastor E., Sarasquete C., and Crespo S. 2004. Larval organogenesis in common dentex *Dentex dentex* L.(Sparidae): Histological and histochemical aspects. Aquaculture. 237(1): 207-228.

Sarasquete, M., Polo A., and Yúfera M. 1995. Histology and histochemistry of the development of the digestive system of larval gilthead seabream, *Sparus aurata* L. Aquaculture. 130(1): 79-92.

Sasaki, K. 1989. Phylogeny of the family sciaenidae, with notes on its zoogeography (teleostei: Perciformes). Memoirs of the Faculty of Fisheries-Hokkaido University. 36

Savona, B., Tramati C., and Mazzola A. 2011. Digestive enzymes in larvae and juveniles of farmed sharpsnout seabream (*Diplodus puntazzo*)(cetti, 1777). Open Marine Biology Journal. 5: 47-57.

Schmidt-Nielsen, K. 1997. Animal physiology: Adaptation and environment. Cambridge University Press.

Schulze, A. D., Alabi A. O., Tattersall-Sheldrake A. R., and Miller K. M. 2006. Bacterial diversity in a marine hatchery: Balance between pathogenic and potentially probiotic bacterial strains. Aquaculture. 256(1): 50-73.

Segner, H., Storch V., Reinecke M., Kloas W., and Hanke W. 1994. The development of functional digestive and metabolic organs in turbot, *Scophthalmus maximus*. Marine Biology, 119(3): 471-486.

Souza, D. M. d., Martins G. B., Piedras S. R. N., Pouey, Juvêncio Luís Osório Fernandes, Robaldo R. B., and Leite F. P. L. 2012. Probiotic actions of *Bacillus cereus* var. toyoi and *Saccharomyces boulardii* in silver catfish (*Rhamdia quelen*) larvae culture. Revista Brasileira De Zootecnia. 41(3): 815-819.

Suzer, C., Aktülün S., Çoban D., Okan Kamacı H., Saka Ş, Fırat K., and Alpbaz A. 2007a. Digestive enzyme activities in larvae of sharpsnout seabream (*Diplodus puntazzo*). Comparative Biochemistry and Physiology-Part A: Molecular & Integrative Physiology. 148(2): 470-477.

Suzer, C., Çoban D., Kamaci H. O., Saka Ş, Firat K., Otgucuoğlu Ö, and Küçüksari H. 2008. *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: Effects on growth performance and digestive enzyme activities. Aquaculture. 280(1): 140-145.

Suzer, C., Kamaci H. O., Coban D., Saka Ş, Firat K., Özkara B., and Özkara A. 2007b. Digestive enzyme activity of the red porgy (*Pagrus pagrus*, L.) during larval development under culture conditions. Aquaculture Research, 38(16): 1778-1785.

Suzer, C., Kamacı H. O., Çoban D., Yıldırım Ş, Fırat K., and Saka Ş. 2012. Functional changes in digestive enzyme activities of meagre (*Argyrosomus regius*; asso, 1801) during early ontogeny. Fish Physiology and Biochemistry : 1-11.

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

Vine, N. G., Leukes W. D., and Kaiser H. 2006. Probiotics in marine larviculture. FEMS Microbiology Reviews. 30(3): 404-427.

Voet, D., Voet J, and Pratt C. 2013. Fundamentals of biochemistry: Life at the molecular level. John Wiley &Sons.

Wang, Y. 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei.* Aquaculture. 269(1): 259-264.

Watanabe, Y. 1982. Intracellular digestion of horseradish peroxidase (as a marker protein) by the intestinal cells of teleost larvae and juveniles. Bulletin of the Japanese Society for the Science of Fish. 48: 37-42

Whitfield, A. K. 1999. Ichthyofaunal assemblages in estuaries: A South African case study. Reviews in Fish Biology and Fisheries. 9(2): 151-186.

Wold, P., Hoehne-Reitan K., Cahu C. L., Infante J. Z., Rainuzzo J., and Kjørsvik E. 2007. Phospholipids vs. neutral lipids: Effects on digestive enzymes in atlantic cod (*Gadus morhua*) larvae. Aquaculture. 272(1): 502-513.

Wu, G., Cao M., Chen Y., Liu B., and Su W. 2008. Leucine aminopeptidase from red sea bream (*Pagrus major*) skeletal muscle: Purification, characterization, cellular location, and tissue distribution. Journal of Agriculture and Food Chemistry. 56(20): 9653-9660.

Yanbo, W., and Zirong X. 2006. Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. Animal Feed Science and Technology. 127(3): 283-292.

Zambonino, J. Z., and Cahu C. 1994. Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae. Fish Physiology and Biochemistry. 12(5): 399-408.

Zambonino, J., and Cahu C. 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 130(4): 477-487.

Zambonino, J., and Cahu C. 2007. Dietary modulation of some digestive enzymes and metabolic processes in developing marine fish: Applications to diet formulation. Aquaculture. 268(1): 98-105.

Zambonino, J., Cahu C., Péres A., Quazuguel P., and Le Gall M. 1996. Sea bass (*Dicentrarchus labrax*) larvae fed different *Artemia* rations: Growth, pancreas enzymatic response and development of digestive functions. Aquaculture. 139(1): 129-138.

Zambonino, J., Gisbert E., Sarasquete C., Navarro I., Gutiérrez J., and Cahu C. 2008. Ontogeny and physiology of the digestive system of marine fish larvae. Feeding and Digestive Functions of Fish: 281-348.

Zouiten, D., Khemis I. B., Besbes R., and Cahu C. 2008. Ontogeny of the digestive tract of thick lipped grey mullet (*Chelon labrosus*) larvae reared in “mesocosms”. Aquaculture. 279(1): 166-172.